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Production of vaccines and vaccine components in corn

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Production of vaccines and vaccine components in corn

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Abstract

Newly emerging infectious agents along with already existing ones, extended life-span and increased population in the modern age, and the threat of biological warfare increase the demand for high amounts of pharmaceutical/diagnostic recombinant protein production. Plants are safe and cost efficient for production of recombinant pharmaceuticals for humans and livestock. This work investigated potential of corn to serve as a production and delivery vehicle for subunit vaccines and vaccine components. Highly immunogenic antigens LT-B and CT-B from enterotoxigenic strains of *Escherichia coli* (*E. coli*) and *Vibrio cholera* (*V. cholera*) were used in this study as vaccine/component candidates.

This study indicated that corn derived LT-B is capable of inducing immune memory formation. Mice were immunized with LT-B corn through feeding when they were young. They showed a rapid immune memory response in the form of elevated levels of LT-B specific IgG and IgA antibodies upon receiving a booster dose 11 months after initial immunization. This work provided evidence that immunization through feeding with antigens produced in palatable parts of plants would be effective as conventional subunit vaccines in terms of immune memory formation.

The B subunit of cholera toxin (CT-B) was expressed in transgenic corn seeds at high levels. Biochemical and immunological analysis indicated that corn derived CT-B, like the bacterial protein, is assembled into pentamers and binds to G_{M1} gangliosides. Although the bacterial signal peptide of CT-B appears to not cleaved in corn seed tissue, unlike its counter part LT-B, it is able to induce murine immune system to produce CT-B specific antibodies. This work also provided evidence that administration of a mixture of corn derived CT-B and LT-B together has potential to induce higher levels of specific antibodies than when they

were administered alone. This finding opens the possibilities of using these two corn derived antigens in combination and may lead to more efficacious vaccines against both cholera and enderotoxigenic *E. coli* induced diarrhea.

To use the potential adjuvant and carrier molecule properties of the B-subunits, we attempted to produce the LT-B and CT-B subunit fusion protein with an epitope that may function as a vaccine. A 42 amino acids epitope of amyloid beta (Aβ), a protein involved in pathogenesis of Alzheimer's disease (AD), was used as a case study. Two strategies, LT-B/Aβ C-terminal fusion and CT-B internal fusion, were used. LT-B/Aβ fusion resulted in pentameric assembly of LT-B alone in corn callus tissue, but the fusion protein was not detected. On the other hand, the CT-B internal fusion resulted in no detection of CT-B or the fusion protein. While these B-subunit protein fusion strategies were successful in producing a number of B-subunit/epitope fusions in bacteria and some plants, this study taught us that current ability to predict a successful fusion is limited and there is a need to evaluate each fusion on a case by case basis in their host organisms.

The immunogenic potential of corn derived LT-B in pigs was also investigated. Oral administration of corn derived LT-B to newly weaned pigs resulted in elevation of LT-B specific IgG and serum IgA in 50% of the subjects. This result expands possibilities for utilization of corn derived LT-B to prevent *E. coli* induced diarrhea in pigs and as a mucosal adjuvant for unrelated antigens.

This work indicated that a major food and feed crop corn holds great potential to be utilized as a production and delivery vehicle for production of vaccine antigens and components which could be used for prevention of both human and animal diseases.

Chapter 1: Introduction

1.1 General Introduction

More than 10,000 years ago, descendants of highly advanced apes started to utilize other living organisms through domestication of plants and animals. Knowledge regarding functioning of living organisms, accumulated since then, allowed modern man to use organismal and cellular paradigms as part of agriculture, medical sciences, industrial applications, environmental and wild life management, forensic science and paleontology. Plants have been used for making clothing and shelter and in medicine since ancient times in addition to being a primary source of food for survival (Gurib-Fakim, 2006). Today, as a result of advancements in biotechnology, plants are used to produce pharmaceuticals including antibodies, antigens and diagnostics, veterinary and industrial products. As of 2005, there are four plant-derived vaccines that are close to commercialization for treatment of diarrhea, hepatitis B and rabies (Ma et al, 2005). Following initial introduction of the concept by Mason et al (1992), a number of antigens have been produced in plants including tobacco, spinach, potato, carrot, lettuce, alfalfa, tomato, and corn (Mason et al, 1992, Tregoning et al, 2005, Karasev et al, 2005, Tacket et al, 2005, Dong et al, 2005, Chikwamba et al, 2002). Seeds are attractive organs in which to produce antigens because they store high amounts of proteins allowing a high level of recombinant protein expression. Seeds also provide stable dry environment which is a major advantage for long term storage and long distance delivery. Also, seeds are used as feed and food; they can readily be used as delivery vehicles for the antigens. In addition to being produced worldwide, corn is an attractive

expression system for plant-derived vaccine production among other seed crops. In this work, we used corn as a vehicle for production of vaccine and vaccine components and also addressed related issues regarding long term analysis of immune response to oral administration of corn-derived antigen and also assessment of allergenic potential of genetically modified corn.

Infectious diseases are responsible for one quarter of all deaths worldwide annually. Most mortality occurs among children in developing countries who are not adequately immunized (Arntzen 2005). Cholera is an enteric disease which is considered to be endemic in Sub-Saharan Africa, South East Asia and South and Central America. Enterotoxigenic *E. coli* (ETEC) is another enteric disease with similar pathology and symptoms; which also has high morbidity. Production of plant-derived vaccines for enteric diseases is desirable because antigens produced in edible parts of the plants can be administered orally which could provide immediate protection at the site of infection.

Pathogenesis of both cholera and ETEC involves intestinal colonization followed by release of toxins; LT (labile toxin) and CT (cholera toxin). Both LT and CT are made of two subunits; toxic A subunit which is internalized by binding of nontoxic B subunit to GM1 gangliosides residing on intestinal epithelial cells. The B subunit is highly immunogenic. Vaccines made of B subunit could elicit immune response and anti-XB (X: LT or CT) could interfere with entry of the toxin to epithelial cells. In addition to being potent immunogens, CT-B and LT-B also non-specifically activates the immune system therefore they could serve as adjuvants in vaccine preparations targeting other diseases. Moreover, CT-B and LT-B could serve as carriers for other antigens through translational fusions.

In this study, we investigated different aspects of production of vaccines in corn. Our first project assessed long term and booster effects of orally administered LT-B corn with a pre-clinical trial conducted on mice. Anti-LT -B antibodies were detectable over an eleven month period in sera of mice fed with LT-B corn when they were young and booster administration of LT-B corn resulted in rapid and strong memory reaction in the form of antibody production.

Our next project focused on production of a transgenic maize line expressing a non-toxic subunit (CT-B) of cholera toxin in its kernels. This was achieved by expression of synthetic CT-B gene under control of the seed specific 27 kDa γ-zein promoter. To ensure expression, possibly at high levels, the synthetic CT-B gene was formulated based on maize preferred codon usage. Expression and native conformation of CT-B in maize kernels were demonstrated by ELISA and immunogenicity was determined by feeding experiments in mice.

In order to test the carrier potential of CT-B and LT-B, fusion genes with gene sequence encoding for amyloid beta protein which is involved in pathogenesis of Alzheimer's disease were created. Even though correctly folded CT-B pentamers with the fusion were not detected, pentameric LT-B were detectable. However, in either case, expression of amyloid beta protein and its epitopes were not achieved.

Although genetic modification of food crops holds great promise for the society in terms of better agricultural management, increased yield and enhanced nutritive quality, all of which could help to ensure food security, the technology also raised fundamental human health concerns. These include potential toxicity and allergenicity of the transgene product (Malarkey, 2003), the use of suicide genes and the possible risk of transmission of resistance

genes to neighboring plants (Metcalfe, 2003). In this study, a preliminary work was also conducted to determine a strategy for complete analysis of transgenic corn lines used and generated in this work.

The last project described in this dissertation evaluated antibody induction potential of LT-B corn in a preliminary pig feeding trial.

1.2 Dissertation organization

This dissertation aims at exploring different aspects of plant-derived vaccines. The following chapters describe projects conducted in my PhD study. Dr. Kan Wang, my major professor, has provided overall supervision to the program. Dr. Joan Cunnick, one of my POS committee members provided the guidance on experiments involving immunization.

Chapter 1 is a literature review. It focuses on achievements and challenges in the production of plant-derived vaccines and the unique advantages that they promise. Rationale of using corn to generate plant-derived vaccines, role of mucosal immunization in development of novel drugs against different classes of diseases, introduction to LT and CT structure and functioning and their potential as mucosal adjuvant and carriers are also given. A neurodegenerative disorder Alzheimer's disease is given as a case study as an introduction to our attempt to generate a plant-derived vaccine for AD. Finally, this chapter is concluded by human health concerns, and allergenic potential in particular, raised by genetically modified crops.

Chapter 2 is the analysis of long term immune response of mice to orally administered LT-B and effect of booster administration. This work has been published in the

journal ***Molecular Biotechnology***. Dr. Joan Cunnick provided hands-on training on animal handling methods. Jennifer McMurray provided technical assistance for sample collection.

In Chapter 3 expression of CT-B in corn seeds is described. This manuscript reporting complete analysis of corn-derived CT-B including a mice feeding experiment indicating its immunogenic activity is in preparation for submission to a peer-reviewed journal. Throughout this work undergraduate assistant Chad Niederhuth and graduate assistant Swetha Gottimukkula assisted me on routine analysis. Jennifer McMurray and Chad Niederhuth helped with sample collection during feeding experiment in mice.

Chapter 4 presents our efforts to generate a plant-derived vaccine against Alzheimer's disease by using LT-B and CT-B as carriers.

Chapter 5 presents analysis of the allergenic potential of transgenic corn lines used and generated in this study (LT-B corn and CT-B corn). Results of this analysis were used to determine a strategy for complete analysis of these transgenic lines.

Work presented in Chapter 6 is carried out as an initial testing of LT-B-corn in pigs for its ability to induce immune response. This work is the collaboration between Dr. Wang, Dr. Cunnick and Dr. Harris from Animal Science. I carried out the initial experimental design of the pig feeding test. This involved determining treatment groups, dose administered and feeding days. I also analyzed sera for determining levels of LT-B specific IgG and IgA antibodies. The pig feeding and sample collection was done by Dr. Mathew Erdman.

1.3 Literature Review

1.3.1 Why Plants

In the past, therapeutics were relatively small molecules produced by chemical synthesis or produced in microorganisms. These pharmaceuticals include antibiotics,

hormones, diagnostics and others. Recently, much attention has focused on small peptides and proteins of varying sizes. Due to their inherent physiological and structural roles in organisms, utilization of proteins as therapeutics holds great promise for treatment and prevention of a number of diseases. Over 20 years after commercialization of the first peptide therapeutic agent, insulin, more than 40 out of 200 best selling drugs were made of peptides or proteins in 2004 (http://www.srinstitute.com). These include the recently commercialized 36-amino-acid synthetic peptide drug Fuzeon® (enfuvirtide) that has been approved by FDA (Food and Drug Administration) for treatment of HIV infection (Jamjian and McNicgoll 2004).

Recombinant proteins can be produced in a variety of systems. Cultured mammalian cells (Derouazi et al., 2004), insect cells (Altmann et al., 1999), bacteria (Eko et al., 2004), yeast (Porro and Mattanovich, 2004), moss (Decker and Reski, 2004), recombinant animals (Dalyrymple and Garner 1998) and plants (Wang et al., 2004) have been widely used. Each of these systems has inherent advantages and disadvantages. Microbial systems are well established however, they are expensive to meet the increasing demand for peptide/protein therapeutics. They can not produce complex eukaryotic proteins. Mammalian cell cultures and transgenic animals have the machinery for producing complex proteins However using these systems involves therapeutic risk because even after purification, the product might be contaminated with viruses, oncogenes and endotoxins. Furthermore, it takes longer time to obtain products with these systems (Goldstein and Thomas, 2004).

A number of recombinant proteins have been synthesized in plants. These include a variety of antibodies and vaccine antigens, adjuvants and blood substitutes (Goldstein and Thomas, 2004). Plants have unique advantages over other expression systems. One of the

main advantages of using plants to express peptides and proteins is that; plants can synthesize short peptides, longer polypeptides and also form multimers (Ma et al, 2003). In addition as their eukaryotic counterparts, plants also perform posttranslational modifications such as phosphorylation and glycosylation which enable transgene products to have their native structure and function. Allowing multimer formation and posttranslational modifications are particularly important for antibody production.

The most important advantage of using plants as bioreactors is that plants essentially are free of human and animal pathogens. In addition, plants are easy to produce and bulk up, which reduces cost of production. In addition to ease in scaling up, being able to be administered orally could also decrease the cost of production by eliminating downstream processes such as purification. In the case of plant-derived vaccine administration, this property offers a special benefit; *oral immunization* (Freytag and Clements, 2005). Orally administered antigens could elicit both mucosal and systemic immune response which provides dual protection (Berinstein et al, 2005). Moreover, availability of specific promoters and signals to target the expression of the transgene to desired organ and organelles makes utilization of plants as expression vectors for recombinant proteins more attractive. Among other organs, seeds are particularly practical due to their inherent role as protein storage unit. In addition, they provide stable and dry environment for the proteins making long term storage easier. This is important in that it might eliminate the need for refrigeration of the vaccine during delivery or storage in underdeveloped and developing countries where refrigeration is not always readily available.

Proteins can be expressed in stably transformed plants or transiently transformed plant cells. Due to difficulties in scaling up, transient systems are usually used only for

testing of the constructs. Agroinfiltration, plant viral vectors and biolistic techniques are commonly used for transient expression. Agroinfiltration is relatively a simple procedure. *Agrabacterium tumafaciens* with its T-DNA containing a recombinant gene is infiltrated into plant tissues. This method provides milligram quantities of the target protein in a few days (Vaquero et al, 1999). Plant viral vectors provide rapid and systemic invasion of the plant tissue enabling expression of the desired protein in a large amount of cells transiently in a very short time. This method can not be used as a stable transformation technique, because the viral genome does not integrate itself to the nuclear genome of the host plant. However, the technique is feasible as a screening method for gene constructs for complex proteins which requires assembly. For example, full length monoclonal antibodies can be produced in plants by co-infection with two plant viruses carrying light and heavy chains of the antibody to be produced. Assembly of the chains can be successfully carried out *in planta* (Verch et al, 1998). Gene delivery with biolistic method for transient expression in plants is also commonly used. Leaves, callus, immature embryo end endosperm cells of corn (personal experience), leaves and calli of rice (Morello et al, 2005), shoot epidermal cells of barley (Douchkov et al, 2005) and strawberry and tomato fruits (Agius et al, 2005).

Stable transformation refers to integration of the foreign gene into nuclear, plastid or mitochondrial genome. Each cell in a transgenic plants produced with this method has the foreign gene in them. New generations obtained from these plants are also transgenic because the germ cells also contain the recombinant gene. *Agrobacterim tumafaciens* and biolistic method are used to generate stably transformed plants. Currently pea, soybean, tobacco and Arabidopsis plants are routinely transformed with Agrobacterium infection (Schillberg et al, 2005). Corn and rice can be transformed with both methods (Zhao et al, 1998, Dai et al,

2001). It has been recently indicated that Agrobacterium mediated transformation leads mostly to single or low transgene copy numbers (Zhao et al, 1998, Dai et al, 2001, Shou et al, 2004). This is likley to stabilize expression of the transgene in subsequent generations (Meyer and Saedler 1996). Therefore the Agrobacterium method is becoming more preferred for maize transformation (Shou et al, 2004).

After two decades of recombinant protein production in plants, the technology is coming close to bearing fruits. As of 2005, there have been eight Phase I and three Phase II clinical trials conducted to test the efficacy and safety of plant made pharmaceuticals (PMPs). Out of these eleven trials, two of them were antibodies, one was a therapeutic enzyme, two were dietary supplements and three were vaccine candidates two of which are against enteric disease while one of them were against rabies (Ma et al, 2005). Commercialization of PMPs will depend on resolving issues regarding formulation of the final product, homogeneity and consistent dosing, regulation, biosafety and social and political concerns.

1.3.2 Selection of the plants and plant tissues

Recombinant proteins can be produced in a large number of plant species. Besides model organisms Arabidopsis and tobacco, fruits (tomato, strawberry, watermelon, and banana), vegetables (spinach, lettuce, potato and carrot), legumes (soybean, pea, and alfalfa), and grains (corn, barley, rice, and wheat) are widely used as host for production of target proteins. The choice of plant to be used depends on certain parameters; production, scale up and delivery cost, time required for generating the fully characterized transgenic line, palatability, requirement for downstream processing and related expenses, regulatory steps to

be followed for field releases and geographical location (Schillberg et al, 2005). Therefore, there is no single host species which is superior to others in terms of all the parameters listed.

Tobacco is the most widely used host species among other plant species for production of recombinant proteins and proof of concept. It is easy to transform and propagate, thereby has high biomass yield. Both nuclear and plastid transformation is possible. In some cases with chloroplast transformation very high levels of expression have been achieved (up to 25% of total soluble proteins). One major disadvantage is that even with low alkaloid varieties, the final product will require a certain level of processing to eliminate toxic alkaloids (Ma et al, 2003). Also, high levels of proteolytic and microbial activity in green tissue makes tobacco less practical for post-harvest stages of the process (Menkhaus et al, 2004). Arabidopsis per se has also been used for proof of concept and as a model to express proteins with pharmaceutical value (Schillberg et al, 2005). However, in addition to possessing all the obstacles regarding green tissue as a system to produce recombinant proteins, its low biomass makes it unlikely that it will be utilized for mass production.

A number of fruit and vegetable species have been successfully transformed. One advantage of using fruits and vegetables is the relatively short time required for generation. Fruits and vegetables are also suitable for production of plant derived pharmaceuticals because they can be fed raw or after minimal processing. Among this class of plants, most of the studies including Phase I clinical trials have been carried out with potato. Antibodies, antibody-fusion proteins, milk proteins and cytokines have been successfully produced in potatoes (Ma et al, 2003, Ohya et al, 2001). Another transgenic vegetable that reached clinical trials is lettuce transformed with gene encoding for hepatitis B surface antigen (Kapusta et al, 2001). Transgenic spinach plants that express antigens of HIV (human

immunodeficiency virus), the anthrax bacterium (*Bacillus anthracis*) and rabies virus have also been produced. All three spinach-derived antigens have been shown to be highly immunogenic (Karasev et al, Sussman, 2003, Modelska et al, 1998). Tomatoes also received attention due to their palatability. A tomato-derived rabies vaccine has also been immunogenic in laboratory animals (Ma et al, 2003). However, low levels of transgenic protein accumulation, due to low levels of proteins in fruit, leaves and tubers of vegetables makes utilization of them to produce bulk quantities of target proteins less desirable. Another disadvantage of fruits and vegetables is their short shelf life which makes partial processing necessary. Powder formulations produced by homogenizing and freeze drying would surmount this obstacle and also enable more homogenous preparations for feedings.

As mentioned before, tissues with a high level of moisture such as leaves and fruits do not provide a stable environment for production and storage of recombinant proteins. However, seeds provide dry, stable environment for proteins during long term storage at ambient temperature. Previous studies indicated that antibodies produced in seeds remain stable for three years (Ma et al, 2003). In addition to lack of toxic phenolic compounds, rapid scaling up makes seeds practical for production of PMPs (plant made pharmaceuticals). Currently there are five commercial pharmaceutical products obtained from food crops which are used in research, diagnostics and manufacturing. These are avidin, β-glucorunidase and trypsin (http://www.prodigene.com/pdf/TrypZean(tm)%20Backgrounder.pdf) produced in corn and lactoferrin and lysozyme produced in rice (Humphrey et al, 2002). Research conducted with soybean, peas and alfalfa also have shown to promise. One major advantage of using legumes is minimal need for chemical support in the form of fertilizers due to their innate ability to fix atmospheric nitrogen (Ma et al, 2003).

1.3.3 Selection of the host species: crops or non-crop plants?

Corn is a common grain produced world-wide. It can readily be transformed to produce recombinant proteins. It has been shown to be an ideal production and delivery vehicle for PMPs, in particular plant-derived vaccines, by different studies (Streatfield et al, 2003). Further investigation of possibilities in the area of PMPs with corn and assessment and resolution of safety concerns will enable commercialization of PMPs for human and veterinary medicine.

The major concern about using food crops for pharmaceutical production is the possibility of contaminating the plants grown for food production. This might result from pollen drift or accidental mixing of modified crops not intended for human consumption with stocks used for human food supply. Using male sterile lines and plastid transformation are two systems to surmount this concern. Currently, field release of PMPs with open pollination system is regulated by United States Department of Agriculture-Animal and Plant Health Inspection Services (USDA-APHIS). APHIS requires temporal and spatial confinement for field release of PMP crops. Temporal confinement prevents pollination between PMP crops and nearby conventional crop of the same species. Open pollinated PMP corn lines are required to be planted 28 days earlier or later than commercial varieties planted nearby. Also, test plots must be at minimum of 1 mile distance from commercial corn fields. In addition, 50 feet of perimeter fallow zone around the test plot is required (http://www.bio.org/healthcare/pmp/factsheet4.asp).

1.3.4 Mucosal immunization, oral tolerance and mucosal adjuvants

Infectious diseases continue to be a major cause of death globally. Vaccination is still the most successful way of prevention of communicable diseases. Increased rates of foreign

travel and international trade of foodstuff has changed the perception and reality for endemics to become pandemics. Today, an infectious disease encountered in one country is a global threat. Emergence of two new diseases; SARS in 2003 and avian flu in 2004 created an intense global response and preparation for preventing pandemics, and it continues to be a concern. Along with rapid rate of evolution of resistant varieties of related pathogens and immense global population, development of effective, cheap and easily distributable vaccines is more important than ever.

There has been an enormous effort to change traditional vaccine preparations as well as using alternative routes of delivery. Traditionally vaccines were made of killed or attenuated pathogens and the standard route of delivery was parenteral administration. Recent efforts have focused on subunit vaccines. This class of vaccine is made of non-pathogenic yet immunogenic elements of microorganism causing the disease. This approach is safer and in some cases more effective. Producing these elements in edible parts of the plants and oral administration might be advantageous. The linings of mucosal surfaces are the entry site for many pathogens and therefore play an important role for rendering microorganisms harmless and preventing disease. These mucosal surfaces are respiratory, genitourinary, nasal, oropharyngeal and gastrointestinal mucosa (Freytag and Clements, 2005). The main advantage of mucosal administration of a vaccine is the ability to induce both systemic and local mucosal response in the form of serum IgG and secretory IgA production, respectively (Eriksson and Holmgren, 2002). Secretory IgA antibodies can prevent entry of pathogens to mucosal cells and neutralize bacterial toxins. In addition, the ability of the mucosal immune system to relay information to distant sites, through common mucosal immune system (CMIS), expands the possibilities for administration of the antigens. For example, the oral

route may be effective to induce sufficient amount of secretory IgA production in lungs and may be chosen as a route of delivery for respiratory diseases as well as GI related diseases.

Mucosal administration also offers hope for treatment of autoimmune diseases such as multiple schlerosis (MS), diabetes, rheumatoid arthritis (RA) and allergies (Faria and Weiner, 2005). In autoimmune diseases, a component in the body, in the case of allergies a protein from outside source (food, plants or animals which is tolerated in majority of human population) are attacked by immune system of the individual with the disease. Induction of oral tolerance (a phenomenon which allows human and animals not to attack ingested proteins) to such components have been tested as a means of therapy. Treatment of such diseases has been tested by oral administration of a related protein, in the case of MS myelin basic protein (MBP) for example, through a specific feeding regimen. Although promising, the results of such efforts are not conclusive yet. A significant placebo effect was observed in clinical trials testing the efficacy of such treatment for MS and RA. New trials with modifications in administration regimen are ongoing (Faria and Weiner, 2005).

Although promising there are challenges regarding mucosal administration of antigens. High acidity and digestive enzymes inactivate the majority of the antigens before they reach Peyer's patches which are T-cell and B cell rich immune structures of gastrointestinal tract (Rigano and Walmsley, 2005). In some cases with oral vaccination systemic tolerance has been observed (Simmons et al, 2001). In order to protect orally administered antigens a variety of strategies have been developed. One strategy uses biodegradable antigen carriers including liposomes, chitosan particles, polyacrylamide starch particles, virosomes, immune stimulating complexes (ISCOMs) and latex microspheres (Foster and Hirst, 2005). In addition to all of the advantages of using plants for

pharmaceutical production, plants also offer an advantage for encapsulation of the recombinant proteins in sub-cellular compartments thereby providing better antigen preservation in harsh environment of the digestive tract (Chikwamba et al, 2002).

Co-administration with mucosal adjuvants can enhance immunogenicity of plant derived antigens. Some mucosal adjuvants can also be used as carriers for antigens. Two bacterial enterotoxins LT from *Escherichia coli* and CT from *Vibrio cholerae*, and their subunits LT-B and CT-B, have received a great deal of attention due to their ability to serve as mucosal adjuvants (Freytag and Clements, 2005). In this work, plant derived LT-B and CT-B have been studied for their antigenic, adjuvant and carrier potential, and are discussed below.

1.3.5 E.coli heat labile toxin and cholera toxin as mucosal adjuvants and carriers

E. coli and *V. cholerae* colonize the intestinal system of humans and animals and cause severe diarrhea through similar mechanisms. Both LT and CT are potent immunomodulators. LT and CT share 82% homology in terms of their amino acid sequences (Williams et al, 1999). They are multimeric proteins with approximate sizes of 84 kilodalton (kDa). A 27 kDa toxic A subunit is non-covalently linked to a ring shaped pentamer made of five identical non-toxic B subunits. A 55 kDa B pentamer binds to the toxin receptor, ganglioside GM1 (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), residing on the surfaces of intestinal epithelial cells. Upon binding, exogenous enzymes proteases cleave A subunit into A1 and A2 fragments. Following transport from ER to cytosol, the A1 fragment catalyzes ADP-ribosylation of a G protein which results in chloride ion (Cl^-) efflux. Activated G protein triggers adenylate cyclase leading to increased levels of

cyclic AMP (cAMP). High amounts of cAMP results in opening of a transmembrane Cl^- channel which is followed by efflux of water along with ions into the intestinal lumen which results in the characteristic diarrhea of the disease (Williams et al, 1999).

Potential of LT and CT as mucosal adjuvants was indicated for the first time in the late 1980s (Clements et al, 1988, Elson, 1989). Due to toxicity of holotoxins, the B subunit of both toxins received attention for utilization as mucosal adjuvants. Several other studies investigated separating toxicity from immunogenicity by creating mutant toxins (Verweij et al, 1998). In addition to their adjuvant properties, B subunits can be used as vaccines against toxin induced diarrhea because they induce secretory IgA and serum IgG that protect against subsequent challenge (Millar et al, 2001). CT-B and LT-B have been produced in a variety of plant species. Transgenic potato, tomato and tobacco plants producing recombinant CT-B (Arakawa et al, 1997, Jani et al, 2002, Wang et al, 2001) and transgenic corn, tobacco, potato and ginseng plants (Chikwamba et al, 2002, Streatfield et al, 2001, Kang(a) et al, 2004, Mason et al, 1998, Kang(b) et al, 2005) producing recombinant LT-B formed the basis for plant-derived vaccines against diarrhea induced by these two toxins.

In order to use CT-B and LT-B as carriers for targeting unrelated antigens to mucosal surfaces, a number of fusion proteins were produced in plant and non-plant systems. These include CT-B fusions with anthrax lethal factor protein (Kim et al, 2004a), simian-human immunodeficiency virus 89.6p Tat protein (Kim et al, 2004b), simian immunodeficiency virus mac Gag p27 capsid protein (Kim et al, 2004c), and rotavirus VP7 protein (Choi et al, 2005). Similarly, a few LT-B-antigen fusion proteins have been produced in plants. These include neutralizing epitope of porcine epidemic diarrhea virus expressed in tobacco (Kang(c) et al, 2005) and a tuberculosis antigen produced in *Arabidopsis thaliana*

(Rigano et al, 2004). In this study, a human protein amyloid beta was chosen to produce fusion proteins with LT-B and CT-B due to its potential to treat and prevent Alzheimer's disease (Schenk et al, 1999).

1.3.6 Case study: Alzheimer's disease

Alzheimer's disease is a severe neurodegenerative disorder seen in elderly. It affects more than 12 million people worldwide (Gelinas et al, 2004). Symptoms of the disease are progressive loss of memory and cognitive decline, behavioral changes and language problems. Current therapies are mainly aimed at symptom control. Five classes of drugs used in AD treatment are cholinesterase inhibitors, glutamatergic neurotransmission suppressers, vitamins and antioxidants, non-steroidal anti-inflammatory drugs (NSAIDs), and drugs that are used to manage neuropsychiatric symptoms (Lleó et al, 2005). All of these drugs provide cognitive, behavioral and motor function benefits to a certain extent; however they do not affect disease progression. Therefore the search for AD treatments that are going to prevent and also slow down the progression of the disease are still continuing.

Morphological hallmarks observed in AD patient's brains are amyloid beta plaques and neurophirillary tangles. Lesions caused by amyloid beta plaques and neurophibrillary tangles lead to death of neurons. Amyloid beta plaques are formed of 40 and 42 amino acid long peptides called amyloid beta. The 42 amino acid long version is more fibrillogenic and is the product of improper cleavage of amyloid beta precursor protein (APP) by β-secretases and γ-secretases. Unlike amyloid beta deposits, neurofibrillary tangles are observed intracellularly, located mostly around the nuclei of neurons. They result from hyperphosphorylation of a structural protein, tau, which normally functions in assembly of

microtubules. Mutations in APP and components of the enzymes processing APP lead to formation of both amyloid beta plaques and tau tangles. However mutations in tau only result in formation of tangles (Walker et al, 2005). This indicates a primary role for amyloid beta in disease pathogenesis, which is referred as 'amyloid beta cascade'. This hypothesis implies that accumulation of fibrillar amyloid beta is followed by neurofibrillary tangle formation, inflammation, death of neurons which then triggers formation of other lesions observed in AD brains. Therefore, much of the recent efforts for treatment of AD have focused on reducing the amount of amyloid beta plaques.

Current strategies targeting prevention of amyloid beta deposition are; inhibition of amyloid beta protein generation, inhibiting formation of multimers and induction of degradation of the plaques via immune system involvement. The first approach involves inhibition of enzymes (secretases) that generate the fibrillar form of amyloid beta. Even though this method has been shown to be successful in terms of reducing the amount of amyloid beta in experimental animals, severe side effects of the inhibitors make their utilization as drugs impossible. Due to their central role in AD pathogenesis, highly toxic oligomeric forms of Amyloid beta have also been targeted in the search of finding a cure for the disease. However, difficulties involved in inhibition of protein interactions *in vivo* slow down the progress that can be achieved with this approach (Walker et al, 2005). The last strategy has been shown to be promising through. Parenteral immunization with aggregated amyloid beta prevented occurrences of the plaques and also removed already existing ones when tested in transgenic mice (PDAPP mice) showing AD pathology (Schenk et al, 1999). In addition, further studies carried out following different immunization schemes indicated

that immunization with amyloid beta also improves cognitive abilities of mice as the plaques are removed (Weksler, 2004).

Given these encouraging results a human clinical trial was commenced in 2001. The Phase I trials with 100 AD patients indicated that injections induced antibody response and they were well tolerated. However, Phase II clinical trials conducted by Elan Pharmaceuticals Inc. had to be stopped due to brain inflammation observed in 6% of the 300 subjects (Haas et al, 2003, Walker, 2005). Complications due to aging are likely to cause unexpected results. In aged individuals, the immune system undergoes certain changes that lead to slower immune response, increased autoimmunity following immunization with a foreign antigen (Weksler, 2004) and chronic stimulation of macrophages leading to a 'pro-inflammatory status' (Kipar et al, 2005). Moreover, evidence indicating induction of innate immune response in AD pathology might suggest that inflammation observed following amyloid beta administration may not be a direct result of the immunization.

It has been postulated that amyloid beta plaques stimulate the innate immune response involving activation of microglia, astrocytes and the complement system and free radical formation, as well as release of pro-inflammatory cytokines such as TNF-α and IL-β (Weiner and Selkoe, 2002) and formation of acute-phase proteins (Akiyama et al, 2000). Support for the validity of this hypothesis came from the studies showing that these molecules, which are suggestive of inflammation, are present in AD patient's brain (Akiyama et al, 2000). On the contrary, nonappearance of immunoglobulins in the brain regions suggests that the humoral immune response is not involved in amyloid beta plaque formation (Eikelenboom et al, 1994). Given the fact that inflammation may play a role in the neuropathological injury observed in AD (Weiner and Selkoe, 2002), the exact reason for

inflammation in Phase II clinical trials is unknown. Hence, complications observed in a small percentage of subjects in the trial should not negate the potential of amyloid beta immunization for preventing and treat AD. Modifying immunization schemes and adjuvant combinations could help overcome some of the severe side effects observed with parenteral immunization.

T-helper cells that secrete cytokines that induce macrophage production are classified as Th1 cell, and those releasing cytokines that induce B-cells are classified as Th2 cells. The Th1 response is characterized by cellular cytotoxicity and local inflammation whereas the Th2 response involves induction of opsonizing antibody formation and suppression of the Th1 response thereby inhibiting inflammation (Janeway et al, 2001). Therefore, one modification that could overcome inflammation is testing different routes of delivery. Intranasal and oral administration of antigens preferentially induces Th2 rather than Th1 type immune response (Constant et al, 2000, Itoh et al, 1999). Encouraging results with mucosal immunization have already been observed. A 50 to 60% reduction in amyloid beta burden was indicated in PDAPP mice which were intranasally immunized with the short form of amyloid beta protein ($A\beta_{42}$) for seven months (Lemere et al, 2002). Not surprisingly, antibody response was found to be low (26 μg/ml). Further study conducted by the same group in wild type mice used LT and a non-toxic version of LT (R1192G) as adjuvant. Antibody levels were found to be dramatically increased, especially with non-toxic LT.

Based on these findings, and with the carrier and adjuvant properties of LT-B and CT-B in mind, in the search for a safe and effective vaccine against Alzheimer's disease, we aimed at producing a plant-derived vaccine against AD by transforming corn with fusion genes.

1.3.7 Age related changes in immune reaction and immune memory

Aging leads to deterioration of both innate and specific immune responses through alterations at the organism and single cell levels (Frasca et al, 2004, Nikolich-Žugich, 2005). These changes are altogether named as immunosenescence. Immunosenescence results in reduced response to vaccination and increased vulnerability to infections. Accordingly, morbidity and mortality rates in aged individuals due to diseases, which are not fatal in healthy young adults, are substantial (Miller et al, 1996, Peterson et al, 2003). As well as changes that occur in immune system, functional and structural modifications observed at the port of entry (skin and respiratory surfaces) and destination organs (circulatory and nervous system) and also contribute to this susceptibility seen in elderly (Wick et al, 2000).

At the cellular level, the changes occurring in systemic immunity observed in aged individuals are as follows; decreased antigen presentation, increased constitutive cytokine release, reduced response to antigen and pathogens, impaired macrophage activation, dendritic cell (DC) migration and Toll-like receptor mediated activation, and alterations in T-cell and B-cell regulation (Frasca et al, 2004, Nikolich-Žugich, 2005). Recent research indicates that intestinal mucosal components also undergo deterioration which accounts for increased incidences of gastrointestinal infections seen in elderly (Beharka et al, 2001).

In this regard, it is essential to study the potential of plant-derived vaccines in elderly. Investigating long term efficacy of mucosal administration following initial sensitization early in life and booster response is also critical.

1.3.8 Allergy concerns

Nutritional quality and yield of food crops can be increased via genetic modification. Biotechnology also enables production of vitamins, antigens and hormones in plants.

Genetically modified food crops with natural pesticides and increased resistance to drought are already part of common agricultural practice (Metcalfe, 2003). Food crops with their allergenic components rendered inactive could also be produced through genetic modification.

In spite of the great promise that it offers, application of biotechnology to food crops initiated concerns regarding potential toxicity of recombinant proteins and unintended environmental consequences of the technology in the long run. Major health concerns regarding GM food crops are potential toxicity and allergenicity of the recombinant protein and the risk involved in rendering any endogenous component of the food crop being modified to be toxic or allergenic.

Symptoms of food allergies involve classic allergy reactions: hives, swelling of parts of body, usually lips, tongue and eyes, vomiting, diarrhea and asthma are observed shortly after ingestion of the allergenic food. Such reactions to foods can be IgE mediated or non-IgE mediated. Since IgE mediated reactions are more common and severe, research regarding allergenic potential of GMO foods has been focused on IgE mediated reactions (Metcalfe, 2003).

No single predictive test is available to test allergenic potential of a given protein. The International Food Biotechnology Counsel and the International Life Sciences Allergy and Immunology Institute formed a decision tree approach to identify allergens in GMOs either before or after production. Final version of the strategy was determined by Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (Poulsen, 2004). The first step is to perform a homology search against amino acid sequences of known allergens. Currently, there are a number of allergen databases. Efforts

are under way to unite these to create a high validity public allergen database (Poulsen, 2004). The second step involves specific or targeted serum screening. Specific serum screening is testing of a protein extract of the GMO food with sera of individuals known to be allergenic to the source of the gene. Positive results of such testing indicate that the newly introduced gene encodes for an allergenic protein. If the result is negative, further testing with a panel of sera that are raised against different groups of organisms is performed. This testing is called as 'targeted serum screening' and provides more broad screening of all potential allergens. Positive result of this test also indicates that the protein is an allergen. The third level of testing is required if targeted serum screen yields negative results. One common property of allergenic proteins is their resistance to digestion. Therefore, a pepsin-resistance test is performed. If an extract from transgenic product is found to be more resistant than its non-transformed counterpart, it might indicate allergenicity. In order to reach a final conclusion, animal models are used to test immunogenicity of the transgenic product.

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Chapter 2: Analysis of immune response in young and aged mice vaccinated with corn-derived antigen against *E. coli* heat labile enterotoxin

Molecular Biotechnology 2006: 32; 31-42

Sule Karaman, Joan Cunnick and Kan Wang

2.1 Abstract

Enterotoxigenic strains of *E. coli* produce a heat labile holotoxin (LT), which causes diarrhea. We engineered corn seeds to produce LT-B, the non-toxic subunit of LT, to serve as a plant-derived vaccine to traveler's diarrhea and as an adjuvant for co-administered proteins. We previously demonstrated that a strong mucosal and systemic antibody response is elicited in young mice upon oral administration of corn-derived LT-B. The present study examined systemic and mucosal antibody responses to LT-B in young and aged mice, and recall responses to oral administration and injection of LT-B in aged mice. Specific IgA and IgG antibodies were detectable over an 11-month period even though the concentration of antigen specific antibodies declined gradually. Booster by feeding or injection dramatically increased the concentration of specific IgA over that seen in young mice. Specific IgG levels were boosted to concentrations similar to those in young mice. This effect may be age-dependent and related to prior immunization exposure. Analysis of the antibody response of naïve aged mice against corn derived LT-B demonstrated an age related suppression in specific IgG

production, but not specific IgA. These results may provide important information for edible vaccine strategies for young and aged individuals.

2.2 Introduction

Mammals are exposed to a vast majority of infectious agents that enter the body through the mucosal surfaces of the upper respiratory, gastrointestinal and urogenital tracts. To protect the animal from pathogens at this first line of contact, mucosal surfaces are armed with immune structures: mucosal-associated lymphoreticular tissues (MALT) including Peyer's Patches in intestines and nasopharyngeal-associated lymphoreticular tissue (NALT) in the upper respiratory tract. Immune stimulation at these locations induces memory B cells and T cells to the common mucosal immune system (CMIS) resulting in increased levels of antigen-specific secretory IgA both at the inductive site where the pathogen is encountered first and in the effector sites throughout the CMIS (McGhee et al, 1998).

The ability of the mucosal system to produce antigen-specific responses is the driving force for attempts to develop edible vaccines by which antigenic proteins are synthesized in edible parts of plants and administered orally to animals. Many studies show that recombinant proteins produced in plants are immunogenic when given orally and also provide protection in animal models of infectious disease (Wang et al, 2004). Phase 1 human clinical trials using three different recombinant antigens produced in plants show similar results as evidenced by the induction of a systemic and secretory antibody response (Tacket et al, 1998, Tacket et al, 2000, Kapusta et al, 1999).

While oral vaccines may stimulate a mucosal response, the extreme low pH and presence of gastric peptidases in the stomach present a challenge for the use of small proteins

as oral vaccines when administered alone. Co-administration of adjuvants can increase immunogenicity of mucosally administered antigens (Fujihashi et al, 2000, Kanellos et al, 2000, Choi et al, 2002). Two such mucosal adjuvants, heat labile enterotoxin (LT) from enterotoxinogenic strains of *Escherchia coli* and its homologue cholera toxin (CT) from *Vibrio cholera,* are strongly immunogenic when administered via the intestinal tract, respiratory tract, or parenteral route (Elson and Ealding, 1984, Clements et al, 1998, Xu-Amano et al, 1993). Both toxins cause diarrhea by a mechanism involving binding of the B-subunit of the toxin to the G_{M1} receptors on intestinal epithelial cells. Binding of the B-subunit enables entry of the enzymatically active A-subunit to the cytosol. This results in secretion of water and chloride ions from epithelial cells to the intestinal lumen due to over activation of adenyl cyclase (Haan et al, 1996). Because neither of the holotoxins is suitable for use as a mucosal adjuvant in humans, much effort has focused on the utilization of the nontoxic B subunit (LT-B and CT-B) and attempts to dissociate toxicity from adjuvanticity [Millar et al, 2001, Pizza et al, 1994, Verweij et al, 1998). Besides potential use as mucosal adjuvants, the two B-subunits can also serve as vaccines against diarrhea caused by LT and CT as the B-subunit induces high levels of anti-LT-B and anti-CT-B secretory IgA that provide protection against subsequent toxin challenge (Haan et al, 1996, Verweij et al, 1998, Mason et al, 1998, Chikwamba et al, 2002, Guidry et al, 1997). In addition, our previous studies indicate that oral administration of LT-B corn can induce significant concentrations of anti-LT-B IgG in serum (Chikwamba et al, 2002).

Immunogenicity and adjuvanticity of LT-B and CT-B have been studied extensively in young mice. However, immune responses in aged individuals can be different due to cellular and molecular alterations associated with aging. Examples of age-related changes

include: dysfunctional alterations in mitogenic responses, phenotype, and cytokine expression of T cells. Changes in T-cell responses can result in an altered mode of help for B cells, ultimately leading to impaired antibody responses (Fujihashi et al, 2000). For example, vaccines against influenza and tuberculosis are less efficient in aged individuals when compared to young adults (Mayer, 2000, Czerkinsky et al, 1999). Aged mice that were given CT and CT-B as an adjuvant for the co-administered antigen ovalbumin demonstrate a diminished capacity to produce antigen specific antibodies mucosally (IgA) and systemically (IgG) when compared to young mice (Fujihashi et al, 2000, Nagler-Anderson et al, 1986). However, immunogenicity and adjuvanticity of LT and its components in aged mice remains to be elucidated. Even though LT-B and CT-B share 80% homology in terms of amino-acid sequence, they have distinct biochemical and immunological features. For example, LT-B is more potent than CT-B in terms of induction of an antibody response. This could be due to the greater stability of LT-B at low pH and high temperature (Nedrud and Sigmund, 1991). Therefore administration of LT-B to aged mice could result in a different outcome than that of CT-B and CT.

Earlier studies of recombinant LT-B and CT-B produced from transgenic plants report antibody responses with efficacy in protecting animals from toxin challenge. However, these studies measured antibody responses over a short time interval in young mice (Chikwamba et al, 2002, Brennan et al, 1999, Tuboly et al, 2000, Yu and Langridge, 2001, Lauterslager et al, 2001, Yu and Langridge, 2003). In the present work, we aimed at investigating immune responses to corn derived LT-B in young and aged mice. Our objectives were to carry out a kinetic assay to determine the time interval in which anti-LT-B antibody levels remain significantly elevated after feeding in young mice and to investigate

the effect of booster administration of LT-B using both oral and intraperitoneal injection methods in aged mice. In addition, we measured the systemic and mucosal immune response against LT-B in elderly naïve mice when fed with corn-derived LT-B antigen.

2. 3 Materials and Methods

Preparation of feeding pellets

Transgenic maize seeds (R_4 generation) expressing LT-B were used for making food pellets as described previously (Chikwamba et al, 2002). Each pellet made of ground transgenic maize seeds and phosphate buffered saline (PBS) weighed 1.87 grams and contained 10 μg of LT-B. Pellets were allowed to air-dry overnight. Non-transgenic maize seed powder was used to make pellets of similar weight to serve as a negative control. For each feeding, one extra pellet was prepared to serve as an LT-B dose control to be analyzed at the end of the experiment.

Immunization of mice-young mice study, aged mice study, recall study

All animal procedures were approved through the Iowa State University (ISU) Committee on Animal Care. Ten-week-old female BALB/c mice were obtained from Harlan (Indianapolis, IN, USA). Prior to start of the experiment, mice were allowed a two-week adjustment period with a reverse light-dark cycle (lights-off at 9AM) in the ISU animal facility where they were housed throughout the experiment.

The design of the overall study is given in Figure 1. Mice were fed a basic diet of mouse chow with water *ad libitum*. The mouse chow was removed overnight (during the light phase) prior to feeding corn pellets on days 0, 7, 21 and 35 of the study. Initial LT-B immunization was done when the mice were three months old (young mice study). They

were divided into two groups: the initial control group mice were fed with non-transgenic corn pellets (NT Corn) while the LT-B corn group mice were fed with LT-B corn pellets. Throughout the study two mice were housed in each cage. However, during feeding of control or vaccine pellet they were caged individually and one maize pellet was placed in each cage (without bedding) 30 min before lights off. Mice were allowed to eat the maize pellet in the dark cycle for 3 hours until the pellet was completely consumed and then returned to their home cage with their normal mouse chow.

At 14 months of age, 12 mice from the initial LT-B corn group were divided equally into three feeding groups for the recall study: 1) the Recall/Control group was fed with non-transgenic maize pellets, 2) the LT-B Corn group was fed with transgenic maize pellets containing 10 μg LT-B and 3) the Recall/Injection group was also fed with non-transgenic maize pellets and was injected intraperitoneally (IP) with 10 μg of purified bacterial LT-B (kindly provided by John Clements, Tulane University, LA, USA) in 100 μl of PBS (phosphate buffered saline). All the mice in this study, which did not have LT-B injections, were injected with sterile PBS to ensure consistency of handling for each subject in the experiment.

In the aged mice study, 6 mice (aged 14 months) not previously immunized with LT-B were assigned to two groups (N=3/group), a negative control group and an LT-B corn group. Food pellets from LT-B corn or NT-corn were administered on days 0, 7, 21 and 35 of the study.

Preparation of plasma and fecal samples and determination of anti-CT-B and anti-LT-B antibodies

For the initial LT-B immunization study, fecal and blood samples (approximately 100 µl) were obtained from mice over an 11-month period (days –1, 6, 13, 20, 27, 34, 41, 51, 63, 94, 270 and 320). In the recall study, samples were collected prior to feeding (day 336) and on days 343 and 350 (6 and 13 days post feeding) of the study. For analysis of LT-B immunization in naïve 14-month-old mice, fecal and blood samples were collected weekly from day –1 to day 41. Mice were bled via the sephanous vein. Plasma and fecal samples were stored at -20°C until analysis by ELISA as described before (Chikwamba et al, 2002). Briefly, plates were coated with 5 µg of mixed gangliosides (G2375, Sigma, St. Louis, MO, USA) per well diluted in coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3 (pH:9.6)] for an hour at room temperature. Following blocking with 5% powdered milk (diluted in PBS), plates were incubated with pure LT-B (John Clements) or CT-B (1 µg/well; Sigma) at 37°C for one hour. Next, diluted serum samples and fecal extracts were added and the plates were incubated at 37°C for one hour. Plates were incubated with biotinylated anti-mouse IgG obtained from rabbit sera (B-8520, Sigma, diluted 1:100,000 in PBS containing 1% powdered milk) and anti-IgA from goat sera (B-2766, Sigma, diluted 1:20,000 in PBS containing 1% powdered milk) for one hour at 37°C to determine IgG and IgA levels, respectively. Next, the enzyme conjugate, streptavidin-horse radish peroxidase (554066, BD Biosciences, San Diego, CA, USA), was added to the wells (diluted 1:1000 in PBS containing 1% powdered milk) and the plates were incubated at room temperature for 30 minutes. Finally, wells were coated with ABTS substrate (3-ethylbenzthiazoline-6-sulfonic acid, A-1888, Sigma) in substrate buffer [1.37 M ABTS, 0.1 M citric acid (pH: 4.35)] and

incubated at room temperature for 30 minutes allowing color development. At the end of the incubation period end-point readings were performed using absorbance at 405 nm. Antibody concentrations were determined as μg/ml by using standard curves derived from pure mouse IgG (MOPC 21, M-9269, Sigma) and pure mouse IgA (MOPC315, M-2046, Sigma).

Due to lack of commercially available LT-B, concentrations of anti-LT-B IgG and anti-LT-B IgA in serum were measured by a sandwich ELISA that utilizes CT-B to capture antibodies induced in mice after LT-B corn administration. Fecal anti-LT-B IgA levels were measured by the same ELISA protocol except bacterial LT-B was used to capture anti-LT-B IgA in fecal extracts. We measured specific IgG and specific IgA antibodies in sera of 4 mice from LT-B corn group obtained on days 51, 62 and 94 of initial immunization by using both LT-B and CT-B in the ELISA protocol described above. We obtained the ratios indicated in Table 1. Our analysis indicated that anti-LT-B antibodies cross react with CT-B, although cross reactivity is not 100%. The results indicate that actual IgG anti-LT-B antibody concentrations will be higher than what we report in Figure 1, Figure 2 c and d, and Figure 3a and c due to the use of CT-B for antibody capture.

Determination of total IgG1 and IgG2a levels

For determination of total IgG1 and IgG2a, sera from days -1, 6, 13, 20 and 27 from the initial immunization part of the study and sera from days 6, 13, 20, 27 and 34 from aged mice study were analyzed. A total of 11 serum samples from mice fed with non-transgenic (NT) corn and 15 serum samples from mice fed with LT-B corn in initial immunization part of the study were analyzed along with serum samples from the aged mice study (3 mice in each group; NT corn & LT-B corn). Purified IgG1 (MI10-102, Bethyl Laboratories, Inc.) and IgG2a (0103-01, Southern Biotechnology Associates, Inc) were used as standards. The same

standard range was used for both total IgG1 and IgG2a assays (2.5-0.019 μg/ml). Standards and samples (diluted at 1:150) in sodium carbonate coating buffer (described in previous section) were directly coated on the 96 well high binding ELISA plates and were blocked with 150 μl of 5% milk. Total IgG1 and IgG2a samples were probed with goat anti-mouse IgG1 (M-8770, Sigma- dilution 1/3,000) and biotin conjugated goat anti-mouse IgG2a (1080-08, Southern Biotechnology Associates, Inc – dilution 1/10,000), respectively. For IgG1 assay a secondary antibody conjugated to biotin (A-5420, Sigma-dilution 1/40,000) was used. Incubations with enzyme and substrate and end-point readings were carried out as described in the previous section.

A ratio of [IgG2a]/[IgG1] was used to estimate changes in TH1/TH2 responses. Although, IgG2a/IgG1 ratio is only indicative of the T-helper balance, the isotypes have been correlated with the production of TH1 and TH2 cytokines, respectively [28]. Earlier experiments attempting to stimulate T-cell cytokine production in splenocytes from LT-B immunized mice using purified recombinant bacterial LT-B were inconclusive due to the abundance of endotoxin associated with the LT-B. Thus, we are unable to measure LT-B induced stimulation of antigen specific T-cells and their cytokines at this time, which would provide a direct measure of TH1 vs TH2 responses.

Statistical analysis

Anti-LT-B antibody kinetic data for serum IgG, serum IgA and fecal IgA were analyzed by ANOVA for estimation of mean differences and IgG2a/IgG1 ratio were analyzed by ANOVA to determine main effects of age, pellet type, and the interaction of age*pellet using SAS® V8.2.

2.4 Results

Antibody response in mice upon initial immunization and a booster LT-B administration:

Twelve young mice (3-month-old) were initially fed four times with LT-B expressing transgenic corn pellets (LT-B Corn group) and six mice were initially fed four times with non-transgenic corn pellets (Initial/Control group). When they reached 14 months of age, the twelve mice from the LT-B Corn group were divided equally into three groups to test a booster response. The three groups included: 1) Recall/Control group (fed once with non-transgenic corn pellets), 2) LT-B Corn group (fed with transgenic corn pellets) and 3) LT-B Injection group [fed with non-transgenic corn pellets but injected with purified bacterial LT-B intraperitoneally (IP)]. Figure 2 shows antigen specific IgG concentrations over the 11-month period following the initial immunization regime on days 0, 7, 21 and 35 and the secondary response after a single recall dose of LT-B administered when the mice were 14 months old (day 337). On day 13, specific IgG antibody concentrations were detected at a level significantly higher than that of the control group ($p=0.0034$) and remained significantly higher throughout the remaining assay dates (p's < 0.005). Specific IgG concentrations steadily increased and reached a peak at day 27 with a mean concentration of 226.21±26.71 μg/ml. Specific IgG concentrations decreased gradually after the last immunization (day 35), but did not significantly differ from day 27 until day 270 ($p=0.0037$).

In the recall experiment, 14-month-old mice were given one single dose of LT-B orally (LT-B Corn group) or intraperitoneally (LT-B Injection group) on day 336. We observed remarkably high levels of specific IgG in sera of both groups as early as 7 days (day 343) after the booster antigen administration, while the recall control group showed no change in levels of specific IgG. The recall responses on day 343, one week after a single

dose of LT-B by feeding and injection, were not significantly different from the specific IgG level on day 41. These two dates (day 41 and 343) both represent secondary responses one week after immunization and were significantly higher than specific antibody concentrations observed on days 6 and 13 in young mice (p's <0.0001). Two weeks after the day 336 booster dose, specific IgG concentrations increased with a higher level of antibody in sera of the group that received LT-B by injection. The specific IgG levels in the LT-B Injection group on day 350 were significantly higher than that of the LT-B Corn fed group ($p<0.0014$).

Figure 3 shows concentrations of anti-LT-B specific IgA measured in fecal material (Fig 3 A & B) and serum (Fig 3 C& D) of mice fed or injected with LT-B. As shown in Figure 3A, concentration of LT-B specific secretory IgA in feces increased gradually following the first immunization on day 1. The highest concentration was recorded on days 27 (1.07±0.07 μg/g) and 41 (0.93±0.16 μg/g) one week post feedings. We observed a relatively lower concentration of specific fecal IgA on days 34 and 41 compared to peak level observed on day 27, approximately two weeks post feeding. However, antigen specific fecal IgA remained significantly higher than control through day 62, one month post feeding.

Figure 3B shows the fecal anti-LT-B IgA response after booster antigen administration when the mice were 14 months old. Fecal anti-LT-B secretory IgA was not detectable before the administration of the booster dose. As in the case of antigen specific serum IgG response to recall, a significant increase is observed as early as 7 days following the administration of the antigen through either route (oral or injection). It is noteworthy that in 14-month old mice the specific fecal IgA response to the booster on day 343, 6 days after recall immunization, was more robust than that of specific IgG. The levels of specific IgA were 2.66 and 3.53 times higher (2.84±0.41 and 3.77±0.68 μg/g for recall feeding and recall

injection groups, respectively) than the peak level (1.07±0.07 μg/g) of specific IgA during the initial immunization and represent a significant difference in response ($p<0.0001$). Specific IgG concentrations of the recall response did not exceed responses seen in animals during the initial immunization period. In the recall experiment, the specific fecal IgA concentrations continued to increase for both groups through day 350 (2 weeks post recall exposure), but the LT-B injection group had significantly higher specific fecal IgA compared to the LT-B corn fed group ($p<0.0001$).

During the initial immunization phase, antigen specific serum IgA was first detected on day 13 and reached a peak on day 27 (Figure 3C). As with fecal IgA, we observed a significant decrease on day 34 compared to day 27 ($p<0.0001$) two weeks after a feeding and a large increase on day 41 compared to day 34 ($p<0.0001$) one week after a feeding. Specific serum IgA antibodies declined gradually, but were detectable over an 11-month period. In the recall experiment, a robust specific serum IgA response was observed upon booster dose administration in 14 month old mice. The aged mice had a more rapid and stronger specific serum IgA response as compared to young mice (Figure 3D). In addition, the concentration of specific serum IgA in the LT-B Corn fed group remained constant from day 343 to day 350, while the level of the LT-B Injection group decreased significantly ($p<0.0001$) compared to day 343. This differs from the result seen with the antigen specific fecal IgA response in the recall mice (compare Figs 3B and 3D).

Antibody response in naïve aged mice upon oral LT-B administration:

Two groups (n=3/group) of aged mice (14-month-old), which were never exposed to LT-B when younger, were used in a separate feeding study to elucidate the immune response to corn derived LT-B in aged mice. One of the groups was fed with LT-B corn pellets, each

containing approximately 10 μg of LT-B on days 0, 7, 21 and 35, while the other group was given non-transgenic corn pellets on the same days. Figure 4 displays the results of feeding young and aged mice with LT-B containing corn. We observed a significant effect of age on the antigen specific IgG concentrations ($p<0.0001$). As indicated in Figure 4A, specific serum IgG antibodies were first observed on day 13, however the concentration of specific IgG antibodies in aged mice were significantly lower than in sera of young mice on day 13 ($p<0.001$) and remained significantly lower throughout days 20 to 41 ($p's < 0.05$).

Contrary to the delayed antigen specific IgG response in the aged LT-B fed group; a very rapid antigen specific secretory IgA response was observed as early as day 6 in the fecal extracts of the aged mice in the LT-B treatment group (Figure 4B). The specific fecal IgA concentrations were significantly higher on days 13, 20, 27 and 41 in both young and aged mouse groups compared to pre-feeding ($p's<0.05$). In the overall analysis, we observed no significant age effect for anti-LT-B fecal IgA levels in young vs aged mice. However, day 41 anti-LT-B fecal IgA was significantly higher for young vs aged mice ($p<0.04$).

Figure 4C shows the antigen specific serum IgA concentrations in young vs aged mice during an initial immunization regimen. Serum IgA was measurable in aged mice as early as day 6 following the first feeding and remained relatively constant over 42 days. Peak antigen specific IgA levels in sera of aged mice (4.1 ± 0.02 μg/ml) were comparable to the peak anti-LT-B concentration recorded on day 27 in young mice (4.63 ± 1.76 μg/ml). However, we observed a significant overall age effect for serum IgA levels in young vs aged mice ($p<0.001$) as anti-LT-B IgA antibody concentrations in sera of aged mice were significantly higher than that measured in sera of young mice ($p's<0.05$) on days 6, 13, 20 and 34.

Both in fecal extracts and in sera collected from the mice in this part of the study, anti-LT-B IgA antibodies were observed as early as one week following the initial LT-B corn administration. This rapid specific IgA response led us to preliminarily investigate changes in Th1 and Th2 responses in these mice as a possible explanation for these results. Using the serum samples collected in this experiment we measured total IgG2a (a Th1 antibody) and IgG1 (a Th2 antibody) concentrations. We were not able to measure antigen specific forms of the antibody, due to lack of sufficient serum. Figure 5 demonstrates the ratio of total IgG2a and IgG1 in young and old mice. The IgG2a/IgG1 ratio was significantly lower in sera of LT-B fed mice ($p < 0.01$). The main effect of LT-B feeding was due to the large decrease in IgG2a/IgG1 ratio seen in the aged mice ($p<0.05$) after immunization. A comparison of young mice fed NT corn and LT-B corn did not demonstrate a significant shift in total IgG2a/IgG1 ratio.

2.5 Discussion

In this study, we monitored antigen specific IgG and IgA concentrations in sera and fecal samples of mice immunized with corn-derived LT-B over 11 months. The peak antibody production was reached after a minimum of 3 feedings over 4 weeks in young mice. This is similar to data published previously by ourselves and others using either recombinant bacterial LT-B (Clements, 1990) or plant derived LT-B (Wang et al, 2004). We previously demonstrated that the specific antibodies can provide protection from challenge with whole *E. coli* heat labile toxin (Chikwamba et al, 2002). The present study expanded our previous research to show specific antibody remained elevated over background for an 11-month period following the initial immunization paradigm.

We observed a kinetic difference in the recall response for anti-LT-B IgG and IgA. A rapid and stronger antibody response is a characteristic of immune memory. Upon booster immunization in aged (14-month) mice, our results indicated a rapid recall response for both feeding and injection of LT-B in measurements of antigen specific serum IgG, serum IgA and fecal IgA. While the recall response of specific IgG levels (day 343 & 350) was not greater than that of initial peak response at week 4, the fecal and serum IgA responses were quite robust and 2-5 times higher than initial peak responses in young animals. This IgA response is important in protection against mucosal pathogens and toxins. It should be noted that our robust IgA response was seen subsequent to both recall immunization using feeding and injection of LT-B.

Others have reported a shift in the type of immune response in aged animals (Wakikawa et al, 1997, Robinowich et al, 1985, Kurashima et al, 1995,). They have noted that aged animals respond with more of a Th2 type immune response and have a decreased Th1 response in comparison to young mice. This shift may help explain our robust IgA (Th2 type) response in aged mice. Th1 cells support production of serum IgG2a while Th2 cells induce production of IgG1 serum antibodies (Kaplan et al, 2002). We measured total IgG2a versus IgG1 in the serum samples from immunized young and aged mice as an indicator of the Th1:Th2 balance. Immunization at the mucosal surface favors a Th2 type of response (Hiroi et al, 1995). Thus, it is not surprising that our analysis indicated that IgG2a/IgG1 ratio of the LT-B corn treatment group is significantly lower than that of non-transgenic control group. The suppression of the IgG2a/IgG1 ratio was significant in the immunized aged mice, but not in the immunized young mice. This result is indicative of a shift towards a Th2 type

response in the aged mice which could account for the rapid production and high levels of antigen specific IgA antibodies.

In addition to a shift from Th1 to Th2 responsiveness, there have been reports of a general decline in immune responses to antigen in the elderly (Schmuker et al, 1997, Koha et al, 2000). In this study we also compared the initial immune response against corn-derived LT-B in young and aged naïve mice. Aged mice immunized with LT-B for the first time demonstrated a delayed and lower peak anti-LT-B IgG production through 4 feedings and 7 weeks of monitoring as compared to young mice. In contrast to the delayed IgG response, the aged mice demonstrated maximal specific IgA production by day 6 after the first feeding. This is much sooner than the IgA response detected in young mice fed LT-B. One other difference between the initial antigen specific IgA response of young and aged mice was that the response of young mice appears to increase one week after each feeding and decrease at 2 weeks after each feeding. This corresponds well with the half life of IgA (6 days in serum). In aged mice there was no fluctuation of antigen specific IgA concentrations seen in serum or in fecal samples in relation to feeding. These differences may be due in part to age related changes in immune regulation.

Although the concentrations of specific IgA in serum of young and elderly mice were comparable, the fecal IgA levels in aged mice did not reach the same concentration observed in young mice on day 41. This observation is not surprising as other researchers have noted increased serum IgA in elderly people (Arranz et al, 1992, Beharka et al, 2001). Schmuker et al. (1997) suggests that the detection of higher serum immunoglobulin levels in the elderly could be due to altered epithelial cell transport to mucosal surfaces.

To date, a significant amount of evidence indicates that edible plant-made antigens

can be a part of clinical practices in the foreseeable future. Corn and its products are a major food source for humans and animals. Moreover, production of edible vaccines in corn is highly desirable in terms of safety, cost efficiency and high yield of transgenic protein. This study demonstrates that corn derived LT-B can elicit high levels of systemic and secretory antibody levels which persists for over 11 months. Re-administration of the antigen does not lead to immune tolerance, but instead boosts the immune response significantly indicating induction of a strong memory cell component. However, immunization of aged mice with LT-B for the first time does not result in a robust mucosal IgA or serum IgG response, indicating that use of LT-B as an adjuvant in aged animals may not be as beneficial as in young animals. These findings may be useful for determining edible vaccine strategies for both young and aged animals.

2.6 Acknowledgements

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Bleed days	Anti-LT-B IgG /Anti-CT-B IgG	Anti-LT-B IgA /Anti-CT-B IgA
Day 51	2.9	6.6
Day 62	2.7	12.7
Day 94	2.8	5.6
Average	**2.8±0.1**	**8.3±2.2**

Table 1. Specific antibody ratios. Sera from LT-B corn group analyzed for anti-LT-B and anti-CT-B antibodies on the indicated days.

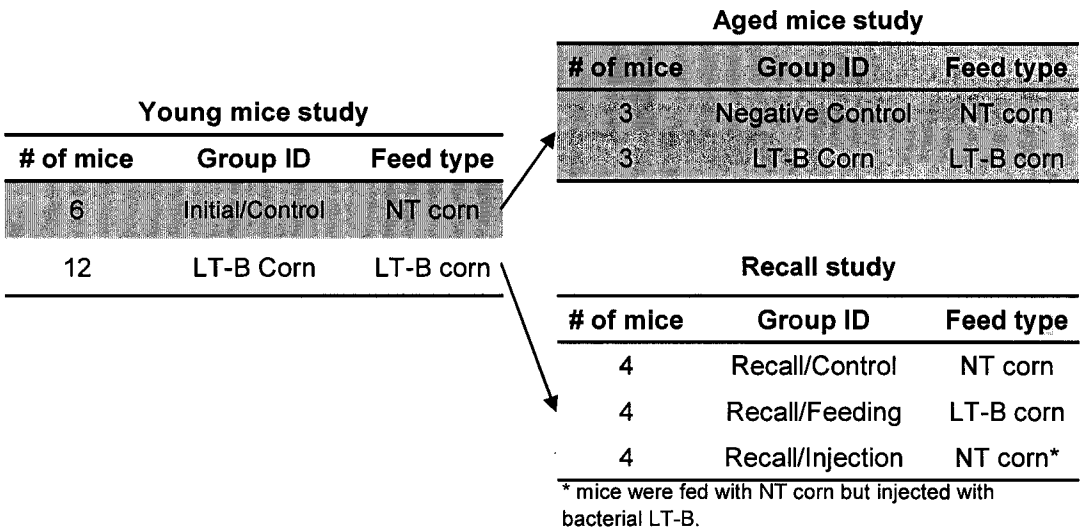

Figure 1. Study design for mouse immunizations.

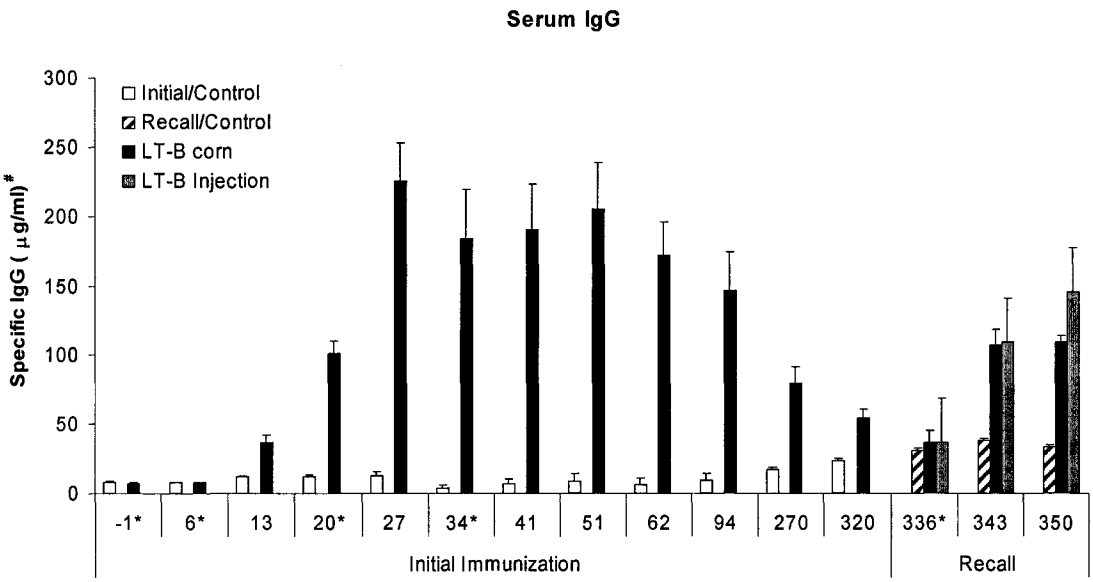

Figure 2. Antigen specific serum IgG concentrations in mice orally immunized with corn derived LT-B and recall to re-immunization through oral and IP administration of the toxin. The X axis are days of initial immunization and recall study. Initial immunization regime involved feeding on days 0, 7, 21 and 35. A booster dose was administrated once (day 337) and antibody response was monitored for two weeks. * indicates the blood collection day immediately prior to a feeding or injection. #Specific IgG measured with bacterial CT-B. Antibody concentrations are presented as mean±SE.

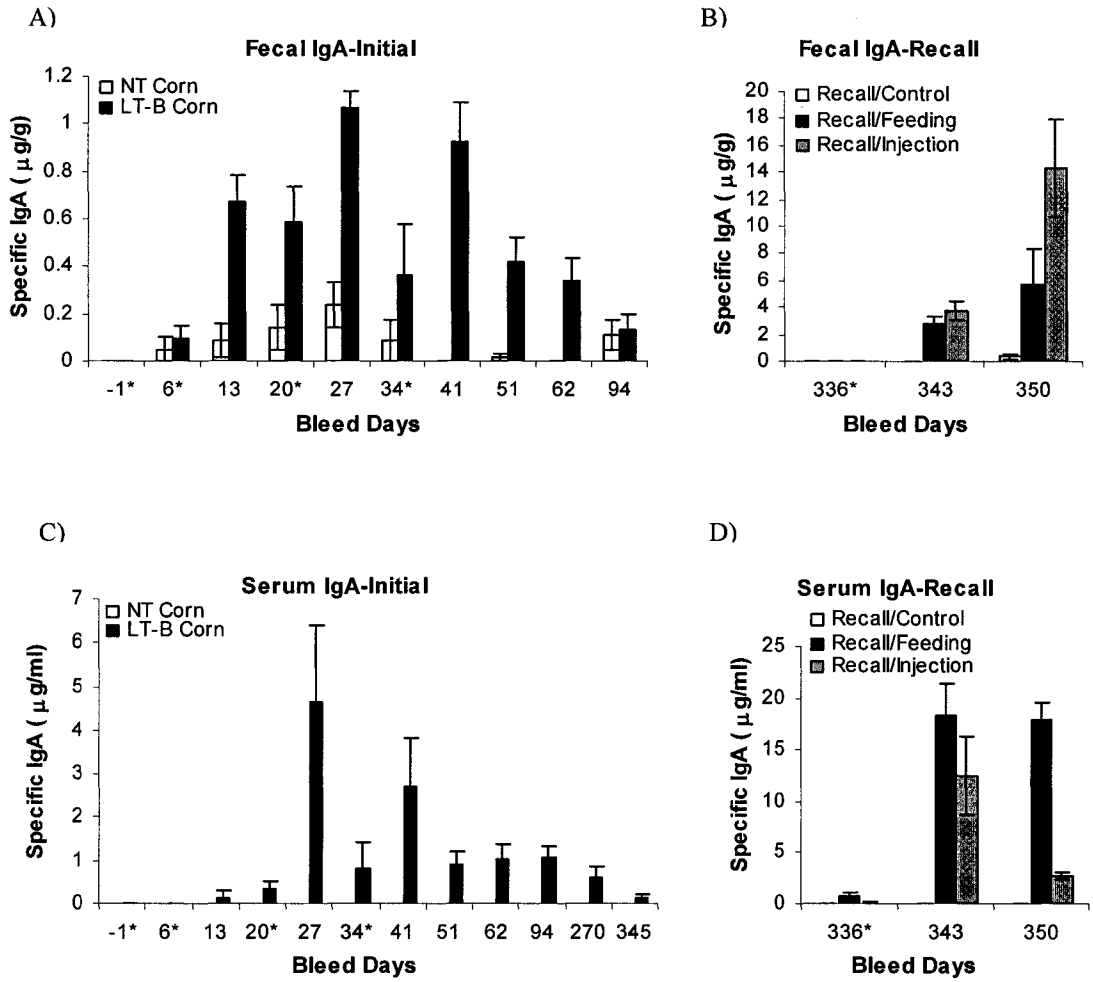

Figure 3. LT-B specific fecal IgA (A & B) and serum IgA (C& D) concentrations for initial immunization in young mice (A & C) and recall in aged mice to re-immunization (B & D). In initial immunization study, mice were fed with meal pellets from non-transgenic corn (NT Corn) and transgenic corn expressing LT-B (LT-B Corn) on days 0, 7, 21 and 35 when mice were three-months old. Mice were given a booster dose of the toxin through oral route and through injection when they were 14 months old (day 337). * indicates sample collection day immediately prior to a feeding and injection. # Specific fecal IgA measured with bacterial LT-B (A&B), specific serum IgA measured with bacterial CT-B (C&D). Antibody concentrations are presented as mean±SE.

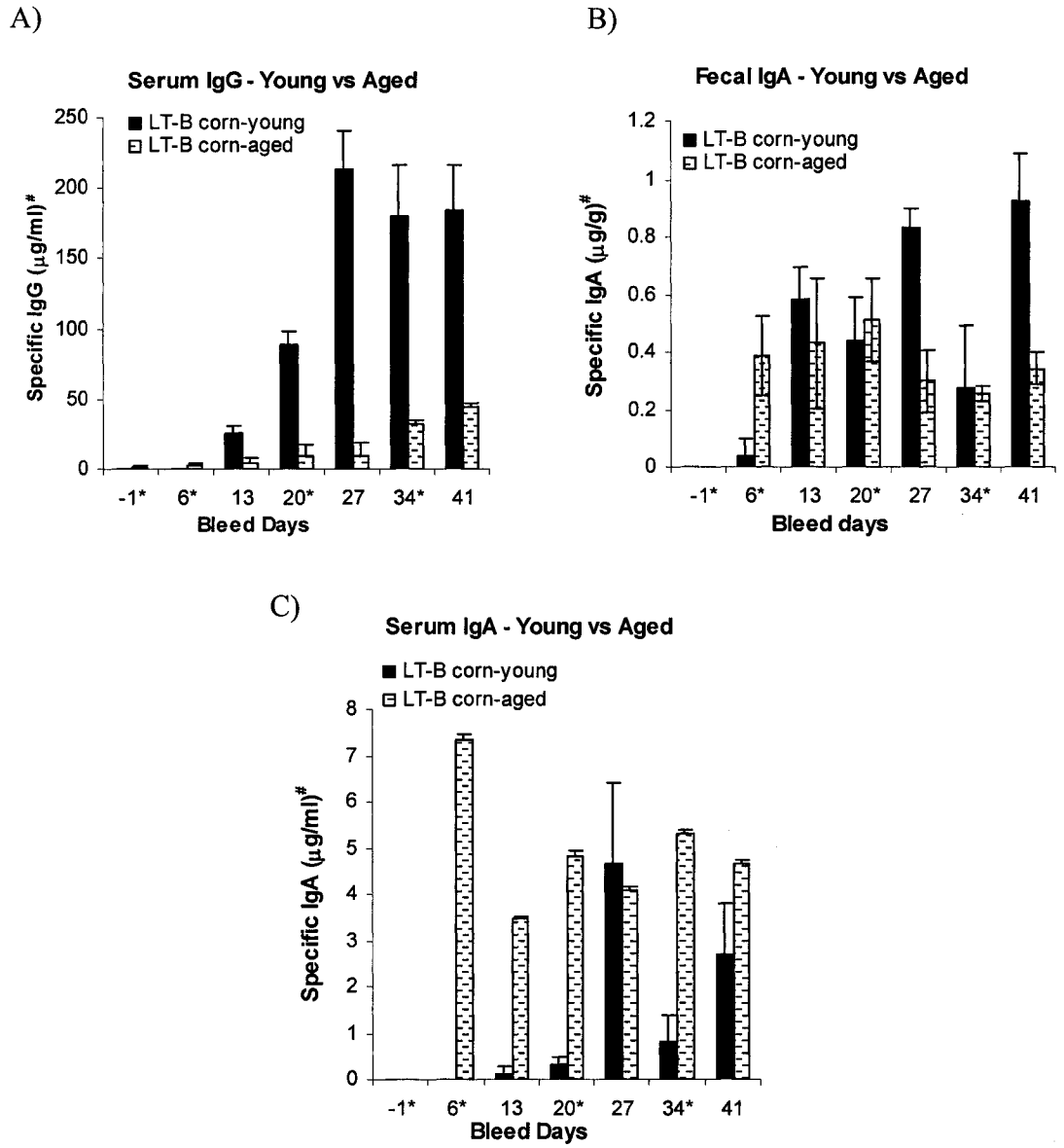

Figure 4. Antibody concentrations of initial oral immunization in aged mice and young mice. Young mice (LT-B corn-young) and aged mice (LT-B corn-aged) were fed with corn meal pellets from transgenic corn on days 0, 7, 21 and 35. (A) Concentration of specific serum IgG, (B) concentration of specific fecal IgA and (C) concentration of specific serum IgA. #Specific serum IgG and serum IgA (A&C) measured with bacterial CT-B, specific fecal IgA (B) measured with bacterial LT-B. Antibody concentrations are presented as mean±SE.

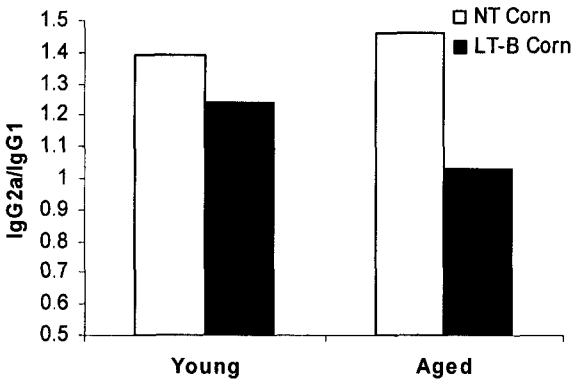

Figure 5. Comparison of IgG2a/IgG1 ratios in young and aged mice.

Chapter 3: Expression of B sub-unit of cholera toxin (CT-B) in corn seeds and evaluation of its immunogenicity *in vivo*

Manuscript in preparation

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3.1 Abstract

The Non-toxic B subunit (CT-B) of cholera toxin from *Vibrio cholerae* is a strong immunogen and amplifies the immune reaction to co-administered or conjugated antigens. Plants are attractive vectors for recombinant protein production. In this work, a synthetic gene encoding for CT-B was expressed under control of a γ-zein promoter in transgenic corn seeds. Levels of CT-B in corn plants were determined via ganglioside dependent ELISA. The highest expression level recorded in R1 generation seeds was 0.0014% of total aqueous soluble protein (TASP). Expression level of the same event in the R2 generation was significantly increased to 0.0197% of TASP. Using Western analysis, we found that, unlike corn-derived LT-B (Chikwamba et al., 2003), the bacterial signal peptide of CT-B was not cleaved in corn seed tissue. Immunogenicity of corn derived CT-B was evaluated by immunizing mice through feeding. Mice immunized with CT-B corn (5 or 10 μg/g per dose) have elevated anti-CT-B specific antibody levels in their sera on day 27 after three feedings. When mice were administrated with an equal amount (5 μg/g each) of mixed corn-derived CT-B and LT-B (Chikwamba et al, 2002), the specific antibody levels were moderately enhanced compared to either the same or a higher amount of CT-B alone. The mice were

partially protected against holotoxin challenge with either CT or LT. These results suggest that a synergistic action may be achieved using a CT-B and LT-B mixture that may lead to a more efficacious combined vaccine against both cholera and diarrhea induced by enterotoxigenic strains of *Escherichia coli.*

3.2 Introduction

Expression of antigens in plant systems for oral vaccine production has been studied extensively. A number of antigens have been produced in plant systems and a few of them are in Phase I and Phase II clinical trials (Ma et al, 2005). Antigens produced in plants can be purified from plant tissue; however there are a number of advantages using plant tissue as delivery vehicle. Seeds for example provide a dry and stable environment for long term storage. As specialized organs for protein storage, seeds also provide increased capacity for producing high amounts of the antigen in a smal unit. Plants are innately free of animal pathogens. Therefore utilization of plants for sub-unit vaccine production is cost efficient and safer than conventional alternatives.

In this work, we expressed the B subunit of cholera toxin (CT) from *Vibrio cholerae* in corn endosperm. CT is highly homologous to heat labile enterotoxin (LT) from *Escherichia coli.* Both of these toxins are composed of pentameric non-toxic B subunit and toxic A subunit. The action of toxic the A subunit leads to watery diarrhea via a mechanism as follows: the toxic A subunit is linked to the B subunits and is cleaved into A1 and A2 fragments by exogenous proteases. The A2 fragment is linked to the B subunits whereas A1 fragment possesses cytotoxic activity. The B subunits binds to G_{M1} (galactosyl-N-acetylgalactosamyl-syalil-galactosylglucosyl ceramide) receptors on intestinal epithelial

cells. This binding aids the toxic A1 fragment to be internalized into the cytosol. In the cytosol A1 catalyzes ADP-ribosylation of GTP binding and hydrolyzing receptors ($G_{s\alpha}$), activating adenylate cyclase which increases cyclic AMP (adenosine mono phosphate) levels. Elevated cyclic AMP levels leads to phosphorylation of protein kinase A which in turn leads to opening of Cl^- channels. Movement of Cl^- is followed by movement of water to lumen (Williams et al, 1999).

The biologically active form of the B subunits of both CT (CT-B) and LT (LT-B) are pentameric. Although the B subunits are non-covalently linked, they are highly stable due to their structural properties. There is high number of salt bridges between adjacent monomers and two or three anti-parallel β sheet structures interacting with neighboring monomers. Therefore, B subunits are highly resistant to trypsin and proteinase K digestion and low pH (Ruddock et al, 1995). Hence, oral immunization with CT-B and LT-B results in strong humoral and mucosal immune response (Rigano et al, 2003).

CT-B has been shown to be a mucosal adjuvant and a good carrier for targeting unrelated antigens to mucosal immune system (Luci et al, 2006). It has also been shown to be an effective carrier for the antigens that are chemically or genetically linked (Dertzbough and Elson, 1993). It was recently reported that intravaginal (ivag) administration of bacterial CT-B chemically conjugated to ovalbumin (OVA) helps to induce OVA specific $CD4^+$ T cells. In that study neither OVA alone nor OVA co-administered with CT-B resulted in OVA specific $CD4^+$ cells (Luci et al, 2006). In fact, CT-B is used as an adjuvant in an oral cholera vaccine (Dukoral®, SBL Vaccin Sweden - used for human use), the basic component of which is attenuated cholera bacteria (Holmgren et al, 2003). Interestingly, some CT-B conjugates were reported to be highly tolerogenic (a substance which invokes immune non-

responsiveness) when administered orally (Holmgren and Czerkinsky, 2005). This function of CT-B has been successfully exploited to carry out proof of concept studies for treatment of human autoimmune diseases. For example, oral administration of the B chain of insulin conjugated to CT-B led to oral tolerance to the B chain of insulin. This in turn resulted in a lower dose of antigen and administration rate in mice models of diabetes for suppression of the disease (Sadeghi et al, 2002). Similar success was attained against type II collagen-induced arthritis (Tarkowski et al, 1999).

The two internationally used oral cholera vaccines Mutacol® (Berna, Switzerland) and Dukarol® (SBL Vaccin, Sweden) are made of live attenuated cultures of *V. cholerae* and killed whole cell *V. cholerae* in combination with the CT-B subunit, respectively. The presence of CT-B in Dukarol® also provides some immunity to *E. coli* infections causing diarrhea. However, they pose a risk by using the whole cells and they only provide short term immunity (National Advisory committee on Immunization, 2005). They are also expensive. Dukarol® is licensed in Canada and a dose costs 75 Canadian dollars (78 US$). Therefore, there is still need for effective and cheap vaccine formulations for treating cholera.

We have expressed a recombinant CT-B gene (optimized for corn expression system) in endosperm of corn kernels to provide a cheaper source for cholera vaccine formulations. We monitored the transgene expression level for two generations. We tested in vivo immunogenicity of corn derived CT-B in mice with an oral feeding trial. In addition to test anti-CT-B antibody levels of mice fed with corn derived CT-B, we also evaluated the specific antibody levels of the mice that were fed with a mixed CT-B and LT-B. This is to investigate whether the combination of these two B-subunits can elicit higher levels of

specific antibodies than either B-subunit alone, thus providing a better protection against both cholera and traveler's diarrhea.

3.3 Materials and Methods

Plasmid Construction

The gene segment encoding for CT-B (PubMed Accession #: U25679) optimized for corn expression system was synthesized and cloned into the plasmid pUC57 (GenBank/EMBL Accession #: Y14837) at *XmaI* site by Genescript Corp. gene synthesis company (New Jersey, USA). The resultant plasmid was named as pUC57-CTB (Figure 1A). CT-B gene was then cloned under 2x35S CaMV promoter (Figure 1C) and an endosperm specific γ-zein promoter (Figure 1E). For constructing the 35S promoter construct, the CT-B gene was isolated from pUC57 with *NcoI* and *KpnI* restriction digestions and inserted into the *NcoI* and *KpnI* sites (replacing the sLT-B gene) in the plasmid pTH210 (Figure 1B). The newly constructed plasmid was named as pCTB-BS (Figure 1C). For constructing the γ-zein promoter construct, the *NcoI* and *EcoRI* fragment carrying the sCT-B and VSP terminator was inserted into the plasmid pRC4 (Figure 1D) to replace the sLT-B and VSP fragment. This plasmid was given the name pCTB-BSG (Figure 1E). Both pTH210 (Mason et al., 1998) and pRC4 (Chikwamba et al, 2002) were previously used for production of transgenic LT-B corn plants (Chikwamba et al, 2002).

Maize transformation

Embryogenic maize Hi II callus was transformed using microprojectile bombardment as described by Frame et al. (2000). The plasmids pCTB-BS and pCTB-BSG were each co-

transformed with the selectable marker gene construct, pBAR184 (Frame et al, 2000). pBAR184 confers resistance to the herbicide bialaphos by expression of the bar gene. Transgenic calli and plants obtained via transformation with pCTB-BS and pCTB-BSG were designated as P247 and P248, respectively. Bialaphos resistant calli obtained from pCTB-BS transformations (P247 lines) were analyzed by ELISA for CT-B expression and Southern blot for integration of the CT-B gene. Clones obtained from pCTB-BSG transformations (P248 lines) were analyzed by polymerase chain reaction. CT-B expressing P247 lines and PCR positive P248 lines were regenerated and raised to maturity in the greenhouse. B73 and/or Hi II inbred lines were used as pollen donor for obtaining R1 generation seeds. DNA obtained from leaves of P248 plants were analyzed by Southern blot. Seeds obtained from this line were analyzed by ELISA and Western blot for expression of CT-B gene in corn endosperm.

PCR analysis

The presence of the cassette including the CT-B gene segment in corn genome was verified with PCR analysis conducted on a 100 ng of total genomic DNA obtained from transgenic callus (P248). DNA isolation from calli was performed as following: 0.1 to 0.25 g of bialaphos resistant callus was transferred to 1.5 ml sterile micro-centrifuge tubes. The calli were homogenized by autoclaved pestles (Kontes Pellet Pestle, K749520-0000, Fisher Scientific, PA, USA) until juice exudes. Four hundreds micro liters of extraction buffer [200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA] was added to the tube. The tissue and buffer were mixed gently by inverting the tube a few times. An equal amount of saturated phenol was added to each sample and mixed gently in a similar fashion. The mixtures were centrifuged for 10 minutes at 12,000 rpm at room temperature. The aqueous phase was

transferred to new micro-centrifuge tubes and 350 µl of chloroform: iso-amyl alcohol mix (24:1 v:v) was added to each tube and mixed gently. This was followed by centrifugation for 10 minutes at 12,000 rpm at room temperature. The aqueous phase was transferred to a new tube and 300 µl of isopropanol was added. DNA was allowed to precipitate for 30 minutes. Next, DNA was pelleted by centrifugation for 10 minutes at 12,000 rpm at room temperature. Isopropanol was discarded and pellets were washed with 500 µl of 70% ethanol by centrifuging at the same speed for 5 minutes. The pellets were air dried for 15-30 minutes and re-suspended in autoclaved water. Three sets of primers were used to verify the presence of promoter-leader-CT-B-terminator region in genomic DNA. The primer sets used are as follows: 1) F1: 5'-gct-atg-acc-atg-att-acg-cca-agc-3' and R1: 5'-cat-tct-gta-ctt-ctg-cgt-ggc-tca-3', 2) F2: 5'-tga-gcc-acg-cag-aag-tac-aga-atg-3' and R2: 5'-ggg-tct-tat-tgt-tcc-aca-cgc-ag-3', 3) F3: 5'-aag-acc-cca-cac-gct-att-gct-g-3' and R3: 5'-gaa-ttc-gct-tca-aga-cgt-gct-ca-3'. These primers amplify 925 bp, 738 bp and 629 bp fragments respectively. The relative positions of sequences on pCTB-BSG aligning to these primers are indicated in Figure 1E. The reaction mixtures and conditions were as follows: Total volume of 25 µl containing 100 ng of maize callus genomic DNA, 1 µl of dNTP mix (containing 10 mM of each dNTP), 1 µl of each primer (2.5 µM stock), 1 µl of $MgCl_2$ (50 mM), 2.5 µl of 10X Taq polymerase PCR buffer and 0.2 µl of Taq polymerase (Biolase USA Inc., City, NJ, USA). PCR was initiated at 95°C for 4 minutes. This was followed by 30 cycles of amplification steps of which are; denaturation at 95°C for 1 minute; annealing at 62°C for 40 seconds, and extension at 72°C for 1 minute.

Southern blot analysis

Sothern blot analysis to estimate copy numbers of the CT-B gene in P247 and P248 events was carried out on DNA extracted from leaf tissue of R1 young plants. Approximately 0.2 g of leaf tissue was ground in liquid nitrogen with mortars and pestles. The powder was transferred into a 15 ml test tube (Fisher Scientific Cat # 232917028) and re-suspended in 2.5 ml of 2x cetyltrimethylammonium bromide (CTAB) buffer [100 mM Tris-HCL, pH 8.00, 1.4 M NaCl, 20 mM EDTA pH 8.00, 2% w/v CTAB]. Following incubation at 65°C for 30 minutes, an equal amount of chloroform was added into tubes and mixed well for 5 minutes at room temperature. The samples were centrifuged at 8,000 rpm in a Beckman J-12 rotor at room temperature for 20 minutes. The aqueous phase was transferred to new 15 ml tube and 5 ml of 100% ethanol was added. Samples were mixed gently to allow genomic DNA to precipitate. The precipitate was hooked out with pipette tips and transferred to 1.5 ml micro-centrifuge tubes which containing 1 ml of wash buffer [76% ethanol, 0.2 M sodium acetate pH 5.2]. Pellets were spun down at 14,000 rpm for 10 minutes at room temperature. The supernatant was discarded and pellets were washed with 70% ethanol and spun for 5 minutes at the same speed. The pellets were then air dried for 7 minutes and re-dissolved in 50 µl of TE 10/0.1 buffer containing 2 µl of 10mg/ml RNase A (R-4642, Sigma, St Louis, MO, USA) at 56°C for 10 minutes. Re-dissolved DNA samples were kept in 4°C overnight to ensure complete re-suspension. Ten micrograms of genomic DNA obtained from selected P247 and P248 events was digested with the restriction enzyme *NcoI* at 37°C overnight and resolved on 0.8% agarose gel. The 950 bp fragment containing synthetic CT-B gene and VSP terminator was excised from pCTB-BS plasmid with *NcoI* and *EcoRI* restriction enzymes and

used as probe following labeling with ^{32}P (Figure 1C). DNA blot was carried out following standard procedures (Sambrook et al, 1989).

Protein Extraction from corn seeds

For CT-B expression analysis of R1 P248 seeds, 15-20 mg of endosperm tissue from 20 seeds of each event was collected with a drill (DREMEL, Mutipro-225 T2) as described by Sangtong et al (2001). The powder was transferred to a 1.5 ml tube. For ELISA extraction buffer [25 mM sodium phosphate, pH:6.6, 100 mM sodium chloride, 1 mM EDTA, 0.1% Triton X-100, Leupeptin (10µg/ml), 0.25 mM Pefabloc SC] was added at a ratio of 10µl/mg of powder. The samples were incubated at 37°C on a shaker incubator for two hours. Highest expressers among R1 seeds were planted to obtain high expressing R2 seeds. For Western analysis, ground whole seeds from R2 p248 plants were used. Ground seed mixtures were suspended in extraction buffer [200 mM Tris-HCl, (pH 8.0), 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-Mercaptoethanol, 0.05% Tween-20] at the rate of 10 µl buffer per milligram of powder. The powder was shaken for one hour on a vortex shaker at room temperature and further soaked in refrigerator overnight. Before loading to protein gels, samples were centrifuged on a bench-top micro-centrifuge at maximum speed for 15 minutes to remove cellular debris. The amount of total soluble proteins (TASP) were determined by the Bradford Assay (Bradford, 1976) with the BioRad (Hercules, CA, USA) protein dye concentrate using a standard curve derived from bovine serum albumin (BSA).

Quantification of CT-B expression in corn calli and seeds

CT-B expression in corn calli, endosperm and ground whole kernels were determined by ganglioside dependent ELISA (Mason et al, 1998, Chikwamba et al, 2002). Anti-sera and

CT-B standard were obtained from Sigma chemical company. Each of the ELISA steps were carried out at 37°C and 50 µl volumes were used unless otherwise specified. Wells were washed three times between steps with 400 µl of phosphate buffered saline Tween-20 [PBST; 0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, (pH 7.2), 0.1 M NaCl, 0.05% Tween-20 (v/v)].

High binding microtiter plates (Costar 3590, Fisher Scientific, PA, USA) were coated with Type III mixed bovine gangliosides (G2375, Sigma, St Louis, MO, USA), dissolved in sodium carbonate coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, (pH9.6)] at room temperature for one hour. The wells were blocked with 150 µl of 5% dry milk (w/v, DM, DIFCO, Becton Dickinson, MD, USA) dissolved in phosphate buffered saline to prevent nonspecific binding to the plate. Next protein extracts and CT-B standards were loaded to the wells and incubated for 1 hour. Anti-CT antibodies raised in rabbit (C-3062, Sigma, St Louis, MO, USA) and biotinylated anti-rabbit goat IgG was used as primary and detection antibodies, respectively. Both antibodies were diluted 1/20,000 in phosphate buffered saline. After detection antibody, wells were loaded with streptavidin horse radish peroxidase diluted 1/1,000 (BD Biosciences, 554066, MD, USA). The color development was obtained by addition of ABTS substrate (3-ethylbenzthiazoline-6-sulfonic acid, Sigma A-1888, MO, USA) prepared according to manufacturer's instructions. End point readings were measured spectrophotometrically at 405 nm in a PowerWave XS plate reader (BT-MQX200R, Bio-Tek). All samples were measured in duplicates and non-transgenic maize protein extracts were used as negative controls. Calculations of CT-B concentrations were carried out using a standard curve of purified bacterial CT-B (C-9903, Sigma, St Louis, MO, USA).

Western Blot

Ground whole seeds from selected P248 ears (R2 generation) and the meal that was used for making CT-B corn containing pellets were analyzed by Western blot for detection of CT-B pentamers and monomers. Approximately 70 mg of total protein extracted from ground material were separated by SDS-PAGE (Laemmli, 1970). The resolved proteins were transferred to 0.45 μm nitrocellulose blotting membrane (BioRad) using a BioRad Transblot apparatus following manufacturer's instructions. After blocking with 5% milk, membranes were incubated at room temperature on a shaker for 1 h in primary antibody raised against CT (rabbit-anti-CT, Sigma C-3062) diluted 1/3,000 in 1% milk. This was followed by incubation in alkaline phosphatase conjugated secondary antibody diluted at the ratio of 1/5,000 in 1% milk (Sigma A-9919) in a similar manner for 1 h. Detection was performed following procedures of Blake et al, (1984).

Preparation of feeding pellets and immunizations

Among the R2 generation plants, P248 event number 4 was found to be expressing CT-B at high levels. Seeds obtained from four individual P248 R2 ears (P248-4-8-4, P248-4-8-5, P248-4-8-8 and P248-4-8-18) were ground in a coffee grinder (Braun, Type 4041: Model KSM2) and a homogenous mix was prepared. The CT-B concentration in the mixed mill was 8.2 μg/g of ground powder.

Five feed categories were used in this feeding trial. The feeding groups used were referred to as CTB-5, CTB-10, LTB-5, CTB5-LTB5 and WT. Mice in CTB-5 and CTB-10 groups received feed pellets containing 5μg and 10μg of corn derived CT-B, respectively. Mice in LTB-5 group were fed with feed pellets containing 5 μg of corn derived LT-B (Chikwamba et al, 2002). Mice in CTB5-LTB5 group received feed pellets containing 5 μg

of corn derived CT-B and 5μg of corn derived LT-B. Mice in WT group were fed with feed pellets made of non-transformed corn and served as a negative control group. A fixed amount of powder (Table 1) was weighed for obtaining the required amounts of CT-B and LT-B for feeding groups and mixed with ground non-transformed corn kernel when necessary to have the same size of pellet for each treatment (1.22 g). The powder was then mixed with sterile distilled water and the dough was shaped into a cylinder by passing it through a 3 ml disposable syringe barrel with the Leur tip cut off. Non-transformed corn powder was used for feeding pellets to be fed to mice in the negative control group.

Four-week-old female BALB/c mice were obtained from Harlan (Indianapolis, IN, USA). Prior to start of the experiment, mice were allowed a two-week adjustment period with a reverse light-dark cycle (lights-off at 9AM) in the ISU animal facility where they were housed throughout the experiment. All animal procedures were approved through the Iowa State University (ISU) Committee on Animal Care. Eight mice were randomly assigned to each treatment group. Mice were fed a basic diet of mouse chow with water *ad libitum*. Mice were fasted overnight (during the light phase) prior to feeding corn pellets on days 0, 7, 21 and 49 of the study. Throughout the study two mice were housed in each cage. However, during feeding of the corn pellet they were caged individually and one maize pellet was placed on the lid of each cage (without bedding) 30 min before lights off. Mice were allowed to eat the maize pellet in the dark cycle for 5 hours until the pellet was completely consumed and then returned to their home cage with their normal mouse chow.

Preparation of serum samples

Approximately 100 μl of blood was collected weekly starting the day before first immunization over 8 weeks. Blood samples were collected from sephanous vein except for

the day of necropsy. On day 55 mice were euthanized blood was collected from their hearts via cardiac puncture. Sera were isolated from blood samples immediately after collection by centrifugation at 14,000 for 15 minutes and kept at -20°C until analyzed. Anti-LT-B and anti-CT-B antibodies were measured with ELISA as described below. Dilutions ranged from 1/60 to 1/400.

Quantification of anti-CT-B and anti-LT-B antibodies

Quantification of anti-CT-B and anti-LT-B was conducted as described in Chapter 2 with a minor modification. Twenty five ng of pure LT-B (John Clements) and CT-B (Sigma) was loaded in each well instead of 1 μg. This modification was determined based on comparison of different amounts of LT-B and CT-B with sera of known antibody levels (data not shown).

Patent Mouse Assay

To assess protection from toxin challenge a patent mouse assay was undertaken (Guidry et al, 1997). Mice in each group were divided into three groups and subjects were randomly assigned to toxin challenge treatments. Three of them were challenged with 30 μg of LT (John Clements), 3 of them were challenged with 30 μg of CT (Sigma, C3012) and 2 of them were gavaged with an equal volume of (200 μl) water. Toxins were administered in 200 μl of phosphate buffered saline via gavaging. The mice were fasted overnight *ad libitum* prior to gavage administration of toxin. Three and half hours after toxin administration mice were euthanized and their carcasses were weighed. Following dissection, guts were removed from duedonum to anus and weighed separately. The gut/carcass ratios (Richardson et al., 1984) were calculated to determine the amount of water influx into the gut.

Statistical Analysis

Data were analyzed by Analytical Software Statistix® 8 (Tallahassee, FL). ANOVA and repeated measures analysis of variance tests were used for analysis of gut:carcass ratios and IgG levels, respectively.

3.4 Results

Analysis of transgenic corn lines carrying CT-B gene under a constitutive promoter

The CT-B coding sequence was synthesized by GenScript DNA Synthesis Company with a codon bias for optimizing expression in corn and cloned under enhanced CaMV35S promoter and a TEV leader sequence (Figure 1C). This construct was co-transformed into maize embryogenic callus along with plasmid pBAR184 carrying the bar gene. Transgenic events obtained were designated as P247 (Figure 1). Twenty one out of 75 putative transformants analyzed were expressing CT-B at varying levels (0.00092-0.009% of total aqueous soluble proteins). CT-B levels were determined by ganglioside dependent ELISA (Haq et al., 1995; Chikwamba et al., 2002). Because only the pentameric form of CT-B can bind to gangliosides, the ganglioside dependent ELISA values are reflective of their biological active quantities. The level of CT-B expression was also analyzed in young leaf tissues of putative P247 transformant lines (R0). As can be seen in Table 2, five of eleven lines showed enhanced CT-B expression (16.6 – 57.5 fold increase) in leaf materials compared to calli tissues. Three of eleven had marginal increase (1.2 – 3.6 fold increase) and another three lines showed no or decreased expression in leaves. These results indicate that

the transgenic CT-B protein was assembled into a pentameric and potentially biological active conformation in both corn callus and leaf tissues.

Southern blot analysis was used to characterize 13 P247 lines. Genomic DNA was extracted from leaves of R0 plants and digested with restriction enzyme NcoI that cuts at 5' end of CT-B gene. ^{32}P-labeled 950 bp fragment carrying CT-B and VSP terminator (Figure 1C) was used as probe. Our results indicated integration of CT-B into the corn genome (Figure 2). Event specific integration was observed. Copy numbers ranged from 1 to 7, which is typical with biolistic gun-mediated transformation (Christou, 1996).

Analysis of transgenic corn lines carrying CT-B gene under a seed specific promoter

After validating functionality of synthetic CT-B gene by constitutive expression in corn calli and leaf tissue, a plasmid (pCTB-BSG) was constructed by cloning CT-B gene under endosperm specific γ-zein promoter (Figure 1E). Transgenic events transformed with pCTB-BSG along with pBAR184 (carrying a selectable marker gene) were referred as P248. The putative transformant (herbicide resistant) calli were screened with PCR for the presence of CT-B gene. Seven out of nine bialaphos resistant events were shown to carry the intact CT-B gene. R0 plants were regenerated from these events and Southern analysis was carried out on DNA obtained from young leaves of six lines in a similar fashion to P247 R0 plants. The Southern results are shown in Figure 3. Event specific integration of the CT-B gene was observed. Half of the events had high copy number insertion (> 10 copies).

R0 young plants were grown to maturity to obtain R1 seeds. Event P248-3 failed to produce any seeds. A total of 25 ears representing 6 events were obtained. Selected ears from each event were analyzed for expression of CT-B in endosperms of R1 seeds. Endosperm

tissue was obtained from 20 seeds for each ear by drilling as described Sangtong et al. (2001).

Expression levels of CT-B were determined by ganglioside dependent ELISA. As summarized in Table 3, four out of six lines were expressing CT-B at varying levels. Among 4 plants generated from line 2, only one ear was shown to be expressing CT-B at low levels. The drilled seeds from ears P248-1-1, P248-1-7 and P248-4-8 were germinated and grown to maturity to obtain R2 seeds. The choice of specific lines for further analysis was based on their CT-B expression levels and seed quantity at the time of analysis. A total of 28 R2 ears representing two independent events were obtained. Forty seeds from each R2 ear were ground to obtain a homogenous mix and analyzed by ganglioside dependent ELISA. One of two lines (P248-1) has stable transgene expression and the other line (P248-2) has 14 folds increase of CT-B in R2 generation (Table 3). The enhanced transgene expression in some events in advanced generations was observed previously with LT-B expression levels in corn seeds (Chikwamba et al, 2002).

Western analysis was also performed in protein extracts obtained from R2 seeds of two events (P248-1 and P248-4). Figure 4A shows the results with non-boiled protein samples while 4B shows the boiled sample results. The bacterial pentameric CT-B has an approximate size of 55 kDa while monomers have approximate size of 11.6 kDa (Zhang et al, 1995). Non-boiled bacterial CT-B used as control (Lane bCTB, Figure 4A) showed multiple bands, suggesting that the commercial bacterial CT-B may contain a mixture of CT-B multimer conformations. This phenomenon was observed by other groups (Arakawa et al, 1997, Jani et al, 2002, Jani et al, 2004). The second strongest band closest to 56.7 kDa marker was considered as the pentamer (indicated by an open arrow). As shown in Figure

4A, three ears of P248-4 and R2-Mix have a band (indicated by a black arrow) cross reacted to CT-B antibody. This is due to lower level of expression in this event. The Lane "R2-Mix" represents the ground corn meal mixture of P248-4 used for mice feeding. No bands were observed in lanes loaded with extracts from P248-1 ears. Interestingly, this band is approximately 20 kDa heavier than that of the bacterial CT-B standard. This result was also reported in recombinant CT-B produced in potatoes (Arakawa et al. 1997), tobacco (Jani et al, 2004) and tomato (Jani et al, 2002). It was suggested that plant tissues were unable to cleave the bacterial signal peptide of CT-B. This was also confirmed by a parallel experiment using the boiled protein samples (Figure 4B). Corn-derived monomeric CT-B moves with an apparent size of 15 kDa while its bacterial counterpart moves with 11 kDa apparent size.

In addition to non cleaved CT-B protein in non-boiled samples, we also observe bands representing CT-B monomers (Figure 4A, Lane R2mix), suggesting instability of CT-B in corn tissues.

Evaluation of immune response of mice fed with corn-derived CT-B and LT-B

It has previously been shown that corn-derived LT-B can elicit anti-LT-B specific antibody in mice orally administered 10 μg/g of corn pellets (Chikwamba et al., 2002). It has also been shown that CT-B produced in potato (Arakawa et al, 1998) and tobacco (Jani et al, 2004) can elicit anti-CT-B specific antibody in animals. Therefore, it is expected that corn-derived CT-B should be able to stimulate anti-CT-B antibody in mice fed with CT-B corn. In addition to evaluating the immunogenecity of CT-B corn in mice, we are also interested in investigating the potential adjuvencity or synergistic effects when CT-B and LT-B are combined.

Mice were assigned to five treatment groups and were administered feed pellets either made of transgenic corn or non-transformed corn (Table 1). There were eight mice in each treatment group. Treatment groups were as follows: The CTB-5 and CTB-10 groups were fed with pellets containing 5 or 10 μg of corn-derived CT-B, the LTB-5 group was given pellets containing 5 μg of corn-derived LT-B, and the CTB5-LTB5 group was administered pellets containing 5 μg of corn-derived CT-B and 5 μg of corn-derived LT-B. The NT group was administered pellets made of non-transformed corn and served as negative control. The data are presented as mean±SE (standard error) in μg/ml (Figure 5).

As shown in Figure 5, anti-CT-B and anti-LT-B antibodies in sera of all groups were measured. For both analyses there was significant feed effect (p values < 0.05). Anti-CT-B antibody levels observed in sera of all treatment groups were lower than anti-LT-B levels observed in sera of LTB-5 and CTB5-LTB5 groups (Figure 5). While anti-LT-B antibodies cross react with CT-B (Figure 5A), anti-CT-B antibodies were found to not cross-react with LT-B (Figure 5B). Starting from day 13, anti-CT-B levels in sera of LTB-5 group (111.58±6.6 μg/ml, Figure 5A) were significantly different than that of WT group (37.29±3.13 μg/ml) throughout the study (p's <0.05). However, as it can be seen in Figure 5B, anti-LT-B antibody levels measured in sera of mice received 5 and 10 μg of corn-derived CT-B were not different than that of WT group at any bleed day (p values for these comparisons >0.05).

Anti-CT-B antibodies in sera of mice in CTB-5 and CTB-10 groups (Figure 5A) were moderately different than that of WT group starting from day 27 ($p=0.0241$) until day 55 ($p=0.02$). Anti-CT-B antibody levels induced by 5 and 10 μg of corn-derived CT-B were not different until day 55. On day 55 anti-CT-B antibodies detected in sera of CTB-10 group

(179.73±25.75) was marginally higher than that of CTB-5 group (110.08±22.26, p=0.04). Anti-CT-B antibody levels in sera of LTB-5 group were found to be significantly higher than that of CTB-10 group on days 13 (p=0.0001) and 20 (p=0.0001). However, on days 27 and 34 the differences were not significant (p=0.84, p=0.6, respectively). Interestingly, anti-CT-B antibody levels in sera of the LTB-5 group exceeded that of CTB-10 group and were moderately larger on days 41 and 55 (p=0.07, p=0.04, respectively). On days 34, 41 and 55 anti-CT-B antibody levels in sera of CTB5-LTB5 group were significantly higher than that of LTB-5 group (p's=0.0061, 0.0008, and 0.001, respectively). On days 34 and 41, anti-CT-B antibody levels in sera of CTB5-LTB5 group were moderately higher than that of CTB-10 group (p=0.02 significant and p=0.076 marginal, respectively).

Analysis of anti-LT-B IgG levels indicated significant treatment effect throughout the study. However, anti-LT-B antibody levels in sera of CTB-5 and CTB-10 groups were not significantly different than that of WT group on any bleed day (p values > 0.05). On the other hand, anti-LT-B levels in sera of LTB-5 and CTB5-LTB5 groups were significantly higher than that of other three groups (p values >0.05) starting from day 13 until the day of necropsy (day 55). Anti-LT-B antibody levels in sera of LTB-5 and CTB5-LTB5 were not significantly different on any bleed days (p values>0.05).

Responses of immunized mice to CT and LT toxin challenges

In order to evaluate whether immunization with corn derived CT-B (at two different doses) and combined treatment (along with corn derived LT-B) provides protection against challenge with CT or LT we conducted the patent mouse assay (Guidry et al, 1997). These toxins cause fluid flow from the body to the lumen of the bowel (Williams et al, 1999). The secreted fluid can not be re-absorbed (Mason et al, 1998) thus accumulates in the gut. This

results in the weight of gut to increase relative to the weight of the body. Therefore the gut: carcass ratios serve as a measure of response toward toxin challenge. If immunizations provide protection we would expect similar gut:carcass ratios with unchallenged control mice that were given water in place of toxin. Larger gut:carcass ratios indicate diarrhea therefore no protection of the treatments.

On day 55 we divided the feeding groups into three sub-groups. Three mice in each feeding group received 30 μg of CT while three different mice received 30 μg of LT. Two mice in each group received water as a negative control. Three and half hours after toxin challenge, mice were euthenized, weighed and dissected. The guts were removed and the gut:carcass ratios were calculated. The results are displayed in Figure 6. Analysis of variance indicated significant toxin effect ($p=0.0001$). However, the feed x toxin interaction for overall data set was not significant ($p=0.28$).

Toxin challenge of the group which received non-transformed corn (WT) throughout the study indicated significant effect of toxins (p values of CT-30 versus water and LT-30 versus water were 0.0322 and 0.0175, respectively). Significant protection against CT was observed in the group which was immunized with 5 μg of corn derived CT-B (group CTB-5, p value for comparison of CT in this group versus CT in WT group is 0.0009). When LT challenge in CTB-5 group is compared to all water treatments a moderate protection was recorded ($p=0.0584$). Interestingly when mice in CTB-10 group were challenged with CT and LT there was no protection (p values for comparisons with water treatment are 0.0006 and 0.0035, respectively). Similarly mice challenged with CT and LT in LTB-5 group were also not protected when compared to water treatment (p values for these comparisons are 0.0004 and 0.0044, respectively). Mice in CTB5-LTB5 group, when challenged with CT

(compared to water treatment p value is 0.005) were not protected however mice challenged with LT in this group were moderately protected (compared to water treatment p value is 0.532).

3.5 Discussion

We have expressed a synthetic gene encoding for B subunit of cholera toxin (CT-B) from *Vibrio cholerae* in corn seeds. We have also showed its G_{M1} binding capacity via ganglioside dependent ELISA and its immunogenicity through a feeding trial using mice. We have detected significant levels of anti-CT-B IgG antibodies in sera of the feeding groups that we utilized. Moreover, we have challenged mice which were fed with transgenic corn with CT and LT toxins and observed moderate protection from toxin challenge in some of the treatment groups.

We have targeted the expression of CT-B to seed endosperms because seeds are tissues which are naturally designated for protein accumulation and storage making them attractive for vaccine expression (Ma et al, 2003). In addition seeds provide a dry stable environment for storing proteins at ambient temperatures. Seeds can also serve as ideal delivery vehicles for plant derived vaccines since they are edible. This would eliminate purification and downstream processing expenses. Expression of CT-B in corn endosperms was achieved by cloning the CT-B gene under control of the seed specific γ-zein promoter which is a strong endogenous corn promoter (Marks et al, 1985). In addition to utilization of a strong promoter, the synthetic CT-B gene used in this study was optimized for corn expression system codon preference (Genescript Corp) to ensure high expression levels.

Seeds obtained from six transgenic lines were analyzed using ganglioside dependent ELISA to determine CT-B expression levels. This analysis has also indicated G_{M1} binding capacity of corn derived CT-B suggesting successful assembly of pentamers. Four out of six lines were found to be expressing CT-B at varying levels. Expression levels in R1 generation ranged from 0.0002 to 0.00128% of total soluble protein. The expression levels within transgenic lines obtained from the same events also varied. The variance among different events might be due to differences in copy numbers of the gene integrated and the position on the genome where the integration occurred. Therefore, integration of the synthetic CT-B gene into the corn genome and copy numbers were also determined by Southern blot analysis. As expected, we observed varying copy numbers in different events. However, the number of events was not enough to make correlations or conclusions regarding copy number and expression level. For example, P248-4 and P248-7 express similar amounts of CT-B in R1 endosperms (μg CTB/g of corn), and their transgene copy numbers were quite different with >10 and 3 copies of CT-B gene, respectively (Figure 3 and Table 3).

Expression level analysis in R2 generation displayed an interesting outcome. R2 seeds obtained from one event contained much higher levels of CT-B compared to their R1 counterparts. A similar trend of increased levels of transgene expression in corn was observed by others. Chikwamba et al (2002) reported increased levels of LT-B expression and Hood et al (1997) reported increased levels of avidin expression in corn seeds in following generations compared to the R1 generation. The reason for this outcome is not known. However, lower performance in R1 generation might be explained by the stress that R0 plants undergo during tissue culture and regeneration.

We had shown via ganglioside dependent ELISA that corn-derived CT-B binds to G_{M1} gangliosides indicating correct folding and pentamer assembly. This suggests that corn derived CT-B is similar to bacterial CT-B in terms of forming pentamers. However our Western blot analysis performed on protein extracts obtained from R2 seeds suggested that bacterial signal peptide may not be cleaved (Figure 4). Our corn-derived CT-B result is in agreement with other plant-derived CT-B work (Arakawa et al, 1997, Jani et al, 2002, Jani et al, 2004) in which uncleaved CT-B products were found. However, this is different from the results obtained with LT-B corn (Chikwamba et al, 2002). Although LT-B and CT-B are highly homologous and the bacterial signal peptide was also used in the case of LT-B corn, bacterial signal was found to be cleaved of the mature LT-B protein in corn tissue. In addition, LT-B corn was found to be localized in starch granules (Chikwamba et al, 2003). We have also detected CT-B in starch isolates (data not shown). It is not clear if the bacterial signal peptide plays a role in localizing LT-B or CT-B in corn tissue. Further experiments are needed to address specific roles of signal peptides of LT-B and CT-B in targeting to sub-cellular organelles in maize endosperm.

We have conducted a feeding experiment with mice to evaluate immunogenicity of corn derived CT-B. In this experiment we also included groups fed with corn-derived LT-B (Chikwamba et al, 2002) to assess potential of mixed administration to lead to enhanced antibody response. We were interested in answering the following questions: 1) are specific antibody levels in CTB5-LTB5 group significantly higher than that of CTB-10 group, 2) are there differences in terms of specific antibody levels in sera of CTB-5 and CTB-10 groups, 3) are anti-LT-B antibody levels in sera of CTB5-LTB5 group different than that of LTB-5

group? All sera obtained from all groups on each bleed day were analyzed for detection of anti-CT-B and anti-LT-B IgG antibodies (Figure 5).

The results were analyzed with repeated measures analysis of variance. Overall analysis of anti-CT-B antibody IgG levels indicated that the treatment effect of CTB5-LTB5 group was different than other treatments. This suggests that in combination corn derived CT-B and corn derived LT-B would work better. We have included two doses of corn-derived CT-B (5 and 10 μg). On none of the bleed days the difference between anti-CT-B IgG antibody levels detected in sera of these two groups were significantly different (p values >0.05). However, on day 55 anti-CT-B IgG levels in sera of the group received 10 μg of corn derived CT-B (179.73±25.8 μg/ml) was moderately higher (p=0.04) than that of the group received 5 μg of corn derived CT-B (110.08±22.3 μg/ml). This suggests that level of anti-CT-B IgG antibodies induced by higher dose (10 μg) of CT-B are primed better although the difference between these two treatments (5 μg vs 10 μg) were not significant in previous bleed days.

Cross reacting anti-CT-B IgG antibodies in sera of LTB-5 and CTB5-LTB5 groups were detectable earlier (day 13) than that of CTB-5 and CTB-10 groups (anti-CT-B IgG antibodies were different than that of wild type as late as day 27). As early as day 13 anti-CT-B IgG levels in sera of CTB5-LTB5 group was higher than that of CTB-5 and LTB-5 alone. On day 27 the antibody levels in all feed groups were similar. However on days 34 and 41 anti-CT-B levels in sera of CTB5-LTB5 were marginally higher than that of CTB-5 and LTB-5. This outcome might suggest that for vaccine formulations which aim prevention of both cholera and ETEC, presence of LT-B in the mix may be of benefit because the induction is faster and anti-LT-B antibodies cross react with CT-B.

Overall analysis of anti-LT-B IgG levels also indicated a significant feed effect. Anti-LT-B antibodies were detectable at high levels as early as day 13 in sera of LTB-5 and CTB5-LTB5 groups. Starting from day 34 anti-LT-B antibody levels in sera of CTB5-LTB5 group appeared to be higher although the differences were not significant. Only on day 34 the difference was marginally significant ($p=0.0854$). Comparison of anti-CT-B IgG antibody and anti-LT-B IgG antibody levels induced by 5 μg of corn derived CT-B and LT-B indicated that antibody induction by CT-B occurs later and the levels are lower. The highest anti-CT-B IgG antibody levels in sera of CTB-5 group was recorded on day 41 with 129.58±22.7 μg/ml whereas the highest anti-LT-B antibody levels in sera of LTB-5 group was observed on day 27 with 640.91±75.1 μg/ml.

Our analysis also indicated that anti-LT-B antibody levels in sera of CTB-5 and CTB-10 groups were not different than that of the WT group throughout the study (Figure 5B). This outcome indicates that anti-CT-B antibodies induced by corn derived CT-B are not cross reacting with LT-B. This finding is different compared to previous reports in which the bacterial CT-B was found to cross react with anti-LT-B antibodies Svennerholm et al, 1983, Lebens et al, 1996). This result also different compared with the observation that protection against ETEC could be achieved when individual was immunized with cholera vaccine Dukarol®. The poor antibody induction and absence of cross reactivity might be explained by signal peptide still being attached to CT-B. Therefore corn-derived CT-B might be behaving differently than bacterial CT-B. Arakawa et al, (1997) indicated that potato derived CT-B might posses signal peptide. When mice were orally immunized with potato derived CT-B, anti-CT-B antibody levels were shown to be significantly lower than these immunized with equal amount of bacterial CT-B (Arakawa et al, 1998). Arakawa et al, (1998) also reported

that 30 μg and 90 μg of potato derived CT-B induced similar levels of anti-CT-B antibodies. These findings may suggest that the presence of a signal peptide might reduce immunogenicity of the CT-B molecule.

We observed mixed results regarding protection from challenge. Mice immunized with 5 μg of corn derived CT-B were moderately protected against both CT and LT challenge. However, we did not record protection against challenge in groups which received 10 μg of corn derived CT-B or 5 μg of corn derived LT-B. Chikwamba et al, (2002) reported protection against challenge when mice were immunized with 10 μg of corn derived LT-B. It is possible that 5 μg of corn derived LT-B is not sufficient to provide protection even though elevated anti-LT-B and anti-CT-B antibodies were observed in these mice. Our observation regarding unexpected responses in CTB-5 and CTB-10 groups might be due to small sample size.

Mice of the CTB-5 and the LTB-5 group were protected against LT however not against CT. Protection against LT was stronger than that observed in CTB-5 group. This suggests that these two subunits work better for providing protection against LT when combined. However the same outcome was not observed with CT.

Enteric diseases cause 1.7-2.5 million deaths each year (Girard et al, 2006). Prevention of enteric diseases is challenging because immunity should be induced at mucosal sites for effective protection (Dougan et al, 2002). Cholera and ETEC are two enteric diseases responsible for high mortality especially in young children in developing countries (Girard et al, 2006) and there is still need for effective vaccines for these diseases. Due to shared mechanism of toxin action, it is desirable and possible to develop combined vaccines which would be effective against both ETEC and cholera. Non-toxic subunits CT-B from

Vibrio cholerae and LT-B from ETEC are strong immunogens and mucosal adjuvants. Therefore their potential as vaccine antigens against cholera and ETEC have been extensively exploited (Lebens et al, 1996, Mason et al, 1998, Chikwamba et al, 2002, Tacket et al, 1998, Tacket et al, 2004). Current ETEC vaccines in development include CT-B in combination with ETEC colonization factors to provide anti-toxic immunity along with antibacterial immunity (Qadri et al, 2003, Savarino et al, 2002). Although CT-B and LT-B share 85% homology (at the amino acid sequence level) in their mature proteins, some neutralizing epitopes in LT-B are missing in CT-B (Lebens et al, 1996). Therefore, utilization of both sub-units in vaccine preparations against both diseases might induce a mixture of antibodies which could neutralize both toxins more effectively.

In addition to being investigated as mucosal adjuvants (Dertzbough and Elson, de Haan et al, 1998, 1993, Bowman and Clements, 2001, Sadeghi et al, 2002, Boyaka et al, 2003, Holmgren et al, 2003), the ability of CT-B and LT-B to bind to intestinal receptors and the carrier function for the A subunit inspired research to utilize them as carriers for unrelated antigens (Kim et al, 2004a, 2004b, 2004c, Kang et al, 2006). Therefore recombinant production of CT-B and LT-B will be important for vaccine and pharmaceutical production in general. Due to high costs involved in current recombinant protein production systems plants are considered to be less expensive and safer alternatives. The subunits could be co-expressed with unrelated antigens in edible parts of plants or as fusion proteins genetically linking them to unrelated antigens could be expressed in plants.

Corn is a major staple feed and food. Recombinant proteins produced in corn have already reached the market. Therefore corn is one of the best crops to be utilized as plant factories for recombinant protein production. Corn can be fed raw. Feed pellets made of

ground raw corn were easily consumed by mice in our feeding experiment. Corn seeds allow high levels of transgene expression which reduces the amount required to be administered through feeding. In our study 1.22 grams of CT-B corn (about 5 corn seeds) provided 10 μg of CT-B.

Our results indicated that recombinant CT-B can be successfully expressed in corn seeds at high levels. We identified in vivo immunogenicity of corn derived CT-B with a mice feeding experiment. We also wanted to address whether corn derived CT-B and corn derived LT-B would induce higher levels on antibodies when given together. Our results were suggestive of this interaction on certain days of sample collection. The bacterial signal for CT-B seemed not being processed in corn tissue according to Western analysis. This suggests that anti-CT-B antibodies induced by corn derived CT-B may be different than that induced by bacterial CT-B. This may also explain lack of cross reactivity between antibodies induced by corn-derived CT-B and LT-B. A new mice feeding experiment including a group with non-transformed corn spiked with bacterial CT-B would help to address this concern. Repeating combined treatments with bacterial CT-B spiked into LT-B corn would also help to deduce more sound results for adjuvant/synergistic actions of CT-B and LT-B*.

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* During mice feeding experiment we have collected fecal samples on each bleed days. On the day of necropsy we collected lung lavages. We are currently analyzing these samples to quantify anti-CT-B and anti-LT-B IgA antibodies. IgA is usually considered to play a major role in protection of mucosa against invading pathogens (Corbeil et al, 2003). Therefore IgA levels would give insight to interpret outcomes observed in response to toxin challenges. When IgA analysis is completed, this chapter will be revised as a manuscript and be submitted for publication.

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3.7 References

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Corn line used	CTB-5	CTB-10	CTB-LTB-5	LTB-5	WT
CT-B-corn (P248)	0.61 g	1.22 g	0.61 g	0 g	0 g
WT-corn (B73)	0.61 g	0 g	0.47 g	1.08 g	1.22 g
LT-B-corn (P77)	0	0 g	0.14 g	0.14 g	0 g
Total (weight of pellet)	1.22 g	1.22 g	1.22 g	1.22 g	1.22 g

Table 1. Amounts of transgenic and non-transgenic (WT) corn used to make feeding pellets for mice feeding experiment.

Event ID	%CTB of TASP in callus	%CTB of TASP in leaves of R0 plants	Fold increase
P247-2	0.0005	0.0266±0.026	51.5
P247-10	0.0107	0.202±0	18.8
P247-11	0.0089	0.0109±0.0009	1.2
P247-12	0.0019	0±0	0.0
P247-13	0.0005	0.0018±0.00045	3.6
P247-25	0.0003	0.0162±0.01	57.5
P247-35	0.0006	0.0311±0.013	52.3
P247-48	0.0033	0.0093±0.00045	2.8
P247-50	0.0021	0.0344±0	16.6
P247-53	0.0012	0±0	0.0
P247-72	0.0009	0±0	0.0

Table 2. Comparison of expression levels of CT-B in calli and young leaves of selected P247 events. Calli expressing CT-B at varying levels were regenerated to obtain R0 plants. CT-B expression levels in leaves of two to four R0 plants for each event were analyzed by ganglioside dependent ELISA. Results are presented as mean±SE. TASP; total aqueous soluble protein.

Event ID	R1 generation		R2 generation	
	%CTB of TASP	µg CTB/g of corn	%CTB of TASP	µg CTB/g of corn
P248-1	0.0007±0.0003	0.02±0.008	0.0002±0.00007	0.04±0.02
P248-2	0.0002±0.00006	0.009±0.003	not followed	not followed
P248-4	0.0014±0.00012	0.05±0.00012	0.0197±0.01	1.56±0.44
P248-5	0	0	not followed	not followed
P248-6	0	0	not followed	not followed
P248-7	0.0007±0.00012	0.045±0.01	not followed	not followed

Table 3. Summary of expression level analysis in R1 and R2 generations. Four out of six transgenic lines producing seeds expressed CT-B in their kernels at varying levels. Expression levels in R1 seeds were determined by quantifying CT-B expression in endosperms of 20 seeds from each ear analyzed. Different number of ears/event was analyzed. Results are presented as mean±SE. CT-B positive seeds from two high expresser lines (1 and 4) were grown to maturity and R2 seeds were obtained from these seeds. R2 expression levels were measured in extracts obtained from whole ground kernel mixes. Averages of CT-B expression levels measured in 9 ears obtained from line 4 and in 11 ears obtained from line 1 are presented as mean±SE. TASP; total aqueous soluble protein.

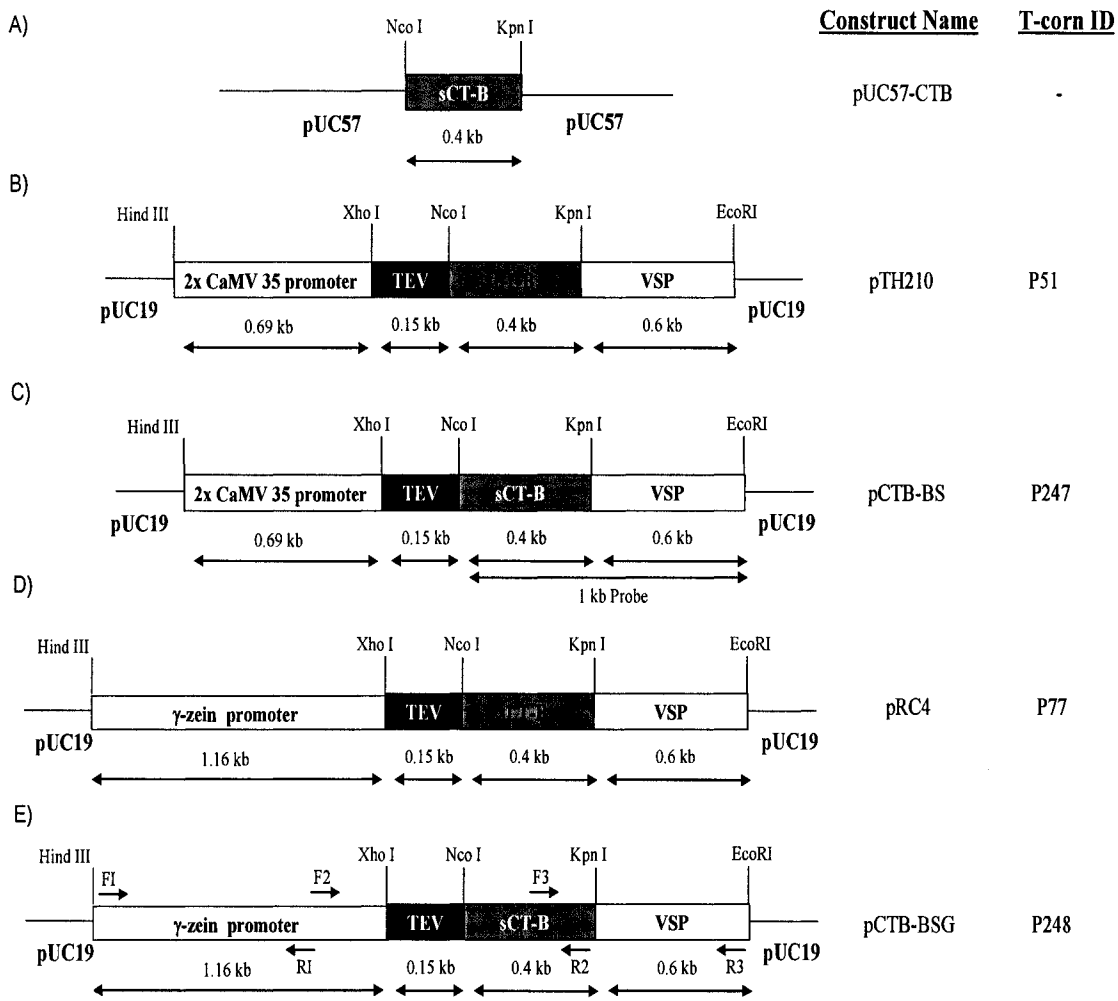

Figure 1. Schematic representation of constructs, their IDs and PTF nominations. Primers used for PCR analysis of P248 events (E) and probe used in Southern analysis (C).

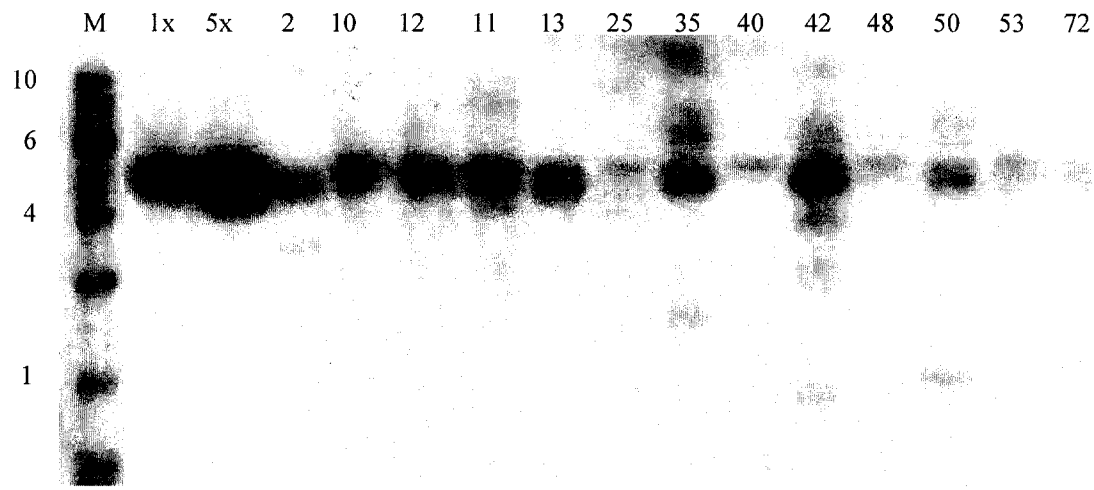

Figure 2. Southern blot analysis of selected P247 transgenic events (2, 10, 12, 11, 13, 25, 35, 40, 42, 48, 50, 53 and 71). Approximately ten micrograms of total leaf genomic DNA obtained from young R0 plants was digested with NcoI, a restriction enzyme with a single cut within at 5' end of the sCT-B gene (Figure 1). DNA samples were resolved in a 0.8% agarose gel overnight at 23 V and blotted onto a Zeta-probe nitrocellulose membrane (BioRad). ^{32}P-labeled 1 kb fragment containing CT-B gene and VSP fragment was used as probe. M indicates molecular weight marker (in kilobases). 1x and 5x indicates 1 and 5 copies of the plasmid pCTB-BS which was used as positive control. Lanes 4 through 16 were loaded with genomic DNA obtained from respective transgenic events.

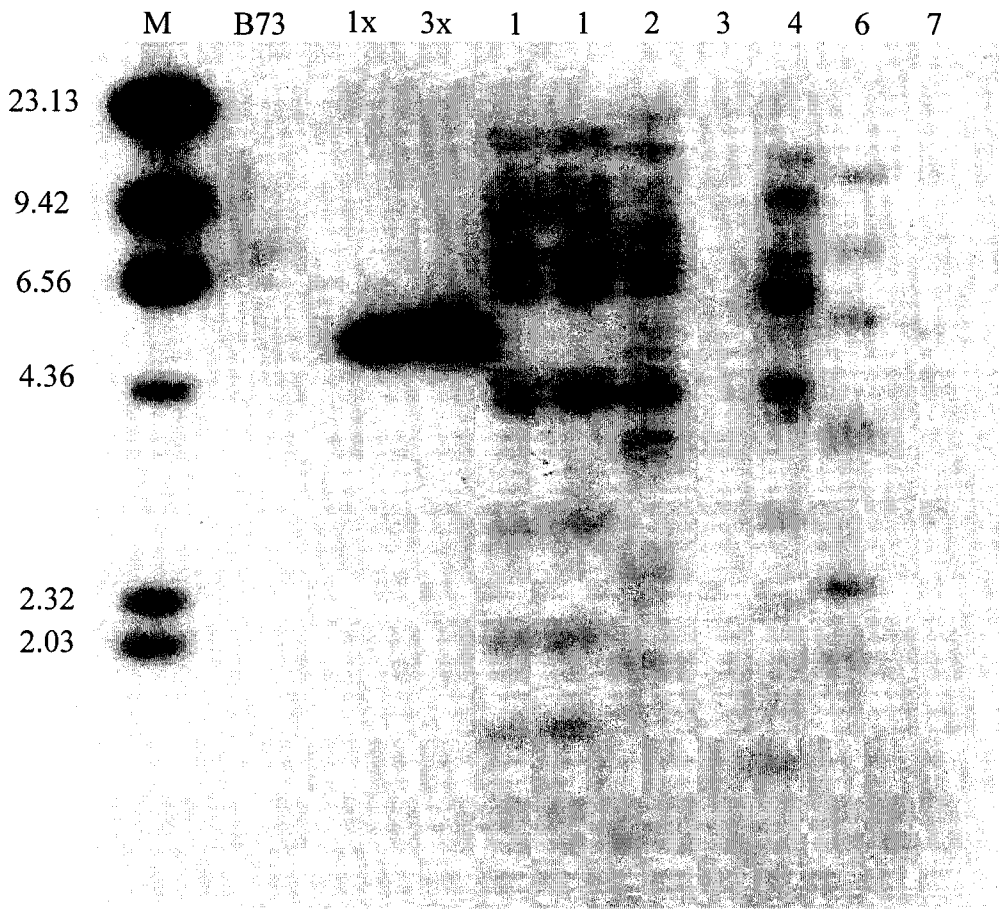

Figure 3. Southern blot analysis of selected P248 transgenic events (1, 2, 3, 4, 6 and 7). Approximately ten micrograms of total leaf genomic DNA obtained from young R0 plants was digested with NcoI, a restriction enzyme with a single cut within at 5' end of the sCT-B gene (Figure 1). DNA samples were resolved in a 0.8% agarose gel overnight at 23 V and blotted onto a Zeta-probe nitrocellulose membrane (BioRad). ^{32}P-labeled 1 kb fragment containing CT-B gene and VSP fragment was used as probe. M indicates molecular weight marker (in kilobases). Non-transgenic B73 corn DNA was used as negative control. 1x and 3x indicates 1 and 3 copies of the plasmid pCTB-BS which was used as positive control. Lanes 5 through 11 were loaded with genomic DNA obtained from respective transgenic events.

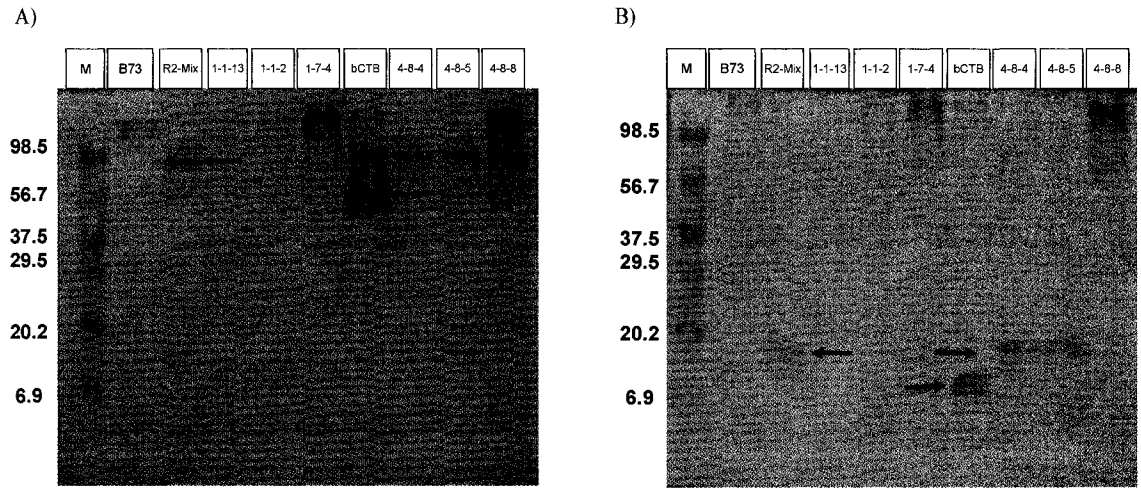

Figure 4. Western blot analysis of P248-1 and P248-4 R2 seeds. Protein extracts were separated in a 12% denaturing acrylamide gel and transferred to 0.45μ nitrocellulose membrane (BioRad). Following blocking in 5% skim milk for one hour at room temperature, the membranes were incubated in rabbit anti-CT- antibody (Sigma) diluted 1/3,000 in 1% skim milk for 1 hour at room temperature. Next, membranes were incubated in alkaline phosphatase conjugated detection antibody goat anti-rabbit IgG (Sigma) diluted 1/5,000. The color development was generated by Alkaline phosphatase substrate kit (BioRad) following manufacturer's instructions. M indicates molecular weight marker (in kilodaltons). Protein extract of non-transgenic corn was used as negative control. Bacterial CT-B (Sigma) was used as positive control and indicated as bCTB. R2-M refers to mill made of R2 seeds which was used in feeding experiment. Black and red arrows indicate bacterial CT-B and corn derived CT-B, respectively. CT-B pentamers (A) and monomers (B) were detected in extracts of event P248-4 unlike that of P248-1.

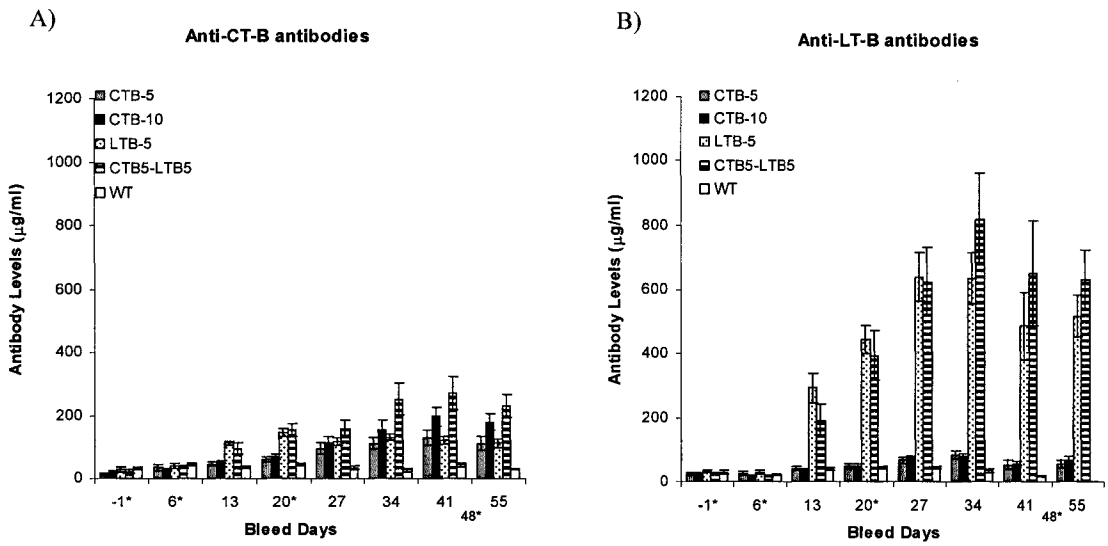

Figure 5. Anti-CT-B (A) and anti-LT-B IgG antibodies (B) measured in sera of all feeding groups over 55 days. Eight mice in each feed group were fed four times with transgenic and non-transgenic (WT) corn on days 0, 7, 21 and 49. Mice in CTB-5 and CTB-10 groups received 5 and 10 µg of corn derived CT-B, respectively. Mice in LTB-5 group received 5 µg of corn derived LT-B. Subjects in CTB5-LTB5 group received feeding pellets containing 5 µg of corn derived CT-B and µg of corn derived LT-B. *indicates the day before feedings.

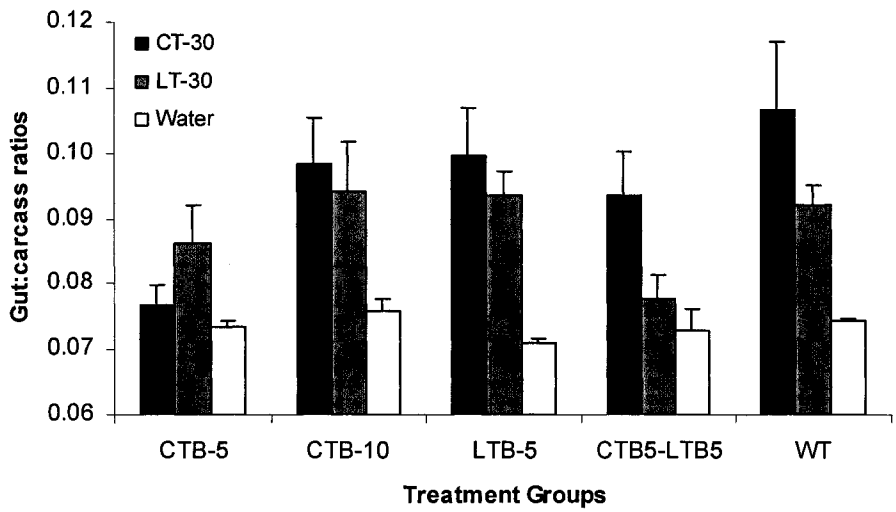

Figure 6. Gut:carcass ratios of immunized mice following toxin challenge. On day 55 three mice from each feed group were gavaged with 30 μg of CT or LT. Two mice from each group were gavaged with water and served as negative control. Gut:carcass ratios were obtained 3.5 hours post-necropsy.

Chapter 4: Attempts for the production of corn derived oral vaccine against Alzheimer's disease

Sule Karaman, Joan Cunnick, Kan Wang

4.1 Abstract

Carrier activity of non-toxic B subunits of *E. coli* heat labile toxin (LT-B) and cholera toxin (CT-B) for targeting unrelated antigens to mucosal immune system has been well established. In an effort to generate corn-derived orally administered vaccine against Alzheimer's disease we fused amyloid beta gene to 3' end of LT-B and we introduced two major epitopes of amyloid beta into internal permissive site of CT-B in three different constructs. The constructs were expressed in transgenic maize callus under the regulation of the CaMV 35S promoter. Analysis of calli transformed with LT-B-$A\beta_{42}$ indicated LT-B pentamer formation. We observed mRNA formation for the whole fusion gene however we failed to detect amyloid beta protein in corn calli. Transformation with the latter two constructs harboring CT-B-epitope fusions did not yield any detectable pentameric CT-B expression.

4.2 Introduction

ADP-ribosylating enterotoxins LT from enterotoxigenic strains of *E. coli* and CT from *Vibrio cholerae* have been shown to be strong immunogens and mucosal adjuvants. Non-toxic subunits of these toxins LT-B and CT-B retain most of these functions

(Czerkinsky et al, 1999). The adjuvant effect of LT-B has been well established, however there have been conflicting reports regarding adjuvant effect of CT-B (Freytag and Clements, 2005). Recently it was shown that CT-B chemically conjugated to ovalbumin (OVA), when administered through vagina (ivag), helps to induce OVA specific $CD4^+$ T cells. In this study, neither OVA alone nor OVA co-administered with CT-B resulted in OVA specific $CD4^+$ cells indicating antigen specific response (Luci et al, 2006). It has been also shown that CT-B induces oral tolerance to linked antigens (Li et al, 2005) suggesting usefulness of CT-B as a carrier to treat human autoimmune diseases. Promising results have been obtained with oral administration of B chain of insulin conjugated to CT-B. Oral tolerance to B chain of insulin was induced, using a lower dose of antigen and administration rate in NOD mice for suppression of diabetes (Sadeghi et al, 2002). Similar success was attained against type II collagen-induced arthritis (Tarkowski et al, 1999).

A number of studies were carried out involving expression of LT-B and CT-B fusions with unrelated antigens in plants (Kim et al, 2004a, Kim et al 2004b, Kim et al, 2004c, Choi et al, 2005, Kang et al, 2006, Walmsley et al, 2003, Rigano et al, 2004) The present work aimed at utilizing LT-B and CT-B as carriers for a human protein 'amyloid beta' and its epitopes to generate an orally administered plant-derived vaccine against Alzheimer's disease (AD). Alzheimer's disease is a neurodegenerative disorder characterized by cognitive decline, memory loss, language deficits and personality changes. Physiological hallmarks of AD are senile plaques made of 40-43 amino acid long amyloid beta protein (Gelinas et al, 2004) and neurofibrillary tangles (NFT) made of hyper-phosphorylated tau proteins (Walker et al, 2005). Amyloid beta protein produced at varying length (40-43 amino acids long) derives from proteolytic cleavage of a membrane spanning amyloid precursor protein (APP).

In healthy individuals, the most abundant form of amyloid beta is $A\beta_{40}$ (Gelinas et al, 2004). $A\beta_{42}$ is more fibrillogenic (Selkoe 2001) and aggregates into a neurotoxic form rich in β sheet structure forming the senile plaques (McLaurin et al, 2000). Figure 1 indicates the sequence of $A\beta_{42}$ and its epitopes.

Given the critical role of amyloid beta deposition in AD pathology, modification of amyloid beta aggregation became the target of a number of therapy approaches. Both active immunization with amyloid beta and passive immunization with anti-amyloid beta antibodies have resulted in prevention of Aβ deposition, clearance of deposits if immunization was done after the onset of the disease, and improvement of cognitive abilities in several transgenic mice models of AD (Schenk et al, 1999, Sigurdson et al, 2002, McLaurin et al, 2002, Lemere et al, 2002, Bard et al, 2000, Pfeifer et al, 2002, Janus et al, 2000, Kotilinek et al, 2002). However, active immunization with $A\beta_{42}$ during Phase II clinical trials resulted in brain inflammation (encephalitis) in 6% of patients which required termination of the study (Weksler 2004). Inflammation has been indicated to be part of the disease process by varied groups (Eikenbloom & Veerhius 1996, Maccioni et al, 2001). The relationship with immunization and enhanced local inflammation in affected patients in the trial still remains to be explained. Most parenteral vaccines induce both T-helper 1 lymphocytes (Th1) and T-helper 2 lymhocytes (Th2) (Friedman and Weiner, 1994). Th1 lymphocytes activate macrophages and macroglial cells which trigger inflammation, while Th2 lymphocytes activate B-cell antibody responses which down regulates the Th1 response (Janeway et al, 2001). Mucosally administered antigens induce anti-inflammatory IL-4/IL-10 (Th2 response) and tumor growth factor (TGF-β) (Th3 response) (Chen et al, 1994). This suggests that mucosal administration of antigens can prevent inflammation.

In this study, we aimed at producing a corn-derived vaccine which would be orally administered, against Alzheimer's disease. We attempted to create fusion proteins with $A\beta_{42}$ to use LT-B and CT-B as carriers. To use LT-B as a carrier, we fused the coding sequence of $A\beta_{42}$ to 3' end of the gene encoding for the LT-B subunit. To use CT-B as a carrier, we inserted two epitopes of $A\beta_{42}$ (in two different constructs) to a surface exposed region of CT-B subunit (Bäkström et al, 1995). Although we detected LT-B pentamers in transformed corn calli, we failed to detect $A\beta_{42}$. Analysis of transgenic corn calli transformed with CT-B- $A\beta_{42}$ fusions did not result in production of either CT-B pentamers or expression of epitopes.

4.3 Materials and Methods

Plasmid Construction: LT-B-$A\beta_{42}$ fusion

A gene fragment encoding for $A\beta_{42}$ (optimized for corn codon preference) was generated by annealing four synthetic oligomers synthesized in DNA Synthesis and Sequencing Facility at Iowa State University. The following oligomers were used: 1; 5'-ccg-ggt-cca-gac-gcc-gag-ttc-cgc-cac-gac-agc-ggc-tac-**gag**-gtg-cac-cac-cag-aag-ctg-gtg-ttc-ttc-gcc-ga-3', 2; 5'-g-gac-gtg-ggt-tcc-aac-aag-ggc-gcc-atc-atc-ggc-ctg-atg-gtg-ggc-ggc-gtg-gtg-atc-gcc-taa-gtc-ttc-ggt-ac-3', 3; 5'-ggt-gca-cct-cgt-agc-cgc-tgt-cgt-ggc-gga-act-cgg-cgt-ctg-gac-3', 4; 5'-cga-aga-ctt-agg-cga-tca-cca-cgc-cgc-cca-cca-tca-ggc-cga-tga-tgg-cgc-cct-tgt-tgg-aac-cca-cgt-cct-cgg-cga-aga-aca-cca-gct-tct-ggt-3'. Annealing and ligation was carried out as described by Brousseau et al, (1982). Figure 2 indicates the positions and orientations of each oligomer used to form double stranded DNA coding for $A\beta_{42}$ with modified ends. Briefly, 500 pmols of each of four oligos were mixed in a test tube and the total volume was brought up to 50 µl with sterile distilled water. The tubes were then wrapped with teflon

sealing tape and immersed in a boiling water bath for five minutes. The mixture of oligos was then cooled down to room temperature overnight. Annealed fragments were ligated by using 0.5 units of T4 DNA ligase (15224, Invitrogen CA, USA). The final gene fragment contained a partial *XmaI* site in 5' end and *KpnI* site at the 3' end ready for ligation. *XmaI* site was generated in a way that half of the hinge (GPGP) was introduced in 5' end of Aβ42 gene fragment. Amyloid beta coding sequence was first cloned into pBlueKSP(+) at *XmaI* site. The DNA of pBlueKSP(+) (8.5 μl) was cut with *XmaI* and *KpnI* yielding sticky ends. Since the gene was designed in such a way that it posses the ends in cut form, it did not require digestion. The gene was cloned into pBlueKSP(+) following *XmaI* and *KpnI* digestion of the vector. The newly constructed plasmid was called as pSK2. In order to fuse the gene that encodes for amyloid beta protein and half of the hinge to C-terminal end of LT-B, the ends of the LTB gene had to be modified in a way to carry other half of the hinge and an *XmaI* restriction site at 3' end and a *NotI* restriction site in its 5'end. This modified LT-B was produced by PCR amplification on pTH210 (Figure 3a) by using the following primers: 1; LTBNotIF2: 5'-attacgagcggccgcccatggt-3', 2; SK-LTBR-1: 5'-ctactacccgggccgttctccatg-3'. Modified LTB, following *NotI* and *XmaI* digestion, was inserted into pSK2 at the N terminal of modified Aβ42. This construct containing the fusion gene from start to stop codon was named as pSK3. The fusion gene was cut from pSK3 (Figure 3b) with *NcoI* and *KpnI* and cloned under enhanced 35SCauMV promoter by replacing LT-B in pTH210. The final construct to be used for maize transformation was named as pSK4 (Figure 3c).

Plasmid Construction: Insertion of $A\beta_{42}$ epitopes into an internal permissive site of CT-B

pCTB-BS (see Chapter 3) which has synthetic CT-B gene (optimized for corn expression system) under enhanced CaMV 35S promoter was altered via site directed mutagenesis by GenScript

Corp. gene synthesis company (New Jersey, USA) to introduce the N-terminal or middle epitopes of $A\beta_{42}$ into an internal permissive site of CT-B. The constructs carrying N-terminal epitope and middle epitope of $A\beta_{42}$ were named as pCTB-AB1 and pCTB-AB2, respectively (Figure 3d). Amino acid sequences of synthetic CTB and modified CTB genes are given in Figure 4.

Maize transformation

For expressing LT-B-$A\beta_{42}$ embryogenic maize HiII callus and for expression of CT-B-Aβ epitopes embryogenic maize HiII calli was transformed using microprojectile bombardment as described by Frame et al. (2000). The plasmids pSK4, pCTB-AB1 and pCTB-AB2 were each co-bombarded with the selectable marker gene construct, P2. P2 plasmid confers resistance to the herbicide bialaphos by expression of bar gene (Frame et al., 2000). Transgenic calli transformed with pSK4, pCTB-AB1 and pCTB-AB2 were designated as PC214, PHC268 and PHC269, respectively. PC214 clones were analyzed using the polymerase chain reaction (PCR) for presence of the LT-B-$A\beta_{42}$ fusion gene, reverse transcriptase polymerase chain reaction (RT-PCR) for presence of messenger RNA, enzyme linked immunoabsorbtion assay (ELISA) for detection of LT-B pentamers and $A\beta_{42}$ and Western blot. PHC268 and PHC269 calli were tested with ELISA.

PCR analysis

One hundred ng of total genomic DNA obtained from transgenic callus (for DNA isolation from corn calli see Chapter 3 in this dissertation) PC214 were used to amplify transgenes in each construct. For amplification of LT-B-$A\beta_{42}$ fusion gene, following primers were used: PSK4-F2: 5`-cga-acg-ata-gcc-cat-ggt-gaa-gg-3` and PSK4-R2: 5`-cgg-cca-gtg-aat-tcg-ctt-caa-ag-3`. These primers amplify 813 bp from 5' end of TEV leader to 3' end of VSP terminator spanning whole fusion gene (Figure 3c). The reaction mixture and conditions

were as follows: Total volume of 25 µl containing 100 ng of maize callus genomic DNA, 1 µl of dNTP mix (containing 10 mM of each dNTP), 1 µl of each primer (2.5 µM stock), 1 µl of $MgCl_2$ (50 mM), 2.5 µl of 10X Taq polymerase PCR buffer and 0.2 µl of Taq polymerase (Biolase USA Inc., City, NJ, USA). PCR was initiated at 95°C for 4 minutes. This was followed by 30 cycles of amplification steps of which are; denaturation at 95°C for 1 minute; annealing at 58°C for 40 seconds, and extension at 72°C for 1 minute.

Detection of Expression of LT-B and CT-B pentamers with ELISA

Expression of LT-B and CT-B pentamers in transformed corn calli were detected by ganglioside dependent ELISA previously described in Chapter 3. Protein extracts obtained from calli were diluted 10, 20 or 40X for different clones assayed. For monomeric CTB detection, the assay was modified such that the protein extracts were coated on to the plate instead of gangliosides capture.

Detection of Expression of $A\beta_{42}$ and its epitopes with ELISA

To detect expression of $A\beta_{42}$ 96 well microtiter plates (Costar 3590, Fisher Scientific, PA, USA) were coated with mixed gangliosides (100µg/ml) (Type III from bovine, Sigma G-2375, MO, USA) in coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, (pH: 9.6)]. After blocking with 5% milk, calli extracts were loaded to the wells. Gangliosides bind to LTB pentamers in the extracts. To capture amyloid beta protein, a biotinylated monoclonal anti-amyloid beta antibody (Mouse monoclonal against human amyloid beta protein, Clone 4G8, Signet Laboratories Inc.9220-02, MA, USA) was used at 1/1,000 dilution. For standards; 31.25, 16.1, 8.625, 4.3 and 2.15 ng/ml of amyloid beta protein (Beta-Amyloid (1-42) human, American Peptide Co. Inc. CA, 62-0-80, USA) were sandwiched in duplicates in

between two mouse monoclonal anti-amyloid beta antibodies raised against different epitopes of $A\beta_{42}$. Mouse monoclonal antibody which is raised against the N-terminal epitope of $A\beta_{42}$ (Mouse monoclonal against human amyloid beta protein, Clone 6E10, Signet Laboratories Inc.9320-02, MA, USA) was used at 1/100 dilution to coat the plate and clone 4G8 which is raised against middle epitope of $A\beta_{42}$ was used as detection antibody at 1/1,000 dilution. Antibodies were diluted in 1% milk in PBS. Calli extracts were diluted 1, 10, 20 and 40X. In all ELISA analysis that is described above, the wells to be used for samples were coated with gangliosides based on the assumption that amyloid beta should be linked to LT-B. In case the fusion protein was broken, calli extracts were also analyzed as standards were developed (with 6E10-4G8 sandwich) without ganglioside coating. After detection antibody, wells were loaded with streptavidin horse radish peroxidase diluted 1/1,000 (BD Biosciences, 554066, MD, USA). The color development was obtained by addition of ABTS substrate (3-ethylbenzthiazoline-6-sulfonic acid, Sigma A-1888, MO, USA) prepared according to manufacturer's instructions. Fifty µl of reaction volume was used except for blocking step. One hundred fifty µl of 5% milk diluted in PBS was used for blocking. Coating and blocking steps were done at room temperature for one hour. Sample, standard, and antibody incubations were done at 37°C for one hour. Plates were incubated for 30 minutes at room temperature for enzyme and substrate steps. Absorbance at 405 nm was read, a standard curve was generated and sample calculations were carried out.

For detection of N-terminal and C-terminal epitopes of amyloid beta inserted into the permissive site of CTB, protein extracts from calli were directly coated on the plate wells. For detecting epitopes 6E10 and 4G8 were used at dilutions indicated above for pHC268 and pHC269 respectively.

RT-PCR Analysis

RNA extraction from transgenic calli was carried out as follows. Approximately 100 mg of callus tissue was transferred to a 1.5 ml RNase-free test tube and ground with a peroxide treated (pestles were placed in a 50 ml falcon tube and incubated in 30%H_2O_2 for 10 min at room temperature and then washed with DEPC treated water several times) sterile Kimble pestle. One ml of Trizol (GIBCO BRL, Life Technologies, Inc.) reagent was added and samples were incubated for 5 minutes at room temperature. Next, 0.2 ml of chloroform was added and tubes were capped securely. Tubes were shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2 minutes. After centrifuging at 12,000 rpm at 4°C for 15 minutes, supernatant was transferred into a fresh tube. Isopropanol (0.5 ml) was added to each tube and incubated at room temperature for 10 minutes. Tubes were centrifuged at 12,000 rpm at 4°C for 10 minutes to precipitate RNA. Supernatant was removed and RNA pellets were washed with 1 ml of 75% ethanol. Pellets were re-suspended in ethanol and centrifuged at 9,000 rpm for 5 minutes at 4°C. Ethanol was removed and pellets were air-dried. RNA pellets were dissolved in 30 µl of DEPC (diethylpyrocarbonate) treated, RNase free water. In order to eliminate false positive results in RT-PCR analysis due to DNA contamination, RNA samples from clones #7, 12, 16, 17 and 18 were treated with Deoxyribonuclease I (DNaseI, 18068-015, Invitrogen, CA, USA). In a total of 10 µl, 1 µg of RNA, 1 µl of 10x DNaseI reaction buffer and 1 µl of DNaseI (1u/µl) were mixed well and samples were incubated at room temperature for 15 minutes. DnaseI was inactivated by addition of 25mM EDTA solution and heating at 65°C for 10 minutes. Samples were kept at -20°C until analyzed.

RT-PCR was carried out using the Qiagen OneStep RT-PCR kit (210210, Qiagen Sciences Inc. MD, UA). 3 sets of primers were used for this analysis. The first set of primers was designed using the 5` and 3` regions of promoter. This served as a DNA contamination control since this part of the cassette is not transcribed. The second set of primers was designed using the sequences that reside only in the LT-B gene. Since some of the clones were positive for LT-B ELISA analysis, this set served as internal control. The third set was designed using the sequences residing within the amyloid beta and LTB gene segments so that the PCR product would contain hinge region. The following primers were used for testing DNA contamination: 1; RT-PR-F1: 5`-gcc tgc-agg-tca-aca-tgg-tgg-3` and 2; RT-PR-R1: 5`-gga-tag-tgg-gat-tgt-gcg-tca-tcc-3`. Primers used as internal control were: 1) RT-LTB-F1: 5`-gcc-cat-ggt-gaa-ggt-gaa-gtg- c-3` and 2; RT-LTB-R1: 5`-tgg-cag-caa-tgg-agt-ttg-gag-tc-3`. These primers amplify 362 nucleotides from the 3' end of the TEV leader to middle of the LTB gene. Primers used for amplifying a 128 base pair long fragment from the 3' end of the LTB gene to the 3' end of the $A\beta_{42}$ coding sequence spanning the hinge region were: RT-AB-F1: 5`-tcc-aga-cgc-cga-gtt-ccg-cc-3` and RT-AB-R1: 5`-cga-tca-cca-cgc-cgc-cca-3`.

The RT-PCR reaction mixture contained the following in total volume of 50 µl: 100 ng of total RNA (in 1 µl), 10 µl of 5x Qiagen OneStep RT-PCR buffer, 1.5 µl of dNTP mix (10 mM of each dNTP), 12 µl of each primer (2.5 µM), 1.5 µl of Qiagen OneStep RT-PCR enzyme mix and 12 µl of RNase free water. The reaction was initiated with reverse transcription at 50°C for 30 minutes which generated a cDNA copy of the relevant RNA fragments. This was followed by initial denaturation at 95°C for 15 minutes. Next, 30 cycles of amplification (denaturation at 94°C for 45 seconds; annealing at 60°C for 45 seconds, and

extension at 72°C for 1 minute) followed. After 30 cycles were completed, fragments were allowed to be fully extended at 72°C for 10 minute.

Western Blot

Approximately 200 mg of calli transformed with pSK4 were sampled into 1.5 ml eppendorf tubes for the events 9, 10, 11, 12, 14, 16 and 18. Calli were ground with autoclaved pestles (Kontes Pellet Pestle, K749520-0000, Fisher Scientific, PA, USA) until juice exudes. A 25-35 µl aliquot of extract was analyzed by SDS-PAGE (Laemmli, 1970). Two sets of proteins were prepared for each sample analyzed; samples in one of the sets were boiled for 5 minutes at 95°C. Proteins were run at 100V for 1 hour and transferred to a 0.45 µm nitrocellulose membrane using the BioRad Transblot apparatus following manufacturer's instructions. Rabbit anti-CT IgG (Sigma, C-3062, MO, USA) diluted 1.5/20,000 in 1% milk was used as the primary antibody and goat anti-rabbit AP-conjugate anti-IgG diluted 1/5000 in 1% milk (KPL, 475-1506, MD< USA) was used as secondary antibody. Proteins were evaluated with the expectation of shift to higher molecular weight because of the presence of amyloid beta. Non-transformed callus was used as negative control while p77 seed extract was used as positive control.

4.4 Results and Discussion

Construction of LT-B fusion genes

In this study we attempted to generate a corn-derived vaccine against Alzheimer's disease by fusing amyloid beta protein (42 amino acid long version) to the C-terminal end of LT-B subunit. To give flexibility to the fusion protein we utilized a hinge made of four

amino acids (Glycine-Proline-Glycine-Proline) in between LT-B and $A\beta_{42}$. This hinge was previously used in tobacco and potato for fusion protein expression (Smerdou et al, 1996, Arakawa et al, 1998, Yu and Langridge, 2001).

Analysis of transgenic calli transformed with LT-B-$A\beta_{42}$

The plasmid carrying the synthetic LT-B-$A\beta_{42}$ fusion gene and a plasmid carrying herbicide resistance gene were introduced to maize hybrid Hi II through biolistic transformation (Frame et al, 2000). Eighteen herbicide resistant calli clones were obtained. Fifteen of those clones were shown to carry LT-B-$A\beta_{42}$ fusion gene via PCR analysis. All PCR+ clones expressed pentameric LT-B at varying levels as indicated in Figure 5. However, we failed to detect amyloid beta with ELISA. Our ELISA system for detection of amyloid beta included human amyloid beta (1-42) as standard.

In order to detect mRNA of the fusion gene, RT-PCR analysis was performed. For this analysis, clones numbered 7, 12, 16, 17 and 18 which were among the highest expressers of pentameric LT-B were selected. For amplification of the RNA product of the LT-B gene and amplification of the amyloid beta coding sequence which also spans the hinge region two sets of primers were used. The former primer set amplifies a 362 bp region from 3' end of TEV leader to middle of LT-B. The later primer set amplifies a 128 bp region from 3'end of LT-B gene to 3' end of amyloid beta gene. A third set of primers which amplifies a region in the promoter was used as DNA contamination control. (Figure 7B)

Figure 7A shows fragments obtained by amplification of RNA fragments, obtained from selected LT-B positive calli, with primers that detect hinge and amyloid beta and LT-B gene. Figure 7B indicates amplification products of DNA contamination control from the same LT-B positive clones. RNA obtained from clone 16 was amplified with the primer set

that amplifies LT-B gene as an integral control for validity of the PCR run. Our results showed expected fragments with the right size for both LT-B (lane 9 and 10 in figure 7A) and for hinge and amyloid beta region (lanes 3, 4, 5, 6, 7 in figure 7A). Contrary to our expectations due to negative ELISA result, RT-PCR analysis indicated presence of full length RNA for the whole fusion gene.

Next, to verify pentameric assembly which was shown with ganglioside binding ELISA and to analyze presence of amyloid fusion, western blot was performed. Pentameric LT-B is 55 kDa made of 11.6 kDa monomers. LT-B can only bind to GM1 gangliosides on the intestinal surfaces when it is pentameric. Immunogenicity of the molecule also depends on pentameric native conformation (de Haan et al, 1998). Extracts of LT-B positive calli lines 7, 9, 10, 11, 16, 18 were used for this analysis. We observed a major band between 35.8 and 56.2 kDa standards (figure 6). A second nonspecific band was observed slightly above 56.2 kDa standard in all samples. As also seen in the lane loaded with Hi II non-transgenic negative control, this band was coming from the negative control background. Presence of amyloid beta on each monomer of pentameric LT-B would have caused approximately 20 kDa up-shift in immunoblot assay. In consensus with ELISA results, LTB pentamers were detectable without amyloid beta.

Although mRNA was detectable for the whole fusion gene, we failed to detect amyloid beta protein via ELISA and western blot. Possible reasons for this outcome could be as follows: 1) mRNA would be unstable. 2) An unknown proteolytic cleavage site might have been introduced at the site where hinge links LT-B and amyloid beta. The sequence of the fusion gene was screened for known trouble causing sequences with GCG program (Appendix II). However, it is unlikely that all proteolytic cleavage sites or mRNA

destabilizing sequences in corn have been identified. Once cleaved, the small protein would possibly get degraded in the cell.

Construction of CT-B fusion genes

To utilize CT-B as carrier we applied a different approach. An internal site in the cholera toxin B subunit previously has been shown to allow expression of short epitopes (Bäkström et al., 1995) without affecting pentamer formation of CT-B. In that study epitopes from human immunodeficiency virus-1, hepatitis B virus and enterotoxigenic *E. coli* with varying amino acid length (10-19 aa) were tested. These epitopes were introduced within a surface exposed region of CT-B by performing deletions at that site. Different combinations of deletions/insertions tested. The conclusion from this study was that Pro^{53}, Gly^{54}, S^{55} and Ala^{64} are critical for stable expression of epitopes and the CT-B pentamer. . Authors called the site from S^{55} to Ala^{64} as 'internal permissive site'. Moreover, 10 aa epitopes were found to be more successful in terms of expression of the epitope.

It has been shown that among three epitopes of $A\beta_{42}$, monoclonal antibodies raised against the first one residing in N-terminal had a high solubilization effect (80 to 98%) on preformed fibrillar β-amyloid. It was also shown that anti-aggregating ability of middle epitope was considerably smaller while monoclonal antibodies raised against the C-terminal epitope did not have detectable solubilization effect on the aggregates (Solomon et al., 1997, Frenkel et al., 2004). Therefore, we inserted N-terminal epitope and middle epitope to internal permissive site of CT-B. We created two versions of CT-B Aβ epitope fusion versions. We chose the first 10 amino acids from N-terminal and amino acids from 16-25 to be inserted into the permissive site. Plasmids harboring these genes under 35S cauliflower mosaic virus constitutive promoter were transformed to immature zygotic corn embryos.

Analysis of transgenic calli transformed with CT-B-Ab42 fusions

Plasmids pCTB-AB1 and pCTB-AB2 containing N-terminal and middle epitope of amyloid beta in the internal permissive site of synthetic CT-B gene were introduced to maize calli (Hi II) in a similar fashion as described in previous section. Total of 101 and 54 calli clones were obtained for PHC268 and PHC269, respectively. Pentameric and monomeric CT-B expression was analyzed by ELISA. None of the clones expressed either pentameric or monomeric CT-B. Epitope insertions at the permissive site disrupted native conformation of the CT-B. ELISA results with antibodies raised against the relevant amyloid beta epitopes did not yield significantly positive results, either.

In the original study, Bäkström et al, (1995) reported 13 CT-B hybrid proteins created with the envelope glycoprotein gp120 from human immunodeficiency virus type 1 (HIV-1), 11 amino acid epitope from hepatitis B virus (HBV) and short peptides related to the heat-stable toxin (ST_a) of enterotoxigenic *E. coli*. The size of the oligos inserted ranged from 10 amino acids to 19 amino acids while deletions occurred at varying lengths between 53^{rd} aa and 64^{th} aa of CTB. It was postulated that both size of the insertion and amino acid composition play role in ensuring proper folding of recombinant CTB proteins. Ten amino acid lengths was found to be a working length while presence of cysteine residues was found to have stabilizing effect on the recombinant protein. It was also concluded that Proline at 53^{rd} position and Alanine at 64^{th} are essential for correct folding of CTB. It was also shown that Glycine at 54^{th} position along with Alanine at 64^{th} position is required for stable expression of the hybrids suggested by the varying expression levels of the constructs studied. Moreover, Serine at 55^{th} position along with Alanine at 64^{th} position was found to be allowing expression independent of the amino acid composition. Taking these into account

we deleted eight amino acids in between Ser^{55} and Ala^{64} and replaced them with 10 amino acid lengths for each epitope. Since there was not a Cys residue in the epitopes or in their neighboring amino acids and since there is no technical tool which would predict precisely three dimensional structures of amino acid deletions or insertions into a larger protein we did not add cysteine but kept the original sequences.

4.5 Concluding remarks

We attempted to produce a corn-derived vaccine against Alzheimer's disease using two different protein fusion strategies, 1) fusion of $A\beta_{42}$ to C-terminal of LT-B and 2) insertion of amyloid beta epitopes to an internal permissive site of CT-B. Analysis of calli transformed with LT-B-$A\beta_{42}$ indicated correct folding of LT-B pentamers however we failed to detect amyloid beta proteins. Our second approach did not yield any detectable CT-B pentamers or detectable amyloid beta epitopes. Bäkström et al (1995) also reported failure with three of the constructs out of thirteen analyzed. With these constructs, they failed to detect pentameric CT-B or the epitopes of interest.

With the current technology and tools of protein biochemistry it is impossible to predict the fate of fusion proteins or modified proteins with replaced amino acids. Varying lengths of proteins have been fused to LT-B and CT-B with or without a hinge and expressed in both bacteria and plant systems (Lipscombe et al, 1991, Arakawa et al, 1998, Smerdou et al, 1996, Luci et al, 2006, Li et al, 2005, Walmsley et al, 2003, Rigano et al, 2004). However, success of the fusion protein approach has to be evaluated on a case-by-case basis (Rigano et al, 2003). The only way of getting positive results from such projects will be testing a number of versions (different amino acid composition and number) of the epitopes and also

border regions. Therefore new versions of these fusions and epitopes should be tested. Assaying such constructs in a transient expression system is desirable. This will allow testing of a number of constructs in a shorter time.

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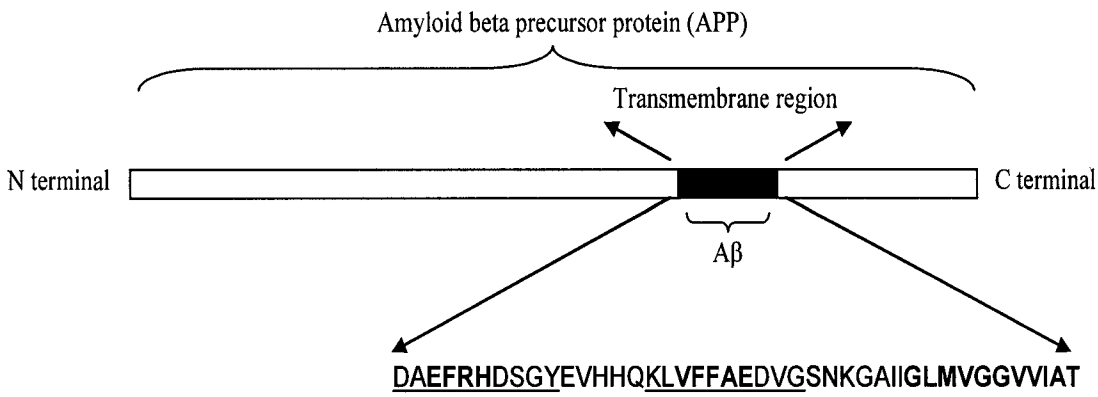

Figure 1. Schematic representation of amyloid beta precursor protein, sequence of amyloid beta (1-42) and epitopes residing on amyloid beta. Bold sequences indicate epitopes. Underlined sequences show fragments inserted into internal permissive sites of CT-B.

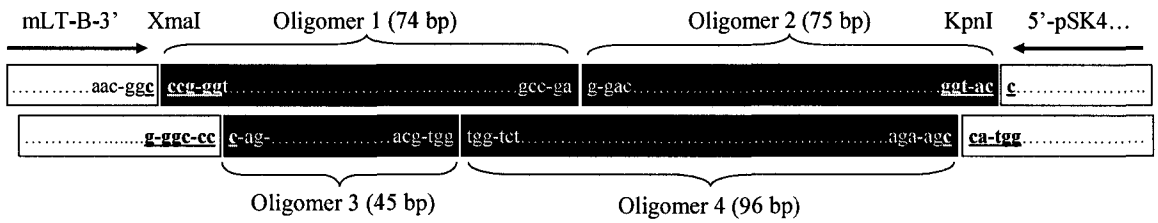

Figure 2. Four synthetic oligomers were used to create synthetic $A\beta_{42}$ which was optimized for corn expression system. 5' and 3' ends of annealed fragments contained portions of XmaI and KpnI restriction sites, respectively. In the final construct (pSK4) an annealed fragment was linked to 3' end of modified LT-B which has the other portion of XmaI restriction site. Underlined sequences indicate restriction enzyme sites. At the 3'end, annealed fragments were inserted to psK4 at KpnI site.

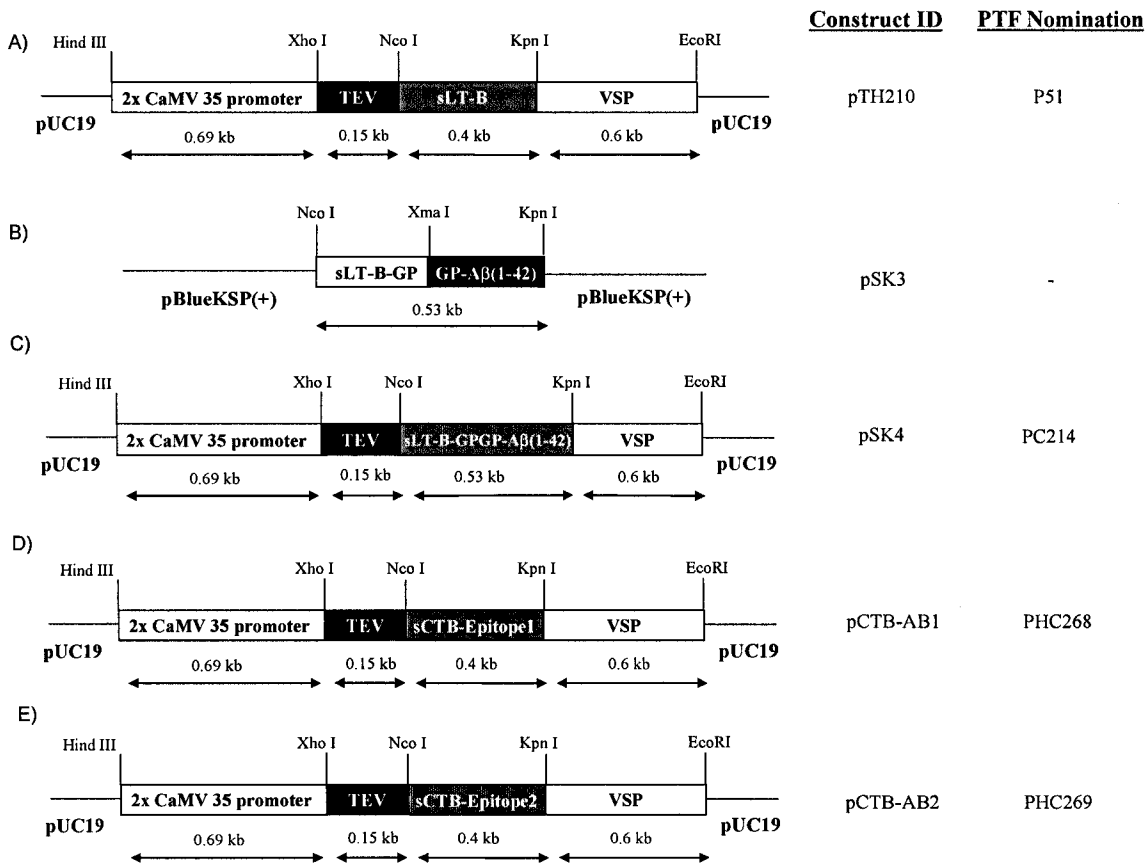

Figure 3. Schematic representation of constructs, their IDs and PTF nominations.

A)

1 MVKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTE
50 SLAGKREMAIITFKNGATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTE
100 AKVEKLCVWNNKTPHAIAAISMAN

B)

1 MVKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTE
50 SLAGKREMAIITFKNGATFQVEVPGSDAEFRHDSGYAIERMKDTLRIAYL
100 TEAKVEKLCVWNNKTPHAIAAISMAN

C)

1 MVKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTE
50 SLAGKREMAIITFKNGATFQVEVPGSKLVFFAEDVGAIERMKDTLRIAYL
100 TEAKVEKLCVWNNKTPHAIAAISMAN

Figure 4. Amino acid sequences of A) CT-B, B) modified CT-B with its internal permissive site replaced by N-terminal epitope of amyloid beta and C) modified CT-B with its internal permissive site replaced by middle epitope of amyloid beta. Sequences in red indicate bacterial signal peptide. Sequences in blue indicate internal permissive site in A) and epitopes from amyloid beta with neighboring amino acids.

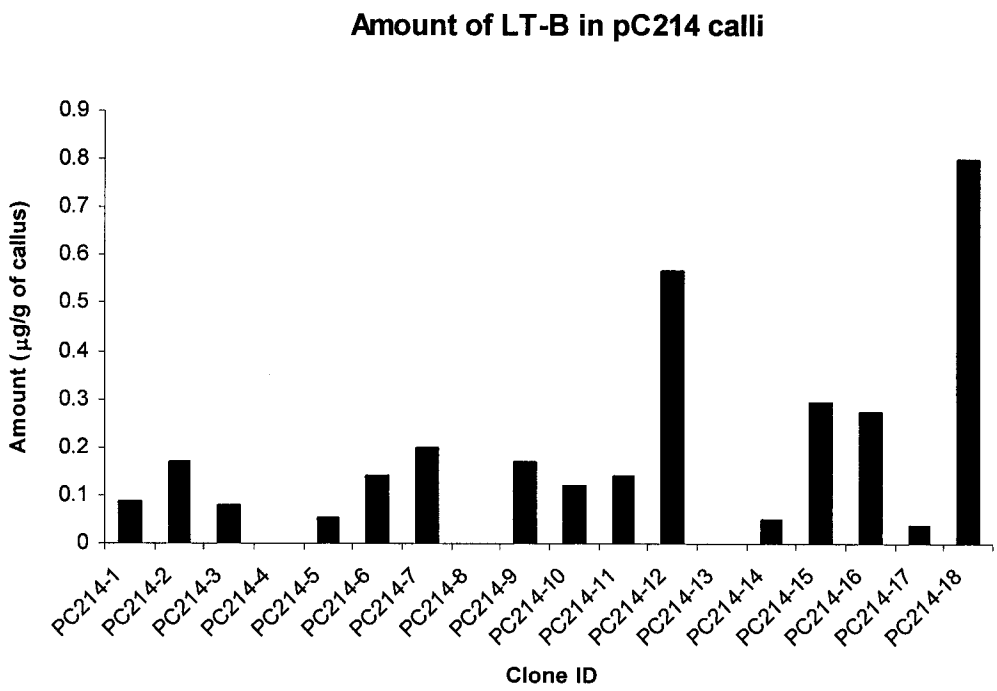

Figure 5. Amount of pentameric LT-B (µg/g) expressed in pC214 calli.

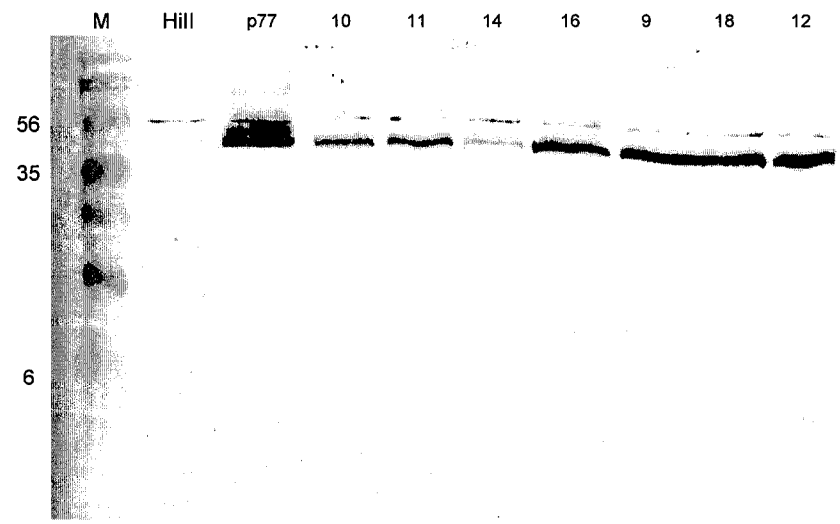

Figure 6. Western analysis of selected transgenic calli; pC214 -9, 10, 11, 12, 14, 16, 18. 30 μg of total protein from calli extracts were separated on 12% SDS PAGE. Rabbit anti-CT IgG and goat anti-rabbit IgG (alkaline phosphatase conjugate) were used as primary and secondary antibodies. The same amount of HiII calli extract and p77 ground whole kernel extract were used as negative and positive controls, respectively. M indicates molecular weight marker.

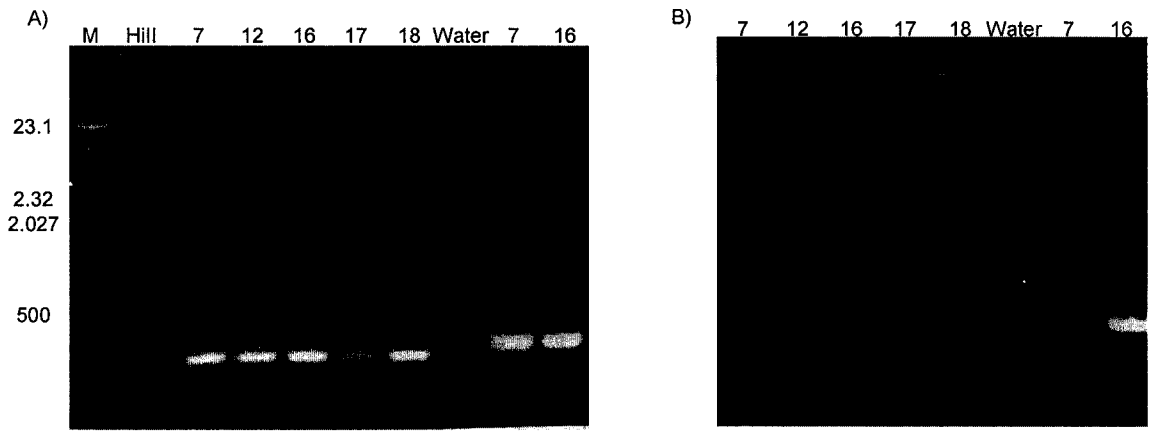

Figure 7. RT-PCR analysis of pC214 calli. ELISA+ selected calli (pC214-7, 12, 16, 17, 18) were analyzed for expression of mRNA for the fusion gene. Panel A indicates amplification of hinge+amyloid beta fragment (pC214-7, 12, 16, 17, 18) and that of LT-B gene (pC214-7 and 16). Panel B indicates amplification results of promoter region from indicated calli. RNA obtained from pC214-16 was amplified with primer sets spanning LTB gene as an internal control for this run.

Chapter 5: Assessment of allergenic potential of corn derived LT-B and C-TB in comparison to Cry 1Ab and cp4 epsps based on their primary sequences

Sule Karaman, Kan Wang

5.1 Abstract

In spite of the great promise it holds for increasing nutritional quality, yield and production of value added crops, genetic modification of food crops has raised safety concerns. One of the human health concerns regarding biotech crops is the risk of allergenicity. Not all the characteristics that make a protein allergenic are known yet. Therefore, there is no straightforward strategy to evaluate the allergenic potential of a given protein. International Food Biotechnology Counsel and the International Life Sciences Allergy and Immunology Institute, Food and Agriculture Organization (FAO), World Health Organization (WHO) and Food and Drug Administration (FDA) all separately initiated studies to develop a system to assess the allergenic potential of biotech food crops by integrating currently available data regarding what constitutes an allergen. These efforts resulted in a decision tree approach. The initial step in decision tree approach is the homology analysis of amino acid sequence of the protein of interest in regard to that of known allergens. Currently, a number of public allergen databases are available. Among those, the Food Allergy Research and Resource Program (FARRP) and Structural Database of Allergenic Proteins (SDAP) provide FAO/WHO allergenicity testing. Using these two

databases we performed potential allergenicity test of B subunits of *E. coli* heat labile enterotoxin and Cholera toxin, LT-B and CT-B, which were used to generate transgenic corn for production of edible vaccines described in this dissertation. We also performed the same test on the sequences of insecticidal protein Cry1Ab and herbicide tolerant enzyme cp4 epsps which are expressed in Bt corn and Roundup ready soybean, respectively. From this analysis we determine future studies are needed to complete the investigation of potential allergenicity of recombinant proteins produced in corn.

5.2 Introduction

Allergies in general are a major public health concern. It is estimated that up to 40% of the population in industrialized countries are affected by allergies towards various environmental or food sources (Hoffmann-Sommergruber and the SAFE consortium, 2005). Specifically, food allergies have been on rise in developed countries within the last 20 years (Sampson, 2005).

Allergy is defined as *hypersensitivity of the immune system* to a natural substance (certain kinds of food, pollen, animal dander and dust) that does not cause symptoms in non-allergic people. People who show symptoms of allergy to any given allergen develop immune response against only a few proteins in the given allergenic substance. Allergic symptoms are mainly mediated through production of IgE antibodies, although other forms of immune reaction that lead to allergic response exist. Following initial exposure (sensitization) to a particular allergen, specific IgE molecules are fixed on basophil and mast cells. Re-exposure to the same allergen activates basophils and mast cells by cross-linking specific IgEs. Activation of these cells results in a set of reactions including histamine

release, production of arachidonic metabolites and immunomodulatory molecules. These molecules interact with target tissues bringing about symptoms of allergies. In the case of food allergies, classical symptoms include itchy mouth, fullness in the throat, shortness in breath, red, itchy rash on skin, difficulty in breathing and vomiting. Some severe allergic reactions might even result in death.

Although no evidence regarding increased allergenicity of genetically modified food crops have been shown, the use of biotechnology in food crops raised health concerns. First, the gene introduced into the crop might be encoding a known allergen or a protein that cross reacts with an allergen. Second, existence of a novel protein in the crop might change the behavior of other proteins in the cell rendering them allergenic or enhancing endogenous allergenicity. Third, novel proteins that we are exposed rarely or none in our daily lives, when introduced to our environment through transgenic crops might cause allergenicity. And fourth, post-translational modifications, such as N-glycosylation, might raise allergenicity concern by altering solubility, stability, size and susceptibility of recombinant proteins to proteases (Lesher and Bannon, 2005, Aalberse, 2000). N-glycosylation occurs on asparagine residues separated by one amino acid from serine or threonine. For example, in a sequence of Asparagine-X–Serine/Threonine), Asparagine usually is N-glycosylated. However, if the amino acid in between is proline or aspartic acid, asparagine residue does not get glycosylated (Lerouge et al, 1998). Glycosylation at this site in plants is critical because α(1-3)-fucosyl and β(1,2)-xylose that are glycosyl groups unique to plants might be included in the final structure creating potential for allergenicity (Goodman et al, 2005). As a matter of fact, bean α-amylase inhibitor, when expressed in peas was reported to be structurally altered and immunogenic (Prescott et al, 2005), while it was not immunogenic in bean.

Elimination of transferring potentially allergenic proteins to food crops is highly desirable. However due to lack of complete information regarding the structural and functional parameters that make a protein allergen, currently there is no straightforward strategy to achieve this goal (Metcalfe, 2003). In spite of the accumulating knowledge regarding allergens and allergenic immune mechanisms, the concepts underlying allergy and allergens are still not very clear. For example, it is well established that allergens must cross-link IgEs. Interestingly, IgE binding to food allergens are observed in persons who are tolerant to the given food allergen (Poulsen, 2004). Therefore a decision tree approach which integrates testing for all known parameters of allergenicity sequentially is applied to evaluate potential risks of recombinant proteins.

The first step of the decision tree approach adopted by FAO/WHO (Metcalfe, 2003) is to perform homology search between amino acid sequence of the protein to be transferred with protein sequences of known allergens. There are a number of publicly available allergen databases (described below). Following that in vitro IgE binding assays are performed. Since most allergens are known to be resistant to proteolytic digestion, the next step of the analysis involves pepsin digestion resistance. The analysis would be completed with information obtained through animal model testing where applicable (Metcalfe, 2003).

In order to assess potential allergenicity of proteins LT-B and CT-B, that are used in the projects described in this dissertation, at the sequence level, FASTA search have been performed by using SDAP and FARRP allergen databases. As control comparison, we also choose two known transgenic proteins Cry1Ab (NCBI Accession #: PAO370) expressed in Bt corn and cp4 epsps (NCBI accession #: Q9R4E4) expressed in Roundup Ready soybean. These two transgenic crops have been studies extensively and deregulated in the United

States. A synopsis of currently available public allergen databases which explains the reason for choosing these two databases for our search is given below.

Allergen Databases:

Rapid increase in number of characterized protein allergens made advanced bioinformatics search tools a necessity. A number of public databases have been generated in US and Europe. These databases typically provide the name, nomenclature and source of the allergen, structural and clinical features and links to references. Two of these databases provide sequence homology search to predict cross-reactivity and potential allergenicity of a given protein. The features of these databases are briefly given below and Table 1 indicates institutes created and maintaining the databases and their URL addresses.

Allergome.org: The list of allergens found in this website is based on selected papers since 1960s. The list is continuously updated. Information provided through this search tool includes source of the allergen, tissue in which the protein is found and the route of exposure (physical contact, inhalation, ingestion, etc). Protein sequence, isoforms, allergenicity of the allergen in its natural conformation and plasmid information in cases where the creation of the allergen occurs through gene recombination are also included (Mari et al, 2004). The allergens included in this database are determined following criteria which are recognized by WHO. Even though it includes all the characterized allergenic molecules, this database however does not provide blast search to determine potential allergenicity of a given protein.

IUIS (International Union of Immunological Societies): The site has been created and maintained by the Allergen Nomenclature sub-committee of IUIS. Two major criteria of the site to include proteins as allergens is more than 5% IgE reactivity and occurrence of IgE in

at least five patients. It merely lists allergens from different organisms and gives references and accession numbers regarding the sequences.

Swiss-PROT index of allergen sequences: This site also contains allergen sequences designated by IUIS. Currently the list of allergens on this site contains 1263 sequences from different organisms.

IFBC-ILSI: This database was formed by the International Food Biotechnology Council (IFBC) and ILSI Allergy and Immunology Institute during joint study that resulted in development of *'decision tree'* approach for assessment of potential allergenicity of GM crops (Metcalfe et al, 1996). This list has not been updated since publication.

CSL Allergen Database: This database was released by the Food Safety and Quality Directorate of The Central Science Laboratory at Sand Hutton York, United Kingdom. The list provides links to sequence and reference information as well as PDP structural data.

PROTALL: The Institute of Food Research of the United Kingdom compiled this database to study issues regarding food allergy. The database contains physical features of allergens such as molecular weight, epitopes, stability and cross-reactivity. This is a searchable database where the query term would either be free text or a specific plant.

ALLALLERGY: This database contains chemical allergens in addition to proteins. It includes more than 4500 entries. The database is currently updated and can be searched using the name or allergen category (grains, fish, pollen etc.).

BIFS Tables: The Biotechnology Information for Food Safety web site lists food allergens, non-food allergens and wheat-gluten proteins. It provides links to sequence information, species name, allergen nomenclature and references. The goal of the project was

facilitating sequence comparisons for potential allergenicity of proteins produced in GM food crops (Gendel, 1998), however it does not provide this function.

Asthma and allergy gene database: This database contains sequence information about linkage and mutation studies regarding asthma and allergy related traits. It was generated by the National Research Center for Environment and Health, Munich, Germany.

SDAP-Structural Database of Allergenic Proteins: SDAP web server was established and is maintained by The University of Texas Medical Branch. This server contains 737 allergens and isoallergens, 829 protein sequences and 22 IgE and IgG epitopes. Allergen list of this server is updated every three months and majority of the allergen sequences are obtained from International Union of Immunological Societies (IUIS). SDAP server provides FASTA search (Pearson and Lipman, 1998) for testing potential allergenicity of new proteins based on FAO/WHO criteria. According to FAO/WHO allergenicity rules addressed in 2001 in a joint report entitled as *'Evaluation of Allergenicity of Genetically Modified Foods'* (http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y0820e/y0820e00.htm), there would be cross-reactivity with a given allergen if there is identity of six adjacent amino acids and over 35% cross-reactivity along 80 amino acid length on the given protein with a known allergen. It is also possible to change these cut-off values if the user desires.

FARRP Allergen Database: Food Allergy Research and Resource Program allergen database contains 1537 entries of all kinds (food, contact, etc.) of allergens and putative allergens and gliadins that cause celiac disease. This database was created and maintained by a group at Monsanto Company. The sequences are obtained from public protein databases (SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and

RefSeq) by using 'allergen' and 'celiac' words as keywords. A minor portion of the entries were identified from Medline which were not entered into any database yet. This database also provides similar FASTA search as SADP does. All the sequences in FARRP are claimed to be unique because duplicate entries and irrelevant sequences are removed from the database. This database has been updated in January 2006.

5.3 Materials and Methods

FARRP and SDAP allergen databases were searched to assess potential allergenicity of LT-B, CT-B, Cry1Ab and cp4 epsps proteins. Both FARRP and SDAP provide *'full FASTA alignment and 80-mer sliding window search'*. Full FASTA alignment considers that two proteins are homologues if e-value is less than 0.01. The alignment of the 80-mer sliding windows in both servers considers cross-reactivity between the query sequence and aligned sequences if there is 35% identity within 80 amino acid portion of the aligned proteins. In addition, SDAP also provides an additional feature which meets the second requirement for fulfilling FAO/WHO allergenicity rules. This server provides *'exact match for contiguous amino acids'* search where the cut-off value is 6 amino acids. We also analyzed the sequences of CT-B, LT-B, Cry1Ab and cp4 epsps for presence of Asparagine-X-Threonine/Serine (where X is not proline:P).

5.4 Results and Discussion

We have analyzed amino acid sequences of antigens; CT-B and LT-B which were used in this dissertation for generation of corn-derived vaccines, to assess their allergenic potential at the sequence level. We also wanted to analyze sequences of proteins which are

expressed in commercial crop varieties and therefore which are already in food supply. We chose insecticidal protein Cry 1Ab expressed in Bt corn and herbicide tolerant enzyme cp4 epsps (5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens*, involved in aromatic amino acid biosynthesis) which is expressed in roundup ready soybean (Fischhoff et al, 1987, Harison et al, 1996).

CT-B & LT-B

FASTA search for finding homologous sequences by using cut off value of $e<0.01$ did not yield any LT-B or CT-B homologous among allergenic proteins listed in either FARRP or SDAP. The 80-mer sliding window search did not yield any hits in the aforementioned databases either. However, as it can be seen from Figure 1a and b, exact match search for contiguous amino acids by using 6 amino acids as cut off value, we found 2 matches for CT-B and 1 match for LT-B, respectively. Figure 1 also indicates relevant potential N-glycosylation sites.

The two allergens that have 6 amino acids exact match to CT-B are **Can f 3** (Genbank accession number: AAB30434) which is dog serum albumin allergenic to human and **Dic v a** (Genbank accession number: 76352) which is a lipid binding polyprotein from lungworm (*Dictiocaulus viviparous*) causing hypersensitivity in cattle. The allergen with a 6 amino acid exact match to LT-B is a wheat protein Tri a TAI. This short segment resides in signal peptide of LT-B. It was shown that the bacterial signal is cleaved in LT-B corn (Chikwamba et al, 2002). However if the signal peptide is not degraded in corn seed after cleavage it might still posses allergenic potential. Assessment of these matches and their possible significance are given in below.

FAO/WHO rules (2001) states that a protein might be an allergen if it shares 35% identity over 80 amino acid window and 6 contiguous amino acids with an allergen. Since these rules were published there have been criticisms over them by the scientific community. Only a small fraction of IgE epitopes have been identified. Given the fact that this characterization is done through *in vitro* mapping assays by using only small number of sera obtained from allergic individuals, what is known about allergic epitopes is far from being complete. It has been indicated by different groups that most known IgE epitopes are at least eight-amino acid long (Chatchatee et al, 2001). It was also shown that high affinity binding which is more relevant to *in vivo* allergenicity also requires at least eight amino acids (Banarjee et al, 1999, Rabjohn et al, 1999). Most striking evidence regarding six-amino acid window search size was shown by Stadler and Stadler in 2003. They indicated that whatever the source of the protein is, 60-80% of all protein sequences have six-amino acid matches to known allergens.

Based on these findings we also searched for presence of eight contiguous amino acids in CT-B and LT-B with known allergens using SDAP and FARRP databases. We did not find any matches for these queries. However as Banerjee et al, also reported in 1999, some epitopes could be as short as five amino acids. Therefore these three matches would still have potential to cause allergy. In addition, presence of two potential N-glycosylation sites in CT-B, and one N-glycosylation site in LT-B (Figure 1) requires thorough assessment of potential allergenicity of recombinant products in corn.

Cry1Ab and cp4 epsps

The same analyses were carried out for these two sequences; FASTA search, 80-mer sliding window search, 6 contiguous amino acid homology and also 8 contiguous amino acid

search with the above mentioned allergen databases. We also analyzed the sequences for presence of potential N-glycosylation sites.

The FASTA search, 80-mer alignment and 8 contigous amino acid searches did not yield any hits. When we used 6 contiguous amino acid search we found nine homologous regions to known allergens in sequences of Cry1Ab and 4 regions for cp4 epsps (Fig 2). Information regarding these allergens (source organism, function of the allergenic proteins if known, etc.) is given in Tables 2 and 3. In addition to these homologous regions, there are also eight and two potential N-glycosylation sites in Cry1Ab and cp4 epsps, respectively.

5.6 Concluding remarks

We analyzed primary sequences of CT-B, LT-B, Cry1Ab and cp4 epsps following FAO/WHO guidelines. Our analysis indicated presence of varying numbers of 6 contiguous amino acids to known allergens and potential N-glycosylation sites. Analysis of extracts obtained from Bt corn and roundup ready soybean with sera of individuals known to have food allergies indicated that these commercial transgenic crops are not allergenic (Batista et al, 2005). As it was mentioned earlier there has been growing doubts regarding value of short amino acid sequence homologies in assessment of allergenic potential of proteins (Stadler and Stadler, 2003, Silvanovich, 2006). Therefore presence of 6 contiguous amino acids and potential N-glycosylation sites in CT-B and LT-B might not render them allergenic. Moreover LT-B is highly resistant to low pH and heat. These findings raise concerns and these transgenic lines should be studied thoroughly. We suggest the following approach.

Following the decision tree approach of FAO/WHO (see Chapter 1, Section 3.8), protein extracts obtained from corn seeds transformed with either CT-B or LT-B should be

evaluated by specific (against sera of individuals and animals known to be allergenic to dog serum albumin, lung worm and wheat) and targeted serum tests. If negative result is obtained from serum screening, pepsin resistance and animal models should be utilized.

For determining whether potential N-glycosylation sites were glycosylated and if they did whether they possess α(1-3)-fucosyl and β(1,2)-xylose groups, extracts should be analyzed by MALDI-TOF-MS analysis. The above mentioned studies are essential for complete characterization of corn-derived CT-B and LT-B to be able to assess safety of the product.

5.7 References

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Database	Institute	URL
Allergome.org	Multiple partners	http://www.allergome.org
IUIS	International Union of Immunological Societies	http://www.allergen.org
SWISS-PROT	Swiss Institute for Bioinformatics	http://www.expasy.org/cgi-bin/sport-search-de?allergen
CSL	The Central Science Laboratory at Sand Hutton York, United Kingdom	http://csl.gov.uk/allergen
PROTALL	The Institute of Food Research of the United Kingdom	http://www.ifr.bbsrc.ac.uk/Protall
ALLALLERGY	German National Research Center for Environment and Health	http://www.allergy.net
BIFS	Biotechnology Information for Food Safety	http://www.iit.edu/~sgendel/fa.htm
Asthma and Allergy	German National Research Center for Environment and Health	http://cooke.gsf.de/asthmagen/main.cfm
SDAP	The University of Texas Medical Branch	http://www.fermi.utmb.edu/SDAP/sdap_ovw.html
FARRP	Monsanto Company	http://www.allergenonline.com

Table 1. Currently available public allergen databases, institutes created and maintaining them and their URL addresses.

Allergen	6 aa match	Organism	Common name	Source or function
Gal d vitollegenin	EFVPGA	*Gallus domesticus*	chiken	Egg yolk
Pha a 1	GSAQGI	*Phalaris aquatica*	grass	Pollen
Cup a 1	GNAAPQ	*Cupressus arizonica*	cedar	Pollen
Jun v 1	GNAAPQ	*Juniperus virginiana*	eastern red cedar	Pollen
Jun a 1	GNAAPQ	*Juniperus ashei*	mountain cedar	Pollen
Jun o 1	GNAAPQ	*Juniperus oxycedrus*	prickly juniper	Pollen
Tri a 1 glutenin	QLGQGV	*Triticum aestvum*	wheat	Glutenin
Hom s 1	LPSAVY	*Homo sapiens*	human	Autoantigen
Chi t 9	YVTLLG	*Chironomus thummi thummi*	midge	Hemglobin component
Asp o 21	TLLGTF	*Aspergillus oryzae*	a kind of fungus	Amylase A
Phl p 11	ESKLKA	*Phleum pretense*	grass (timothy)	Trypsin inhibitor homologue
Eur m 14	VKRAEK	*Euroglyphus maynei*	mites	Apolipophorin

Table 2. List of allergenic proteins with 6 contiguous amino acid homologies to insecticidal protein Cry1Ab expressed in Bt corn (aa stands for amino acids).

Allergen	6 aa match	Organism	Common name	Source or function
Mala s 1	SHGASS	*Malassezia sympodialis*	fungi (molds)	Unknown
Der p 7	VKSEDG	*Dermatophagoides pteronyssinus*	mites	Unknown
Cry j 2	LLVPGS	*Cryptomeria japonica*	Japanese cedar	Pollen
Hor v 1	LAVAAA	*Hordeum vulgare*	barley	Trypsin/alpha-amylase inhibitor

Table 3. List of allergenic proteins with 6 contiguous amino acid homologies to herbicide tolerant enzyme cp4 epsps expressed in roundup ready soybean (aa stands for amino acids).

A)

Signal Peptide

1 MVKLKFGVFFT**VLLSSA**YAHGTPQ***NIT***DLCAEYHNTQIHTLNDKIFSYTE 50
51 SLAGKREMAIITF**KNGATF**QVEVPGSQHIDSQKKAIERMKDTLRIAYLTE 100
101 AKVEKLCVWN***NKT***PHAIAAISMAN 124

B)

Signal Peptide

1 MVKVKCYV**LFTALL**SSLCAYGAPQSITELCSEYRNTQIYTINDKILSYTES 50
51 MAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDTLRITYLTET 100
101 KIDKLCVWN***NKT***PNSIAAISMEN 124

Figure 1. Amino acid sequences of CT-B (A) and LT-B (B). Underlined sequences indicate 6 contiguous amino acid matches to known allergens. Italicized and bold characters indicate potential N-glycosylation sites.

A)

```
   1  MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL   60
  61  VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD   120
 121  PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ   180
 181  RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV   240
 241  LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL   300
 301  NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR   360
 361  TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV   420
 421  PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST   480
 481  NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS   540
 541  IDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID   600
 601  RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC   660
 661  LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV   720
 721  TLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGT   780
 781  GSLWPLSAPSPIGKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFL   840
 841  EEKPLVGEALARVKRAEKKWRDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI   900
 901  AMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNG   960
 961  LSCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVT   1020
1021  IHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSV   1080
1081  PADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETE   1140
1141  GTFIVDSVELLLMEE                                                1155
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B)

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   1  MSHGASSRPATARKSSGLSGTVRIPGDKSISHRSFMFGGLASGETRITGLLEGEDVINTG   60
  61  KAMQAMGARIRKEGDTWIIDGVGNGGLLAPEAPLDFGNAATGCRLTMGLVGVYDFDSTFI   120
 121  GDASLTKRPMGRVLNPLREMGVQVKSEDGDRLPVTLRGPKTPTPITYRVPMASAQVKSAV   180
 181  LLAGLNTPGITTVIEPIMTRDHTEKMLQGFGANLTVETDADGVRTIRLEGRGKLTGQVID   240
 241  VPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTRTGLILTLQEMGADIEVINPRLAGGED   300
 301  VADLRVRSSTLKGVTVPEDRAPSMIDEYPILAVAAAFAEGATVMNGLEELRVKESDRLSA   360
 361  VANGLKLNGVDCDEGETSLVVRGRPDGKGLGNASGAAVATHLDHRIAMSFLVMGLVSENP   420
 421  VTVDDATMIATSFPEFMDLMAGLGAKIELSDTKAA                            455
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Figure 2. Amino acid sequences of Cry1Ab (A-NCBI accession #: POA370) and cp4 epsps (B-NCBI accession #: Q9R4E4). Underlined sequences indicate 6 contiguous amino acid matches to known allergens. Italicized and bold characters indicate potential N-glycosylation sites.

Chapter 6: Preliminary analysis of sera specific anti-LT-B antibodies in pigs administrated orally with corn-derived LT-B

Sule Karaman , Mathew Erdmann, Kan Wang

6.1 Abstract

An oral immunization trial was conducted to test the antigen specific antibody production in pigs against B subunit (LT-B) of *E. coli* heat labile enterotoxin. Transgenic corn producing recombinant LT-B was used as a delivery agent. Pigs were fed with one of two doses of transgenic corn on days 0, 7, 21 and 34. Sera were collected weekly over a 41 day period starting the day before the initial feeding and analyzed for presence of anti-LT-B IgG and serum IgA. Our preliminary results indicated that 50% of subjects in the treatment groups had elevated levels of anti-LT-B IgG in their sera. Also, sera of 50% and 75% of the pigs in 1xLT-B and 2xLT-B groups, respectively, were anti-LT-B IgA positive. This preliminary work investigating dosage requirements for antibody induction against corn-derived LT-B formed a reference for future studies which will evaluate potential of corn-derived LT-B as an adjuvant in pigs.

6.2 Introduction

Infectious diseases cause high mortality and morbidity in livestock including pigs. Porcine reproductive and respiratory syndrome virus (PRRSV), foot and mouth disease virus (TMDV), classical swine fever virus (CSFV) and enterotoxigenic *E. coli* are among the

pathogens that have had important economic impact on the swine industry (Nilubol et al. 2004, Song et al 2005, Maurer et al. 2005, Verdonck et al. 2005, Joensuu et al. 2004). In addition to the economic loss that they cause, the prevalence of these infectious diseases raises concerns about pork safety. Therefore, development of effective, safe and cost-efficient vaccines is essential.

Recently, a significant amount of work has been done on the development of mucosal vaccines for prevention of a number of diseases that affect pigs. Oral immunization of newly weaned pigs with FaeG and that of suckling pigs with F4 fimbriae against *E. coli* (Verdonck et al. 2005, Snoeck et al.2003), oronasal administration of CSFV replicon particles (Maurer et a. 2005), administration of a DNA vaccine against PRRSV to the tongue of pigs via gene delivery gun (Barfoed et al. 2004), oral delivery of recombinant corn-derived spike (S) protein of transmissible gastroenteritis virus (TGEV) in a pre-immunize-oral boosting study (Lamphear et al. 2004) and intranasal and oral vaccination against foot-and-mouth disease (Song et al. 2005) have indicated promising results for success of mucosal vaccination in pigs.

Mucosal vaccination is particularly important and essential for prevention of enteric infections. Antigen-specific antibody induction at intestinal mucosa is required to defeat pathogens that enter the intestine (Porter et al. 1974, Bloom and Boedeker, 1996). The best level of response at intestinal mucosa can be obtained by oral immunization however; most of the protein antigens are not stable in gastrointestinal (GI) tract. Another challenge regarding oral immunization is oral tolerance. There are a few proteins which are stable in the GI tract. These include bacterial toxins such as cholera toxin (CT) from *Vibrio cholera* and its homologue heat labile toxin (LT) from enterotoxigenic strains of *E. coli.* CT and LT

are multimeric proteins made of a pentameric non-toxic subunit B and the toxic subunit A (Freytag and Clements, 2005). B subunits bind to receptors on intestinal epithelial cells and aid in internalization of the toxic A subunit which leads to a sequence of intracellular events resulting in ion and water loss. CT, LT and LT-B are strong immunogens and mucosal adjuvants (Freytag and Clements, 2005). CT-B alone is weakly immunogenic (Hyland et al. 2004). Due to toxicity of the holotoxins CT and LT, which results in severe diarrhea, much of the interest has focused on CT-B and LT-B.

The purpose of this work was to investigate antibody response against corn-derived LT-B in post-weaning pigs. This preliminary work tested dosage requirements for elicitation of LT-B specific antibodies in pigs that were orally administered corn-derived LT-B. The long term goal of this project is to investigate whether corn-derived LT-B can be used as oral adjuvant for pigs. The results presented here form a basis for further studies.

6.3 Materials and Methods

Immunization of pigs with LT-B-corn

All animal procedures were approved through the Iowa State University (ISU) Committee on Animal Care. Naturally farrrowed, cross-bred, weaned pigs were obtained at three weeks of age from a high health status herd located in Iowa. Prior to starting of the experiment, pigs were allowed a one week adjustment period with a light-bulb above their tub to keep the room warm in ISU animal facility where they were housed throughout the experiment with 15/7 light-dark cycle. Pigs were fasted 12 hours prior to syringe feeding.

Table 1 indicates the design of the overall study. Pigs were fed with regular pig feed, starter ration throughout the study. Initial LT-B immunization was done when the pigs were

four weeks old. Feedings were carried out on days 0, 7, 20 and 34. Twelve pigs were divided into three groups of four: 1) wild type (WT) group. Pigs in this group were fed with non-transgenic corn powder suspension. 2) 1xLT-B and 3) 2xLT-B groups. Pigs in these groups were fed with two different doses of LT-B corn suspensions. The 1xLT-B dose corresponds to amount used in a human Phase I clinical trial testing antibody response against potato derived-LTB (Tacket et al, 1998). In that study, the average dose of LT-B used was 750 μg per subject. We generated a dose/kg ratio by considering average weight of a human as 80 kg. We obtained a dose/kg of body weight ratio and used it as 1xLT-B dose in this study (9.38 μg/kg). We included a group receiving double dose to increase the chances of responders due to likely differences in processes in human and swine gastrointestinal systems. On day 0, 1xLTB and 2xLTB doses corresponded to body weight ratios of 9.38 and 18.76μg of LTB/kg, respectively. Due to limited amount of LT-B corn we had, for the last three feedings the doses were 8.83 and 17.76μg of LTB/kg. In order to feed pigs with a constant ratio of LTB, we increased the amount of transgenic corn powder for both group 2 and 3 following initial immunization, based on the expected weights of the pigs (see Table 1). Throughout the study, pigs within a group were all held in a single enclosure.

Both non-transgenic and LT-B-corn powders were re-suspended in Esbilac milk replacer (PetAg, Hampshire, USA) to desired concentration. The suspensions were then delivered to pigs' mouths with 60 ml syringes. Some volume of leakage occurred during each feeding. Therefore the fed volume was always close but approximate.

Collection of blood samples and analysis of sera for quantification of anti-LTB IgG and anti-LTB IgA antibodies

Approximately 3 ml of blood were obtained from the anterior *vena cava* via disposable syringe and 20 gauge needle on days -1, 6, 13, 20, 27, 34, and 40. Blood samples were collected directly into serum separator tubes which were centrifuged in a large, floor model centrifuge at 3500 rpm for 25 minutes at 5°C. Sera were then aliquoted into 1.5 ml test tubes and frozen at -20°C.

For anti-LTB IgG and anti-LTB IgA analysis, high binding microtiter plates (Costar 3590, Fisher Scientific, PA, USA) were coated with mixed type gangliosides (Type III from bovine, Sigma G-2375, MO, USA), 1.5 μg/well diluted in sodium carbonate coating buffer [3.75 mM Na_2CO_3, 8.75 mM $NaHCO_3$, 0.75 mM NaN_3, pH:9.6]. Following incubation at room temperature for 1 h, 25 ng/well pure bacterial LTB (John Clements, Tulane University, LA, USA), diluted in 1xPBS (phosphate buffer saline) were loaded into wells and incubated at 37°C for 1 h. Following this, wells were blocked with 5% dry milk (Difco 232100, MD, USA) for 1 h at room temperature. Serum samples diluted in 1xPBS were loaded and incubated at 37°C for 1 h. Next, plates were incubated at 37°C for 1 h with biotinylated anti-pig-IgG (Bethyl Laboratories Inc. A100-104P TX, USA) and anti-pig-IgA (Serotec Ltd. AAI40P Oxford, UK) diluted in 1% dry milk at 1/20,000 for IgG and 1/10,000 ratios, respectively. Enzyme streptavidin-horse radish peroxidase (554066, BD Biosciences, X, USA) was added to the plates at 1/20,000 ratio diluted in 1% milk and incubated at room temperature for 30 minutes. Color development was obtained by incubating the plates at room temperature for 30 minutes with ABTS (3-ethylbenzthiazoline-6-sulfonic acid, Sigma A-1888, MO, USA) substrate. Incubations with enzyme and substrate and end-point readings

were carried out as recommended by the manufacturer (BD Biosciences). A total volume of 200 µl per well was used at each step except for blocking. Milk 250µl (5%) was loaded into each well for blocking. Anti-LTB antibody levels were calculated as µg/ml by using standard curves developed from pig reference serum (Bethyl Laboratories Inc. A100-104P TX, USA). The standard ranges used were 0.65, 0.325, 0.161, 0.08, 0.04, 0.02 and 0.0 µg/ml and 4, 2, 1, 0.5, 0.25, 0.125, 0.063 and 0.031 µg/ml for IgA and IgG, respectively.

Statistical analysis

Anti-LT-B antibody kinetic data for serum IgG and serum IgA were analyzed with ANOVA for estimation of mean differences in SAS® V8.2.

6.4 Results and Discussion

We investigated the ability of corn-derived LT-B to induce LT-B specific antibody production in pigs. The sequence of the synthetic LT-B gene used to generate a transgenic corn line used in this study came from *E. coli* strain that infects human (NCBI #: J01646). LT-B subunits of heat labile holotoxins obtained from *E. coli* strains infecting pigs (NCBI #: M17873) and human are highly homologous. As indicated in figure 4, these two subunits are 99.97% homologues at amino acid sequence level and three dimensional structures of both versions are considered the same (Spangler 1992). Therefore antibodies raised against different versions cross react (Kazemi and Finkelstein, 1990).

Pigs were orally immunized with ground LT-B corn in a liquid suspension. In this pig feeding trial, two different doses of LT-B corn were used. A dose/body weight ratio based on a human trial carried out with LTB-potato (Tacket et al., 1998) was taken as reference. The ratio used in the trial carried out by Tacket et al., 9.38 µg/kg body weight, formed 1xLTB

group in this study. Since human and pig gastrointestinal tract and immune systems are likely to function differently, a 2xLTB dose group was included in this study to avoid possibility of non-induction of the specific antibodies with 1x dose. LT-B corn was given to pigs on days 0, 7, 21, and 35. Blood samples were collected on days -1, 6, 13, 20, 27, 34 and on day 41. Here we report induction of LT-B specific serum IgG and serum IgA antibodies in pig sera upon oral administration of corn-derived LT-B.

Induction of LT-B specific IgG and IgA in sera of each individual subject:

LT-B specific antibodies were quantified with ganglioside dependent ELISA. Figure 1A shows anti LT-B specific IgG in four pigs of group that were administrated with wild type non-transgenic corn. One of the subjects (pig-3), showed a higher level of specific IgG compared to the other three pigs even before the experiment started (day -1). While it was not clear whether this high level was due to LT-B contamination in the sample or possible passive immunity through maternal pass over, this pig seemed to have higher specific antibody throughout the experiment. Interestingly, when IgA responses were analyzed in this group, pig-3 did not seem to be an outlier (figure 1.D). Instead, in sera of all subjects in WT group a comparable low level of background IgA levels were detected throughout the study.

When IgG levels of individual subjects in 1xLTB group were analyzed, it was observed that, pig-7 showed a slight response on bleed days 27 and 34 (Figure 1.B). Very high levels of anti-LT-B IgA was detected in serum of pig-7 on day 27 following similar pattern that was observed for IgG level of this particular subject. Serum IgA level of pig-5 was also elevated on day 27 even though anti-LT-B IgG was detectable on day 34 for this subject (Figure 1.E&B). Anti-LT-B IgG and IgA levels peaked at different days in sera of the responders in this group. Peak values for both kinds of antibodies were observed on day 27 in

serum of pig-7 and on day 41 in serum of pig-5. The IgA level in serum of pig-7 dropped largely on day 34 and slightly increased after last feeding on day 35. On the other hand, IgA level in serum of pig-5 continued increasing gradually until the end of the study. On day 41, IgA levels of both pig-7 and pig-5 seemed comparable.

As shown in figure 1.Ca, much rapid induction of anti-LT-B IgG were observed in serum of responder pigs in 2xLTB group (pig-10 and pig-12). On day 13, the specific IgG levels in serum of pig-10 was approximately 5.5 times higher than the average of pre-feeding values of this group. Similarly, on day 13, the specific IgG level in serum of pig-12 was also higher (3.4x) than average of pre-bleeding antibody levels in this group. A second peak value was recorded IgG levels in serum of pig-10 on day 34 following administration of corn derived LT-B on day 21.

When serum IgA levels were analyzed in sera of 2xLTB group (Figure 1.F), three of the subjects seem to have increased levels of LT-B specific IgA. These were pig-9, pig-10 and pig-11. Although anti-LT-B IgGs were recorded in serum of pig-12, anti-LT-B IgA levels in serum of this subject were not different than background. The level of IgA in serum of pig-9 began increasing on day 20, continued to increase until day 34, however dropped after day 34 even though the pigs were fed one more time with LT-B corn on day 35. Anti-LT-B IgA levels in serum of pig-10 peaked twice as it was the case for IgG levels in serum of this particular pig. However peaks were observed on days 27 and 41 for IgA levels. Anti-LT-B IgA levels in serum of pig-11 started increasing on day 27 and kept increasing until day 34 which was followed by drop on day 41. It should be noted that even though pig-11 is the subject with the highest level of serum IgA, anti-LT-B IgG level in serum of this subject did not increase compared to the level recorded before the first feeding.

As a result, 2 out of 4 of the subjects in each transgenic corn treatment groups (1x LTB and 2xLTB) showed induction of anti-LT-B IgG antibodies upon oral administration of LT-B corn, with higher levels in 2xLTB group compared to that observed in 1xLTB group. While anti-LT-B serum IgA levels were elevated in 50% of the subjects in 1xLTB group, anti-LT-B antibody levels were detectable in sera of 75% of the subjects in 2xLTB group.

Overall serum IgG analysis:

As it can be seen in Figure 2, anti-LT-B IgG levels in sera of the 2x LTB group began to increase on day 6 upon initial sensitization performed on day -1. The IgG level in 2x group reached a peak on day 13 after first two feedings and began to decline gradually. IgG levels in 1x LTB group and WT group followed a similar pattern and antibody levels until day 34. On day 41 IgG levels in 1xLTB group seemed increasing compared to WT group. If antibody levels were screened during a longer period, an increase in IgG levels in 1x LTB group might have been observed. This delayed response is likely due to the low amount of LTB given with 1x dose, because the increase in IgG levels was observed 7 days after the first feeding for 2xLTB group.

A high background for the level of LT-B specific IgG was observed in wild type group and in sera of treatment groups (1xLTB and 2xLTB) on day -1 prior to initial sensitization with corn-derived LT-B. This might be due to: 1) cross-reactivity of other proteins in sera of pig with secondary antibody used in our ELISA procedure to detect anti-LTB IgGs, 2) the presence of a base level of anti-LTB antibodies in sera of piglets in all groups which might have been acquired through passive transfer of antibodies in maternal colostrums.

While increased anti-LTB antibody levels are seen in 2xLTB group (Figure 2), due to small sample size and large differences between individual subjects, the comparisons among all three groups at any sample collection day did not yield statistically significant results (p's for all pair wise comparisons >0.05).

Overall serum IgA analysis:

In the case of serum IgA overall analysis, a different pattern was observed such that, the highest anti-LT-B serum IgA level was observed on day 27 in 1xLT-B group. As indicated in figure 3, the IgA levels began to rise on day 20 for both treatment groups. The third feeding performed on day 20 boosted IgA levels in both 1xLTB and 2xLTB groups. Unlike 1xLTB group, the highest level of 2xLTB group was observed on day 34. On day 34, anti-LT-B IgA levels of 1xLTB group declined sharply, followed by an increase upon last feeding administered on day 35. Interestingly, the last feeding did not further increase the levels of IgA in sera of 2xLTB group. As in the case of overall IgG analysis (Figure 2), a high level of background was observed. However the level of background was much lower than that of IgGs. This might be due to nature of anti-LT-B antibody-secondary antibody interaction which leads to cross reactivity. Or as stated before in previous section, maternal IgG antibodies might have passed to pigs through breast feeding yielding a baseline of specific IgG antibodies at the start of the feeding trial. It should also be noted that, as in the case of comparison of averages of anti-LT-B IgG levels in all groups, comparisons between treatment groups versus wild type group or comparisons between treatment groups did not yield any significant difference on any given sample collection day.

These preliminary results indicated that two out of four subjects (50%) in treatment groups showed elevated serum IgG levels. Two out of four subjects (50%) of the 1xLTB

group produced detectable levels of serum IgA with respect to WT group. Anti-LTB IgA antibodies were detectable in three out of four subjects (75%) in 2xLTB group.

This trial forms a basis and preliminary data to design a second trial with a larger sample size to obtain more sound results. Several parameters should be considered in the next study: 1) Using pigs from mothers with no anti-LT-B antibodies could decrease the background antibody readings. 2) Adding a higher dose to the study might eliminate occurrence of non-responders because certain individuals might react to higher doses. 3) Monitoring the antibody levels for longer periods could give a better idea to determine the number and timing of antigen administration. 4) Functionality of antibodies should be tested with challenge with enterotoxigenic strain of *E. coli* which causes diarrhea in pigs.

This study suggests that corn-derived LTB can induce serum specific IgG and IgA in pigs. More experiments are needed to confirm whether LTB-corn has potential to serve as a vaccine against diarrhea and it might also be utilized as carrier or adjuvant for antigens to treat other pig diseases.

6.5 Further experiments

Fecal samples, saliva and tears are going to be analyzed by our collaborators Dr. Joan Cunnick and her student April Beyer. In this chapter we report LT-B specific serum IgG and serum IgA responses.

6.6 Acknowledgments

We are thankful to Drs Hank Harris and Matthew Erdman of Iowa State University Animal Science Department for pig feeding and sample collection. We are also thankful to

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Groups	# of pigs	Toxin	Day 0			Day 7			Day 21			Day 34		
			(µg of LTB)	(µg/kg)	(g of corn)	(µg of LTB)	(µg/kg)	(g of corn)	(µg of LTB)	(µg/kg)	(g of corn)	(µg of LTB)	(µg/kg)	(g of corn)
WT	4	WT corn	0	0	5	0	0	5.9	0	0	0	0	0	9.7
1xLTB	4	LT-B corn	68	9.38	5	80	8.83	5.9	120	8.83	8.8	184	8.83	9.7
2xLTB	4	LT-B corn	136	18.76	10	160	17.66	11.72	240	17.66	17.66	368	17.66	19.4
Age of pigs (weeks)			4			5			7			9		
Weight of pigs (lb)			16			20			30			46		

Table 1. Design of the study.

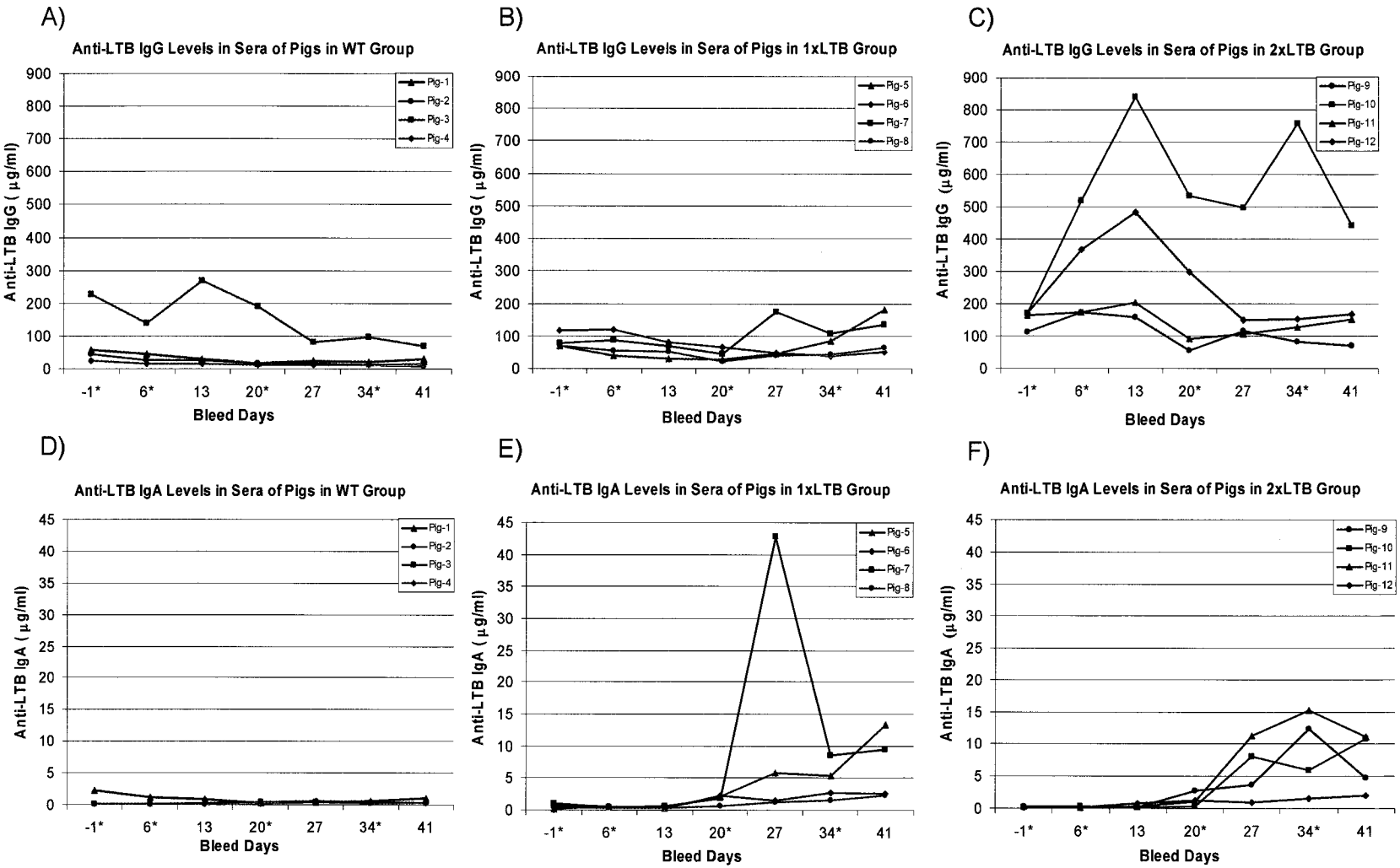

Figure 1. Anti-LT-B IgG and anti-LT-B IgA levels in sera of 12 pigs studied. * indicates the day before administration of LT-B corn. The results are presented as µg/ml. A), B), C) indicate anti-LT-B IgG levels in sera of pigs in WT, 1x LTB and 2x LTB groups, respectively. D), E), F) indicate anti-LT-B IgA levels in sera of pigs in WT, 1x LTB and 2x LTB groups, respectively.

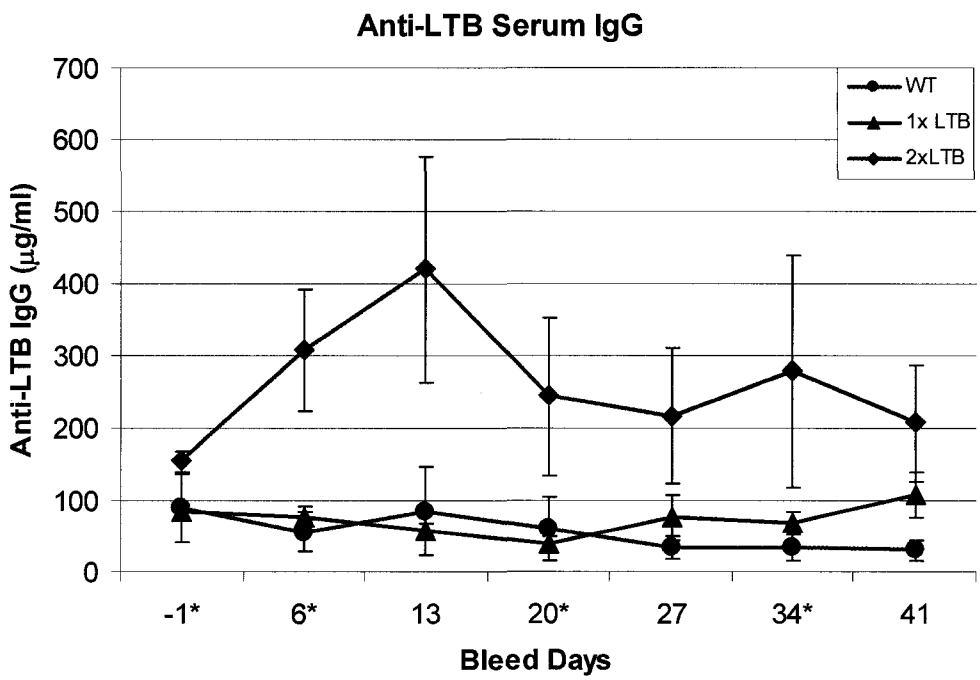

Figure 2. Anti-LT-B IgG levels in sera of subjects in WT, 1xLTB and 2xLTB groups. Four pigs were used in each group as subjects. *indicates the day before administration of LT-B corn. The results are presented as mean±SE in μg/ml.

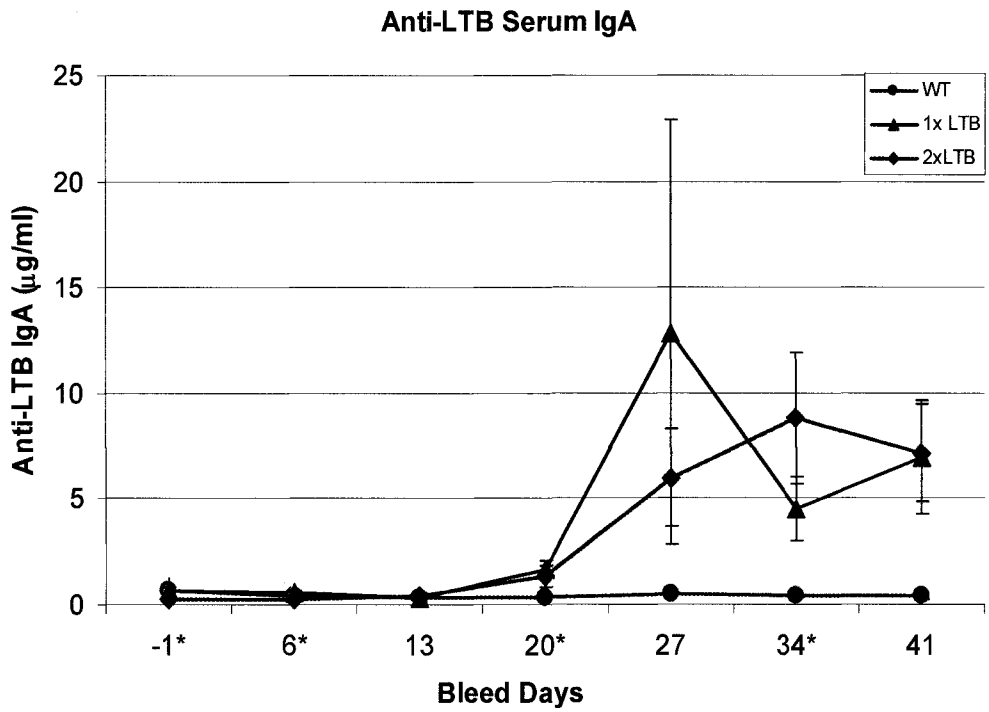

Figure 3. Anti-LT-B IgA levels in sera of subjects in WT, 1xLTB and 2xLTB groups. Four pigs were used in each group as subjects. *indicates the day before administration of LT-B corn. The results are presented as mean±SE in µg/ml.

```
          1                                                  50
LT-B(h) APQSITELCSEYRNTQIYTINDKILSYTESMAGKREMVIITFKSGATFQV
LT-B(p) APQTITELCSEYRNTQIYTINDKILSYTESMAGKREMVIITFKSGETFQV

          51                                                 100
LT-B(h) EVPGSQHIDSQKKAIERMKDTLRITYLTETKIDKLCVWNNKTPNSIAAIS
LT-B(p) EVPGSQHIDSQKKAIERMKDTLRITYLTETKIDKLCVWNNKTPNSIAAIS

          101 103
LT-B(h) MEN
LT-B(p) MKN
```

Figure 4. Amino acid sequences of LT-B subunits from enterotoxigenic strains infecting human (h) and pigs (p). Two versions are 99.97% homologous.

Chapter 7: General conclusions

In this work we examined the potential of corn to be utilized as a vehicle for production of vaccines and vaccine components which would be used in oral immunization regimens for both human and livestock. We used nontoxic, highly homologous B-subunits of heat labile toxin from enterotoxigenic strains of *E. coli* (LT-B) and cholera toxin from *Vibrio cholerae* (CT-B) as model antigens. The following aspects of plant-derived vaccines were explored in this dissertation; 1) investigation of long term antibody and immune memory responses to a booster administration following initial oral immunization with LT-B corn (Chikwamba et al, 2002), 2) generation of transgenic corn producing CT-B in their seeds and structural and immunogenic characterization of corn derived CT-B, 3) using LT-B and CT-B as carriers to produce a corn derived oral vaccine against Alzheimer's disease, 4) evaluation of the allergenic potential of LT-B and CT-B at amino acid sequence level to determine a strategy for complete analysis of these two transgenic corn lines, 5) analysis of immunogenic potential of corn derived LT-B in pigs.

Chapter 2 indicates that plant derived vaccines can induce immune memory formation. Mice were immunized with LT-B corn when they were young and a booster administration was given eleven months after initial immunization. LT-B specific IgG and IgA antibody concentrations were monitored during 11 months. When mice were 14-mo-old, booster immunization through feeding of corn derived LT-B and injection of bacterial LT-B was carried out. A rapid and strong anti-LT-B IgG and IgA response was observed. An accelerated immune response in this case shows that antigens delivered in edible tissues of plants through feeding can elicit immune memory. This finding provides a new evidence for

potential of vaccination with plant derived antigens through feeding. This work also examined responses of aged mice against corn derived LT-B. A lower level of IgG antibodies in sera and lower levels of fecal IgA were recorded in aged mice compared to the response observed in young mice. Serum IgA levels in sera of aged mice were comparable to that of young mice. Interestingly, fecal IgA and serum IgA were detectible in sera of aged mice as early as one week following first immunization. These differences were attributed to alterations that occur in the mucosal immune system of aged individuals. In accordance with observations reported by other studies, we showed by examining levels of total IgG_{2a} and IgG_1 in aged and young mice that aged mice responded with more of Th-2 type immune response. This observation might relate to the faster IgA response in aged mice. The results of this study evidenced the following; 1) immune memory forms upon administration of antigens produced in palatable parts of plants when they are delivered through feeding, 2) lower levels of fecal IgA and serum IgG observed in sera of aged mice suggest that LT-B may not be an efficient adjuvant in aged individuals when used for the first time.

Expression of CT-B in corn seeds, biochemical characterization of corn derived CT-B and its immunogenic properties are reported in Chapter 3. Our results indicated that CT-B can be expressed at high levels in corn seeds. It was shown via ganglioside dependent ELISA that corn derived CT-B forms pentamers as its bacterial counterpart. Corn derived CT-B is also capable of inducing CT-B specific IgG. We also wanted to address whether corn derived CT-B and corn derived LT-B would work synergistically to induce higher levels of antibodies when given together. Our results were suggestive of this interaction on certain days of sample collection. An interesting outcome of our mice feeding study was that antibodies raised against corn derived CT-B do not cross react significantly with bacterial

LT-B. This observation may be explained by possible structural differences of corn derived CT-B when compared with bacterial CT-B. Western blot analysis of corn derived CT-B suggested that the bacterial signal is not cleaved. Therefore corn derived CT-B may have different epitopes leading to presence of new structures on anti-CT-B antibodies which do not recognize the bacterial LT-B. Partial protection against toxin challenges observed in our treatment groups may also be explained due to this structural difference. Further studies need to be conducted to address this observation. To test this hypothesis, a transgenic corn line transformed with CT-B gene encoding a mature peptide (without signal peptide) should be produced and this corn should be evaluated in mice. In this mice experiment one should compare immunogenicity of mice fed with 1) corn-derived CT-B mature protein, 2) corn-derived CT-B (with bacterial signal peptide, as this chapter) and 3) bacterial CT-B spiked corn meal. This comparison will be helpful to address importance of the bacterial signal peptide in immunogenicity of the subunit.

Our efforts to produce a corn derived vaccine against Alzheimer's disease (AD) are presented in Chapter 4. Amyloid beta protein ($A\beta_{42}$) plays a critical role in pathogenesis of Alzheimer's disease. Active immunizations with amyloid beta and passive immunizations with anti-amyloid beta antibodies were shown to treat and prevent induction of the disease in mice models of AD. LT-B and CT-B have been shown to act as carriers for unrelated antigens. We generated fusion genes with amyloid beta protein to use LT-B and CT-B as carrier for this antigen. Analysis of calli transformed with LT-B- $A\beta_{42}$ fusion gene indicated expression of LT-B and correct assembly of pentamers. However although mRNA was detectable for the whole fusion gene, we failed to detect $A\beta_{42}$ protein. Analysis of protein extracts from transformed calli indicated that transgenic protein (product of the fusion gene)

moves along with pentameric LT-B. This suggests that amyloid beta protein was either cleaved from the fusion protein by an unknown protease or the part of mRNA encoding for amyloid beta might not be stable. Our second approach utilized an internal permissive site (a site in surface exposed part of CT-B which allows insertion of foreign epitopes) to express amyloid beta epitopes on CT-B. We generated two versions of the gene with N-terminal and middle epitopes of amyloid beta protein. Our gene design failed to produce pentameric or monomeric CT-B. We also failed to detect epitopes of amyloid beta. While we followed the recombinant protein strategies used successfully by other groups, we did not produce functional fusion proteins that would allow us to continue the experiments. Success of fusion strategies has to be evaluated on a case by case basis. For the LT-B $A\beta_{42}$ fusion a new construct without any hinge and with different hinges might be tested. In addition, N-terminal fusion of $A\beta_{42}$ to LT-B may yield to different outcomes. For insertion of epitopes into the permissive site of CT-B shorter fragments might be tested because we had introduced neighboring amino acids around the epitopes.

Ever since the StarLink corn incidence, the potential of genetically modified food crops to trigger allergies has become a great concern. Analysis of allergenic potential of model antigens used in this study at amino acid sequence level was described in Chapter 5. We wanted to include analysis of transgenic crops which are already in food chain and deregulated to infer insights regarding usefulness of this analysis. Sequences of Cry1Ab (insecticidal protein from *Bacilllus thrungiensis*) and cp4 epsps (herbicide tolerant enzyme, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens*) which are recombinantly produced in Bt corn and Roundup Ready soybean respectively were selected to serve as controls. FAO/WHO guidelines were followed and two publicly available

databases (FARRP and SDAP) were used for the analysis. Potential N-glycosylation sites on the sequences were also determined since these sites might possess allergenic α(1-3)-fucosyl and β(1,2)-xylose glycosyl groups. Varying numbers of 6 contiguous amino acids homologous to known allergens and N-glycosylation sites were found in all sequences analyzed. These findings suggest that further analysis of transgenic corn lines transformed with LT-B and CT-B are required. Protein extract from these lines should be tested against sera of individuals with food allergies. A broader analysis should be performed with sera of individuals who have different classes of allergies. Interestingly, despite the presence of a high number of 6 contiguous amino acids homologous to known allergens and N-glycosylation sites, similar analysis with sera of individuals with food allergies indicated that Bt corn and Roundup Ready soybean protein extracts do not cross react with IgEs. This comparion may suggest that LT-B and CT-B corn itself may or may not cause food allergy. However, because LT-B and CT-B are known to be strong immunogens, similar analysis with LT-B and CT-B corn may yield different outcomes. Therefore full analysis following FAO/WHO guidelines should be conducted. If negative results are obtained from serum screening, pepsin digestion and animal models should be utilized.

Enterotoxigenic strains of *E. coli* is a leading cause for diarrhea in humans, it also causes severe diarrhea in pigs. A pig feeding experiment with LT-B corn was carried out and results are presented in Chapter 6. We fed newly weaned pigs with two doses of corn derived LT-B (1xLTB: 8.83 μg/kg of body weight; 2xLTB: 17.76μg/kg of body weight) four times. Elevated anti-LT-B specific IgG antibodies were detectable in sera of 50% of the subjects in treatment groups. Fifty percent of the subjects in 1xLTB group produced detectable levels of serum IgA with respect to the WT group. Anti-LTB IgA antibodies were recorded in 75% of

the subjects in 2xLTB group. These preliminary results suggest that LTB-corn produced in our lab is capable of inducing serum IgG and IgA in pigs. Therefore LTB-corn has potential to serve as a vaccine for pigs against diarrhea and it might also be utilized as carrier for antigens to treat other pig diseases.

Major conclusions inferred from the work presented in this dissertation can be summarized as follows. Plant derived vaccines are capable of inducing immune memory which strengthens the potential of immunizations through feeding to be part of clinical practices. Administration of corn derived CT-B and LT-B in combination has potential to induce higher levels of protective antigens. This outcome suggests that a mixture of these two components may be used in vaccine preparations targeting protection against both cholera and ETEC. Ability of corn seeds to allow expression of high levels of recombinant proteins and ability of LT-B and CT-B to serve as carrier for unrelated antigens makes them attractive candidates to produce fusion proteins. Many versions of the peptides have to be tested because not all fusion strategies lead to functional proteins. A transient corn expression system for screening of high number of constructs should be developed. Use of corn leaf as transient testing system for this purpose is being investigated in our lab. Amino acid sequences of LT-B and CT-B show similarities to short fragments found in a few known allergens. There are also potential N-glycosylation sites on these two proteins. Complete analysis of allergenic potential of these two transgenic corn lines has to be conducted following FAO/WHO guidelines. Testing immunogenic potential of LT-B corn in pigs has also indicated its usefulness of corn derived LT-B in vaccine preparations for preventing diarrhea in pigs. Our results strengthen the potential of corn to be used as production and delivery vehicle for antigens through oral immunization.