Evaluation of low dose exposure and immunogenicity of transgenic maize expressing the Escherichia coli heat-labile toxin B subunit when fed intermittently and daily

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Evaluation of low dose exposure and immunogenicity of transgenic maize expressing the *Escherichia coli* heat-labile toxin B subunit when fed intermittently and daily

by

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Abstract

Transgenic maize expressing the non-toxic B subunit of the *Escherichia coli* heat-labile toxin (LT-B) in seed has proven to be an effective oral immunogen in mice. Currently, there is considerable concern over accidental consumption of transgenic maize expressing LT-B by humans and domestic animals. While consuming maize-expressed LT-B appears to have no toxic side effects, we have yet to define nonimmunogenic levels of transgenic LT-B when ingested. We also intend to determine if accidental exposure to LT-B could affect a later response to a vaccine containing LT-B as either an antigen or a carrier.

Our first goal was to determine the largest dose of LT-B orally administered in mice that does not result in a measurable immune response. Mice were fed low doses of LT-B intermittently (days 0, 7 and 21) resembling vaccine dose scheduling. To determine the effects of previous exposure on vaccine administration, we fed mice intermittently or daily for 28 days to resemble two distinct inadvertent exposure scenarios. We subsequently boosted all mice with vaccine-level doses of LT-B. To determine immune responses, serum and fecal pellets were collected weekly for measurement of LT-B-specific antibodies.

Mice fed 0.02 µg LT-B intermittently demonstrated immune priming in 62.5% of the animals. Mice that were fed ≤ 0.002 µg LT-B showed no increase in specific antibody nor did they demonstrate immune priming, thus indicating that 0.002 µg LT-B was the highest nonimmunogenic dose tested. Mice that were exposed to maize-derived LT-B, whether daily or intermittently, generate dose-dependent antibody responses to LT-B. All animals that had been previously exposed to LT-B by either intermittent or daily feeding responded strongly to the vaccine-like booster doses, indicating that mice orally exposed to LT-B do not develop oral tolerance to LT-B. Thus, inadvertent oral exposure to LT-B may not negatively impact future vaccinations containing LT-B as either an antigen or carrier.
Chapter 1: Introduction and Literature Review

Thesis Organization

This thesis consists of four major sections. The first chapter includes a detailed review of the literature upon which the subsequent research is based. The following two chapters consist of manuscripts prepared for submission to scientific journals that describe the research conducted and the results obtained. The first manuscript has been published in the journal *Environmental Health Perspectives* (2007 Mar;115(3):354-60). The second manuscript has been prepared for upcoming submission to *Infection and Immunity*. The fourth chapter is a discussion of the findings from the research conducted as described in the previous two chapters.

Literature Review

I. Vaccines

A. Introduction. Development of vaccines is considered one of the most important public health advancements of the 20th century. With the development of childhood vaccination programs in the U.S., there has been a 90 to 100% decrease in death and disease caused by several formerly common childhood infections (Ogra et al. 2001). In the last 50 years alone, the U.S. has seen a 95 to 100% reduction in death and disease due to diphtheria, pertussis, tetanus, poliomyelitis, smallpox, measles, mumps, and rubella (Ogra et al. 2001). The advancements have lead to the worldwide eradication of smallpox and the near eradication of poliomyelitis.

B. Vaccine routes and types. The vast majority of vaccines currently licensed for use in the U.S. are parenterally injected and are composed of whole pathogens, either live-attenuated or killed (inactivated), toxoids or purified capsular polysaccharides (Ogra et al. 2001). These vaccines have reduced or eliminated infections from the associated pathogens.
and undoubtedly saved millions of lives. However, the medical and scientific communities are beginning to see the limitations of parenteral vaccines and current research is now focusing on alternatives to parenteral delivery in order to facilitate vaccination programs in developing countries. One of the problems associated with parenteral immunization is the use of needles, which in developed countries is viewed as unpleasant and something parents want their children to be able to avoid. In developing countries, needles spread blood-borne diseases from person-to-person through their reuse due to short supply as well as through inadvertent needle sticks due to the inability to dispose of them properly (Levine 2003).

Alternatives to parenteral delivery that have been recently investigated include mucosal (most commonly through the oral or nasal routes) and transcutaneous administration. Unlike parenteral immunization, which at best is only able to stimulate serum IgG and cell-mediated immunity (Goldsby et al. 2000), all three of these routes (oral, nasal and transcutaneous) have been shown to elicit both serum and mucosal antibody production in addition to stimulating cell-mediated immunity (Hammond et al. 2001; Levine 2003; Poland et al. 2002). Mucosal antibody, immunoglobulin A (IgA), production is especially important in preventing infection since most pathogens enter the body via mucosal surfaces. All of these immunological benefits are gained while avoiding the use of needles.

In addition to changes in the route of administration, advancements in biotechnology have facilitated the development of vaccine types other than the traditional live-attenuated, inactivated, toxoid or polysaccharide types. Early vaccines relied on culturing pathogens and then modifying or purifying components of them to be used in vaccines (Goldsby et al. 2000). Currently, genetic manipulation provides a means by which the genes for specific antigenic components can be expressed in safe vector systems and subsequently purified (Goldsby et al. 2000). These systems include non-pathogenic bacterial, yeast, or mammalian cell cultures. More recently these genes have also been expressed in edible plants to
facilitate oral vaccination without purification (Mason et al. 2002; Mercenier et al. 2001). Edible plants as oral vaccines will be the primary focus of this review.

C. **Mucosal immunity and oral tolerance.** In order to fully understand the benefits and drawbacks of oral immunization, an understanding of how the gut-associated lymphoid tissue (GALT) differs from systemic immunity is necessary. The systemic immune system functions under normally sterile conditions and thus every foreign antigen is attacked swiftly; however, GALT is in constant contact with foreign antigens such as food proteins and commensal bacteria and thus needs to carefully select which antigens necessitate an immune response. GALT is constantly down-regulating or suppressing effector cells so that excessive inflammation does not result from the constant exposure to foreign antigen (Holmgren and Czerkinsky 2005; Mayer 2000; Mestecky et al. 2005; Mowat 2005). Because of these intrinsic differences, formulating antigen for an oral vaccine is more complex than that for non-mucosal vaccination and generally necessitates mixing your antigen of choice with a mucosal adjuvant (Mowat 2005; Ogra et al. 2001) or conjugating it to a carrier. This adjuvant or carrier would direct the immune cells in the gut towards an immune response and away from suppression.

The typical immune response to oral antigen is described in a review by Mayer (2000) and is summarized briefly below. It begins with the uptake of antigen from the lumen of the gut into specialized epithelial cells called M cells. Antigen passes through the M cell and then is taken up by a macrophage or dendritic cell (DC). The DC or macrophage processes the antigen, travels to the underlying Peyer’s patch, and presents the antigen to lymphocytes inducing an active immune response (Gullberg and Soderholm 2006). The cytokine microenvironment of the Peyer’s patch causes B cells to undergo class-switching towards an IgA isotype as well as to express homing receptors which direct these cells towards mucosal tissues. Activated B cells then travel to the mesenteric lymph nodes, through intestinal lymphatics to the thoracic duct to the vena cava. From here they circulate
in the blood and home to mucosal sites where they often end up in the lamina propria as terminally differentiated plasma cells which secrete IgA into mucosal surfaces.

Two unique features of the mucosal immune system are the common mucosal immune system (CMIS) and oral tolerance. CMIS refers to the way all mucosal associated lymphoid tissues are interconnected due to the mucosal homing receptors expressed after activation (Holmgren and Czerkinsky 2005). The specific mucosal tissues to which effector cells are likely to migrate is generally limited to a certain extent through compartmentalization. For example, oral immunization is likely to induce antibody production not only in the gut, but also in the naso/oropharynx and mammary secretions (Holmgren and Czerkinsky 2005; Ogra et al. 2001). While oral immunization is not strictly limited to antibody production in these two sites, these sites are more likely to secrete protective amounts of antibody. Nasal immunization, on the other hand, is likely to induce antibody production in the lungs, naso/oropharynx and vagina (Holmgren and Czerkinsky 2005; Ogra et al. 2001). Thus, determining the best route of immunization is dependent upon which organ system most needs protective antibody.

Oral tolerance is a phenomenon of the mucosal immune system and refers to the state of systemic tolerance that results from previous oral administration of antigen. Oral tolerance was first described by Wells (1911) who orally administered an egg protein to guinea pigs and was unable to elicit an immune response when he subsequently boostered them systemically by injection. Unlike an active mucosal immune response, the processes of oral tolerance have not been fully elucidated even though the phenomenon has been observed for decades.

From the research that has been performed to date, we are beginning to understand the underlying mechanisms of oral tolerance. When administered orally, soluble antigens tend to induce tolerance more often than particulate antigens (Mayer 2000). Intestinal epithelial cells (IECs) covering the lamina propria are suspected to play a larger role in oral
tolerance than M cells which cover the Peyer’s patch. This is because IECs take up soluble antigen from the lumen whereas M cells typically sample only particulate antigens (Chehade and Mayer 2005; Mayer 2000; Mowat 2005). Rimoldi and Rescigno (2005) suggested that IECs provide the signals necessary to determine whether the response to antigen will be tolerance or active immunity. Their research indicates that in the presence of bacteria, pathogenic or not, IECs produce CCL-20, a chemokine which recruits immature DCs (Rimoldi et al. 2004). Of more importance is that in the presence of pathogenic bacteria, IECs also produce inflammatory mediators such as IL-8. Only DCs that were exposed to the soluble inflammatory mediators such as IL-8 produced by IECs were activated (Rimoldi et al. 2004). Moreover, previous research indicated that immature DCs constitutively migrate to peripheral lymphoid organs where they present antigen and induce tolerance (Steinman et al. 2003). Therefore, they concluded that in a state of inflammation, DCs are activated before presenting antigen and induce active immunity, whereas without inflammatory signals, immature DCs present antigen and induce tolerance. This is merely one pathway for the induction of tolerance, however, as T cells in the Peyer’s patch have also been implicated in oral tolerance (Mayer 2000).

DCs, however, are only mediators in the signaling process and do not actually carry out the effector processes of tolerance. Regulatory T cells of various subsets have been identified as the cells that ensure active immunity is not carried out. The currently known subsets and their suspected mechanisms of tolerance have been recently reviewed by Chihade and Mayer (2005) and will be discussed briefly below. Three subsets of CD4+ T cells, all acting in an antigen-specific manner, have been identified as regulatory cells in the gut: Th3 cells, TR1 cells and CD4+CD25+ T cells. Th3 cells produce transforming growth factor-β (TGF-β) in response to antigen. TGF-β, which has long been known to induce IgA isotype switching in B cells (Mayer 2000), has also been implicated in oral tolerance. TR1 cells are suspected to induce tolerance mainly through secretion of IL-10. In a mouse model, IL-10
was shown to suppress antigen-specific immune responses (Groux et al. 1997). The presence of CD4+CD25+ regulatory T cells is clearly necessary for oral tolerance; however, the exact mechanisms for this tolerance are as yet uncertain. One theory is that their expression of surface bound TGF-β leads to immunosuppression (Nakamura et al. 2001), although another study found that these cells could induce tolerance without TGF-β (Piccirillo et al. 2002) suggesting that these cells induce tolerance through multiple mechanisms.

In addition to CD4+ cells, other types of regulatory T cells have been identified that may play a role in oral tolerance. CD8+ suppressor T cells respond to antigen presented by IECs, which can act as non-professional antigen presenting cells. This appears to lead to suppression of local immune responses although the exact mechanism has not been determined (Bland and Warren 1986) and whether these cells actually play a role in oral tolerance remains controversial (Mowat 2004). Another regulatory T cell is the natural killer 1.1 T (NK-T) cell found in the liver. Small amounts of antigen sometimes pass through IECs, enter circulation via capillaries and travel through the liver where antigen presumably comes in contact with NK-T cells (Chehade and Mayer 2005). Although the exact mechanism of tolerance is unknown, it was demonstrated that animals depleted of these cells suffered from colonic inflammation (Trop et al. 1999).

Other than a state of inflammation, various other factors have been shown to affect oral tolerance induction and each of these will be discussed briefly. The dose of the antigen administered orally appears to affect how tolerance is carried out. High doses of antigen tend to result in clonal anergy or deletion of reactive lymphocytes whereas low doses result in suppression, mediated by activation of the regulatory cells listed above (Blanas and Heath 1999; Chehade and Mayer 2005; Mayer 2000). As mentioned earlier, soluble antigens are more likely to result in tolerance than particulate antigens. In addition to these antigen-related factors, several host-intrinsic factors also seem to affect oral tolerance. Host genetics appear to play a role as certain inbred mouse strains appear to be more susceptible to food
allergy than others (Lamont et al. 1988) and humans with food allergies carry common specific HLA genes. The normal flora of the host may also play a role, although mixed results have been reported. Host age may also be a factor with neonates and infants demonstrating less oral tolerance than adults given the same treatment (Chehade and Mayer 2005).

Oral tolerance, while being a challenge for the development of oral vaccines, has been studied recently as a means by which to treat or prevent the development of autoimmune diseases. The theory is that feeding autoantigens will suppress the immune response that is causing disease symptoms. Studies in animal models have demonstrated more promising results than those in humans (Arakawa et al. 1998b; Bergerot et al. 1997; Holmgren and Czerkinsky 2005; Ogra et al. 2001; Sai et al. 1996; Wardrop and Whitacre 1999). One problem may be that many animal studies have used oral tolerance as a means of preventing disease, whereas most human studies focus on treating an existing condition (Wardrop and Whitacre 1999). Some studies indicate that inducing oral tolerance in a previously systemically primed system is much more difficult than in a naïve system, if not impossible (Leishman et al. 2000; Mestecky et al. 2005). In addition, one study in mice demonstrated that oral administration of ovalbumin lead to the development of active immunity (Blanas and Heath 1999). These data demonstrate that caution must be taken in using oral tolerance as a means of preventing or treating disease in humans as this could instead lead to the development or exacerbation of disease. More information on the basic mechanisms of oral tolerance will likely be necessary in order to develop effective treatment protocols with reduced risk of disease development or exacerbation.

D. Oral vaccines and mucosal adjuvants. Despite that GALT tends towards tolerance to foreign antigen as opposed to active immunity, effective oral vaccines have been approved for human use and more are currently being developed. As mentioned earlier, oral vaccination is highly desirable because the local IgA production that is elicited is able to
neutralize and eliminate antigen before infection is established. Currently, the rotavirus vaccine, RotaTeq, is the only oral vaccine used routinely in the U.S. (CDC 2006). RotaTeq was first licensed in February 2006 after extensive clinical trials demonstrated protection as well as lack of adverse side effects such as intussusception, which was a problem with a previous oral rotavirus vaccine (Parashar and Glass 2006). Other oral vaccines approved, but not routinely used in the U.S., protect against polio (OPV), typhoid, and adenovirus. OPV was developed in the 1960s and was a highly effective vaccine; however, rarely the live virus was associated with causing vaccine-associated paralytic poliomyelitis (CDC 2000). Due to the fact that after decades of polio vaccination the only cause of polio in the U.S. was due to oral vaccination, the Advisory Committee on Immunization Practices (ACIP) recommended in 1997 that all future vaccinations were to use the parenteral, inactivated formulation (IPV) (CDC 2000). Currently OPV is still in use in developing countries that still have outbreaks of wild type polio. In these countries, the benefits of oral vaccination (local antibody response and shedding of attenuated virus to non-vaccinated individuals) outweigh the dangers (CDC 2000). Three vaccine types that protect against typhoid are available, one of which is orally administered (CDC 1994). Typhoid vaccination is not carried out regularly and is only recommended for travelers to regions with endemic typhoid, those in close contact with carriers of Salmonella enterica serovar Typhi, and laboratory personnel who frequently work with S. Typhi (CDC 1994). The adenovirus vaccine is only available for vaccination of military populations (CDC 1991). One commonality for all of these oral vaccine formulations is that they consist of live, attenuated bacteria or viruses.

While few oral vaccines are currently available, much research is focused on developing more. This research is not only focusing on developing oral vaccines to protect against gastrointestinal pathogens such as Escherichia coli, Helicobacter pylori and Shigella species (Khan et al. 2007; Nystrom and Svennerholm 2007; Ranallo et al. 2007; Sack et al. 2007; Weltzin et al. 2000) but also respiratory pathogens such as Haemophilus influenzae,
*Streptococcus pneumoniae* and the influenza virus (Foxwell et al. 2006; Lu et al. 2002; Seo et al. 2002), and urogenital pathogens such as the herpes simplex virus (Mohamedi et al. 2001). While most of these new formulations are either inactivated or subunit vaccines, they all contain an additional component to boost the response and/or target the antigens to the mucosal surface. It is clear that the majority of oral vaccines need an adjuvant of some kind in order to elicit a strong mucosal immune response, especially if live organisms are not used.

As mentioned earlier, in order to avoid inducing oral tolerance, some sort of signal is necessary so that effector cells recognize the antigen as a potential threat. All currently licensed oral vaccines contain live organisms in order to send this signal. However, live attenuated organisms could potentially pose problems for vaccine development. For example, colonization of the gut is typically necessary for a strong immune response; however, colonization is often linked with pathogenicity. Any residual pathogenicity of a vaccine strain is unacceptable and could not be used in infants and immunosuppressed or immunocompromised individuals. In addition, using live organisms runs the risk of genetic recombination within the gut which could cause reversion to virulence (Bouvet et al. 2002).

Because of these challenges to live oral vaccine formulations, much research is focused on using mucosal adjuvants or delivery systems in order to boost the immune response to poorly antigenic proteins. Although none of these are currently approved for human use, those that have been shown to enhance oral immune response to antigens include non-bacteria-derived ISCOMs (immunostimulating complexes) and microspheres as well as bacteria-derived Cpg motifs, monophosphoryl lipid A (MPL) and ADP-ribosylating bacterial toxins.

ISCOMs are spherical structures composed of glycosides which encapsulate antigen. They have been shown to improve active immunity to soluble antigens and may induce both humoral and cell-mediated responses (Ogra et al. 2001). They are able to boost responses by
fusing with the membrane of epithelial cells releasing antigen directly into the cell (Goldsby et al. 2000). Microspheres are composed of biodegradable poly (lactic/glycolic) acid (Poland et al. 2002). Orally delivered microspheres have been detected in Peyer’s patches, mesenteric lymph nodes and the spleen indicating that both mucosal and systemic immune responses could be generated through oral delivery (Ogra et al. 2001).

CpG motifs are unmethylated dinucleotides (cytosine (C) and guanine (G) connected by a phosphate group (p)) within particular base sequences of DNA. Because vertebrate DNA is typically methylated in these regions, these unmethylated portions of DNA are recognized as bacterial by toll-like receptor (TLR)-9, thus inducing the release of inflammatory cytokines (Holmgren et al. 2005). The use of CpG motifs as oral adjuvants has demonstrated the generation of Th1 responses which would be beneficial for vaccines against intracellular pathogens and viruses (Freytag and Clements 2005; Holmgren et al. 2005). Monophosphoryl lipid A (MPL) is derived from the lipopolysaccharide (LPS) of Salmonella and is recognized by TLR-2 and TLR-4 to lead to an inflammatory response and active immunity (Freytag and Clements 2005).

Unlike the previously mentioned adjuvants, the ADP-ribosylating enterotoxins are able to both direct the response towards active immunity as well as deliver conjugated antigen to the mucosal surface via the specific binding of their B subunits. These toxins include cholera toxin (CT) produced by Vibrio cholerae and heat-labile enterotoxin (LT) produced by some enterotoxigenic strains of E. coli. While they are the most potent mucosal adjuvants known, their inherent toxicity has limited their use. Attempts to retain their adjuvanticity while reducing or eliminating toxicity included the development of several mutants. These include recombinant production of their non-toxic B subunits (CT-B and LT-B) as well as whole LT toxin mutants with reduced toxicity. While CT-B has demonstrated potential as an oral adjuvant (Seo et al. 2002), LT-B has shown very weak oral adjuvanticity for urease (Plant et al. 2003) and no oral adjuvanticity for ovalbumin (Weltzin et al. 2000).
Because LT-B has shown little potential for use as an oral adjuvant, non-toxic mutants of LT have been created which have demonstrated potential for adjuvanticity (Dickinson and Clements 1995; Guillobel et al. 2000; Lu et al. 2002).

E. Plant vaccines as oral vaccines. Plants serving as factories for the production of recombinant vaccine antigens are currently an area of major interest. Much attention has been focused on this area of research due to the many benefits of using plants to facilitate oral vaccination. First, plants are much more cost effective in producing recombinant antigens than other systems (Streatfield et al. 2001; Tacket and Mason 1999). Production could be easily scaled up for mass vaccination campaigns by simply increasing acreage and vaccines could even be produced locally in developing countries if the antigen is produced in a food crop and requires no further purification (Mason et al. 2002; Tacket 2005). Second, plant cells offer protection to the antigen to increase stability at ambient temperature for storage and transport (Streatfield et al. 2001). In addition, some plant cell walls may provide protection to the antigen as it passes through the harsh environment of the stomach (Chikwamba et al. 2003) optimizing the immune response. Third, plants as vehicles for oral vaccines offer improved safety. Besides avoiding the use of needles through oral delivery, there are no known human pathogens that also infect plants, eliminating the concern for potential infectious contamination (Streatfield et al. 2001; Tacket 2005).

Along with the many advantages associated with plants as oral vaccines come challenges for vaccine developers. The major one is ensuring that a strong immune response is generated against the vaccine antigen while maintaining tolerance to the food proteins surrounding the antigen (Tacket and Mason 1999). Additionally, many antigens may require the addition of a mucosal adjuvant for the generation of an optimal immune response making tolerance to the food proteins even more difficult to maintain. Despite this hurdle, several plant-based oral vaccines are under development and some have even progressed to human clinical trials, as discussed below.
Many different plant-produced oral vaccines have been developed. Nearly all of them have been tested in animals and many of them have proven to be protective against challenge. Because of the route of entry of the pathogen, it makes sense to use oral vaccination to protect against gastrointestinal illness. Vaccines have been developed against diseases caused by *V. cholerae*, *E. coli*, rotavirus and the porcine pathogen transmissible gastroenteritis virus (TGEV) (Arakawa et al. 1998a; Joensuu et al. 2006; Streatfield et al. 2001; Wen et al. 2006; Yu and Langridge 2001). In addition, plant-based oral vaccines to non-gastrointestinal illnesses have also been developed. These include vaccines against Hepatitis B (reviewed in Kumar et al. 2007), HIV, measles, Severe Acute Respiratory Syndrome (SARS), tetanus, and tuberculosis (Huang et al. 2001; Li et al. 2006; Rigano et al. 2006; Shchelkunov et al. 2006; Tregoning et al. 2005; Webster et al. 2002)). Some of these vaccines were produced in tobacco or *Arabidopsis*, which may result in high antigen yields, but typically require purification of the vaccine antigen before oral delivery; however, many vaccines were developed in food plants such as potato, barley, lettuce, carrot, banana, cherry tomatillo, tomato and maize.

While many plant-based oral vaccines have been developed, only a half dozen have progressed to human clinical trials. These include the *E. coli* heat-labile toxin B subunit (LT-B) expressed in both potatoes and maize (Tacket et al. 1998; Tacket et al. 2004). Both of these vaccines resulted in good serum IgG responses (90% and 78% of vaccinees, respectively); however, they demonstrated only moderate mucosal IgA responses (50% and 44% of vaccinees, respectively). Other vaccines have been developed to protect against Hepatitis B. Those tested in humans were produced in lettuce and potato (Kapusta et al. 1999; Thanavala et al. 2005) and while these vaccine formulations are distinct, they had similar effects on antibody responses (66% and 62% of individuals responded, respectively). A vaccine antigen expressed in spinach to protect against rabies resulted in 55% of volunteers having a significant increase in antibody (Yusibov et al. 2002). While most of
these previous vaccines resulted in poor immune responses, a vaccine expressed in potatoes to protect against Norwalk virus demonstrated much better effects with 95% of volunteers developing significant increases in IgA antibody secreting cells; however, only 20% resulted in significant serum IgG titers and 30% for fecal IgA titers (Tacket et al. 2000).

While the results from human trials thus far have not demonstrated highly effective vaccines, they do stress that antigen-specific immune responses can be elicited in humans from the consumption of transgenic food products. It is likely that in order to obtain optimal immune responses it may be necessary to modify the vaccination protocol in regard to the timing and dose, the particular plant within which the antigen is expressed and administration with buffers and/or addition of mucosal adjuvants.

II. Model Antigen System

A. Introduction. During the development of new vaccine delivery systems, a model antigen is necessary for optimization of the new techniques. Cholera toxin (CT) and the *E. coli* heat labile toxin (LT) and their non-toxic derivatives have been used as model antigens for developing effective mucosal immunization strategies.

Enterotoxigenic *E. coli* (ETEC) is a major cause of diarrheal disease throughout the world, but especially in developing countries where sanitation and clean water are limited. The two populations that are most greatly affected by ETEC are children under 5 years old who live in developing countries as well as travelers (of any age) to these countries (Qadri et al. 2005; Spangler 1992). The World Health Organization (WHO) estimates that ETEC is the cause of 210 million cases of diarrhea and 380,000 deaths every year (WHO 1999). The diarrhea associated with infection is primarily caused by two toxins produced by ETEC: the non-antigenic heat-stable toxin (ST) and the highly immunogenic heat-labile toxin (LT) (Spangler 1992). Because a component of LT has been the focus of my work, the remainder of this literature review will focus on LT and its B subunit (LT-B).
B. Escherichia coli heat-labile toxin. LT is a member of the A-B toxin family (Rappuoli et al. 1999). Other toxins in this family include cholera toxin, pertussis toxin, diphtheria toxin, Pseudomonas exotoxin A, Shiga toxin, shiga-like toxins, tetanus toxin, several botulinum toxins, and anthrax toxin (Spangler 1992). These toxins consist of an enzymatically active A subunit and a non-toxic B subunit (Rappuoli et al. 1999). Of the toxins in this family, LT is closely related to the cholera toxin (CT), with which it shares approximately 80% nucleotide sequence homology, similar three dimensional structures, binding specificities and modes of action (Rappuoli et al. 1999; Spangler 1992). LT is a heterohexameric protein consisting of one A subunit and a B subunit composed of five identical B monomers which bind to one another in a circular doughnut-like shape with an empty center (Fan et al. 2004). The A subunit of LT consists of two domains: A1 and A2 (Spangler 1992). LT-A1 is enzymatically active and is connected to LT-A2 through a disulfide bond (Spangler 1992). LT-A2 serves to connect LTA1 to LT-B by inserting into the center hole of LT-B (Fan et al. 2004; Spangler 1992).

The mechanism of action of LT is highly complex and has been reviewed by Spangler (1992) and Rappuoli (1999) and is briefly discussed below. LT-B facilitates toxin entry into intestinal epithelial cells by binding to the nearly ubiquitously expressed GM1 gangliosides. Each B subunit binds an individual GM1 ganglioside for a total of five bound gangliosides per LT-B molecule; however, only two B subunits are required for binding to GM1 gangliosides (Rigano et al. 2003). Upon LT-B binding, LT enters the cell in vesicles and is transported to the golgi where the A and B subunits dissociate. The B subunit is later degraded. The A subunit is transferred to the endoplasmic reticulum and then released to the cytosol. At some point during transport, A1 and A2 are separated via proteolytic cleavage and disulfide bond reduction. At this point, A1 is able to bind an adenosine diphosphate (ADP) ribosylation factor (ARF) which activates A1. Upon activation, A1-ARF can bind nicotinamide adenine dinucleotide (NAD) and subsequently transfer an ADP ribose (ADPR)
from NAD to Gsα, the GTP-binding protein that regulates adenylate cyclase. This Gsα-ADPR then binds to guanosine triphosphate (GTP) and complexes with adenylate cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Because of the bound ADPR, GTP hydrolysis is prevented and adenylate cyclase remains activated resulting in abnormally high levels of cAMP and the release of salt and water into the lumen of the gut.

Despite the high toxicity of LT, this toxin has been evaluated for use in vaccines. Its unusually high mucosal immunogenicity and adjuvanticity have prompted research that focuses on reducing or eliminating toxicity while maintaining immunogenicity and/or adjuvanticity. As mentioned previously, strategies have focused on retaining the whole LT molecule, but using point mutations to eliminate toxicity either by preventing protease cleavage between A1 and A2 (Dickinson and Clements 1995; Guillobel et al. 2000; Lu et al. 2002) or by mutations within the active site which prevent substrate (NAD) binding (Ryan et al. 2000; Tierney et al. 2003). Certain mutations result in complete loss of toxicity while others result in reduced toxicity and these mutants demonstrate immunogenicity as well as adjuvanticity. Other strategies thus far have focused on recombinantly producing the non-toxic B subunit alone which has proven to be highly immunogenic due to its receptor binding capabilities (Chikwamba et al. 2002a; Guidry et al. 1997; Lauterslager et al. 2001), although not necessarily functional as an adjuvant (Plant et al. 2003; Weltzin et al. 2000). LT-B may not be a successful oral adjuvant; however, because it binds specifically to intestinal epithelial cells it may serve as a successful carrier which would increase the immune response to an unrelated protein antigen if it were physically attached to LT-B.

C. LT-B in vaccines. Because of the great potential of LT-B as a vaccine component, several prototypic vaccines containing LT-B have been developed thus far using both plant and non-plant production systems.
Recombinant LT-B not produced in plants is generally produced in bacterial or yeast production systems and subsequently purified before administration. Several vaccines containing LT-B from a non-plant system will be briefly described below. LT-B vaccines that have been used for potentially protecting against ETEC infection include an oral vaccine consisting of live-attenuated *Salmonella* Typhi expressing LT-B which resulted in 67% of human subjects responding to vaccination (Khan et al. 2007). In addition, an oral vaccine composed of yeast-derived LT-B was shown to be immunogenic in chickens (Fingerut et al. 2005). In this same study, they produced a fusion protein consisting of LT-B fused to VP2, a viral protein from infectious bursal disease virus. They demonstrated that LT-B served as an effective carrier molecule boosting the response to the viral antigen when administered orally in chickens. One study found LT-B to be an effective oral adjuvant for a different chicken vaccine (Fingerut et al. 2006). This vaccine was composed of bacterially-produced LT-B co-administered with rKnob, a recombinantly produced antigen from egg drop syndrome adenovirus, and demonstrated increased antibody production over animals given rKnob alone. Other non-plant-derived LT-B vaccines have used LT-B as an adjuvant or carrier for intranasal delivery of antigen. Those demonstrating intranasal adjuvanticity in mice include a vaccine protecting against ocular herpes simplex virus type 1 (Richards et al. 2001) and influenza (de Haan et al. 1996). LT-B has served as an effective carrier for intranasal vaccination in mice against prion proteins (Yamanaka et al. 2006) and *Mycoplasma hyopneumoniae*, a porcine pathogen (Conceicao et al. 2006).

A variety of different plant systems have been used for the expression of LT-B including both edible and non-edible plant species. While non-edible plants such as tobacco generally require purification of the protein prior to vaccine administration to remove toxic compounds and improve palatability, purified LT-B has been shown to be immunogenic when administered intranasally, inducing high titers of serum IgG (Wagner et al. 2004). *Arabidopsis* has been used as an expression system for a fusion protein consisting of LT-B
fused to ESAT-6, a protective antigen of *Mycobacterium tuberculosis* (Rigano et al. 2006). An immune response was induced after oral administration in mice; however, it was not protective against challenge.

More commonly, LT-B has been expressed in edible plants that do not require purification and can be easily administered orally. Early studies used potatoes as the expression system. Potatoes transgenic for LT-B were shown to elicit serum and fecal antibodies following oral administration and partially protected mice against challenge with LT holotoxin (Mason et al. 1998). The same research group then fed these potatoes to humans as a proof of concept that humans could elicit an immune response to antigens delivered in food (Tacket et al. 1998). They found that 91% of volunteers demonstrated elevated titers for serum IgG, 55% for serum IgA and 50% for fecal IgA. Another study of orally administered potato expressing LT-B demonstrated that mice did not respond unless they had been previously primed with the antigen extract subcutaneously (Lauterslager et al. 2001). Some of the problems associated with potatoes as an expression system include heterogeneous distribution of the recombinant protein, and the need to administer the potatoes raw in order to avoid breaking down the antigen with heat, which is generally not palatable. In addition, potatoes spoil quickly which would make vaccination throughout developing countries difficult.

Other food plants that have been less studied as LT-B expression systems include tomatoes, lettuce and soybeans. Tomatoes were used to express LT-B fused to ZP3, a mouse immunococontraceptive epitope (Walmsley et al. 2003). This protein was successfully produced, but was not tested for immunogenicity or effectiveness. Lettuce also successfully expressed LT-B, but has not been tested for immunogenicity (Kim et al. 2007). Soybeans were recently shown to effectively express LT-B and oral administration provided partial protection against challenge in mice (Moravec et al. 2007).
Maize as a production system for oral LT-B vaccines shows much promise. In addition to the studies conducted in our institution, which will be discussed in detail below, others have independently expressed LT-B in maize and found it to be highly immunogenic. Oral administration of LT-B in maize to mice elicited similar serum antibody responses and better fecal IgA responses than those elicited by purified LT-B (Streatfield et al. 2001). This same study demonstrated that mice orally vaccinated with LT-B in maize were completely protected against challenge with LT. In addition, maize-expressed LT-B has been tested in humans (Tacket et al. 2004). Oral administration resulted in 78% of volunteers demonstrating significant increases in serum IgG and in IgG and IgA antibody-secreting cell numbers; however, only 44% of volunteers had significant serum and fecal IgA responses.

D. LT-B expressed in maize. Maize as a production system for vaccine antigens has several advantages over other plant systems. Each plant produces a large number of seeds which eases scaling up for mass production (Chikwamba et al. 2002a). In addition, antigen expressed in the seed is often stored in storage bodies which provide a stable environment with very little enzymatic activity before germination (Chikwamba et al. 2002a). This enables long term seed storage at ambient temperature with minimal degradation of the vaccine antigen. In addition, maize is a major ingredient in livestock feed and a staple food in many countries (Chikwamba et al. 2002a). Unlike other plant production systems, extensive heating is not required for processing which reduces the chance of antigen denaturation (Chikwamba et al. 2002a).

Our research group has previously demonstrated the expression of LT-B in maize (Chikwamba et al. 2002b). The DNA sequence was modified from bacterial LT-B in order to optimize expression in potato and maize (Chikwamba et al. 2002b). A seed endosperm-specific promoter, the γ-zein promoter, was used in order to focus expression in the edible portion of the plant (Chikwamba et al. 2002b). Further studies demonstrated that the maize-produced LT-B was able to form functional pentamers within the seed as demonstrated by
the ability to bind GM1 gangliosides in an enzyme-linked immunosorbant assay (ELISA) (Chikwamba et al. 2002a). In addition, immunogold labeling and electron microscopy were used to show that the LT-B was localized to the starch granules of the endosperm (Chikwamba et al. 2003). Maize-expressed LT-B is more stable than bacterial LT-B added to ground maize highlighting the protective ability of the plant cell. It appears that LT-B expressed in maize is protected from denaturation due to high temperatures and digestive enzymes (Chikwamba et al. 2003). This protection is likely to increase the amount of functional LT-B that reaches immune cells and possibly result in a slow release of antigen to prolong immune exposure, both of which are likely to enhance immune responses (Chikwamba et al. 2003).

The LT-B transgenic maize was also shown to be highly immunogenic when orally administered to mice in four doses each containing 10 µg LT-B (Chikwamba et al. 2002a). The same study demonstrated that maize-expressed LT-B induced stronger mucosal antibody responses than maize spiked with an equivalent amount of purified bacterially produced LT-B. Not surprisingly, the LT-B-specific antibodies were cross-reactive with the closely related CT-B, and these antibodies protected mice against challenge with both LT and CT holotoxins (Chikwamba et al. 2002a). Further studies of maize-expressed LT-B investigated its immunogenicity when orally administered to young vs. aged mice (Karaman et al. 2006). Mice primed as young adults demonstrated good systemic and mucosal antibody responses after boosters as aged mice. Aged mice that were naïve to LT-B developed less serum IgG, slightly less fecal IgA and increased serum IgA as compared to young mice (Karaman et al. 2006). This suggests that immune regulatory mechanisms change with age and possibly favor IgA responses over IgG. However, in older mice, the high serum IgA and low fecal IgA levels may indicate that less IgA is being transported into mucosal secretions where it is needed (Karaman et al. 2006).
Edible vaccines produced in plants have been shown to be immunogenic in both animals and humans. Plant-produced vaccines show much promise and could facilitate economical vaccine production and distribution in developing countries where vaccination is currently difficult to carry out; however, more research is necessary to optimize protein expression and immune responses. Because plant-produced vaccines are a new area of research, studies involving their safety are also required.

III. Risk Assessment

Some characteristics of optimized plant vaccine delivery systems also necessitate strict regulations during production and in-depth safety studies before vaccine approval, such as high antigen expression levels, high antigen stability and mass production of the product economically in the field.

Inadvertent exposure to plant-derived pharmaceuticals (PDPs), including plant-derived vaccine antigens, at any level is considered unacceptable and is avoided through strict guidelines that ensure that the PDP will be confined at all times. However, because these plants are grown in the field and typically do not look different from their non-transgenic counterparts, the possibility of human error leading to contamination of the food supply exists. Plant expression systems for oral vaccine production have much potential for inducing protective antibodies at the mucosal surface and for making vaccination possible in developing countries; however, this is a new area of research and regulations regarding their safety and handling are still being developed. Currently, assessing the risk of PDPs involves combining procedures put in place for assessing both transgenic plants that serve as food (such as Roundup Ready soybeans, Bt corn and Flavr Savr Tomatoes (Metcalf et al. 2003)) and pharmaceuticals themselves. The two primary categories that need to be assessed are potential exposure to and hazard of the PDP (Wolt et al. 2006).

Exposure to PDPs will need to be evaluated for each individual protein and the plant it is being produced in. The amount of PDP a human is likely to be exposed to involves
several factors including 1) the protein expression levels in the consumable portion of the plant, 2) the amount of the transgenic plant product that contaminates the non-transgenic food supply, 3) the fraction of this contaminated product that is processed into a food product, 4) the amount of transgenic protein that survives processing and digestion, and 5) the amount of this contaminated food product that is consumed (Wolt et al. 2006). A recent risk assessment of maize-derived LT-B suggests that at even the highest levels of predicted LT-B expression in maize, it is highly unlikely that a person would be exposed to more than a functional dose (the amount given in a vaccine dose) and that a more likely exposure scenario would result in very low exposure that is not likely to have any effects (Wolt et al. 2006).

The hazard potential of a PDP is assessed in a similar fashion to transgenic food crops, with appropriate modifications as PDPs are likely to be consumed in much smaller quantities and with much less frequency than transgenic food crops, whether used therapeutically or ingested inadvertently. The first analysis is to determine substantial equivalence of the PDP to its non-transgenic counterpart. This includes comparing the composition of the transgenic plant to its non-transgenic equivalent in terms of nutrients, anti-nutrients, fatty acids and amino acids (Metcalfe et al. 2003). This process has not yet been completed for maize transgenic for LT-B (Wolt et al. 2006). Substantial equivalence also involves comparing plant-produced LT-B to bacterially produced LT-B (Metcalfe et al. 2003). This has been completed for LT-B activity and physical characteristics with no differences identified (Chikwamba et al. 2002a; Wolt et al. 2006).

The second analysis involves testing for protein safety, including allergenicity and toxicity (Metcalfe et al. 2003). Testing for allergenicity is a complex process that evaluates whether a protein is similar to known allergens and thus likely to induce allergy. First, the source of the protein is evaluated. If the protein originated from a source known to be allergenic, testing immediately proceeds to in vitro and in vivo testing which will be
described below (Metcalf et al. 2003). If the source is not known to be allergenic, the protein’s amino acid sequence and protease stability are compared to those of known allergens. If a protein has less than 50% homology it is not likely to be allergenic, but if it has more than 70% homology to a known allergen it is likely to be allergenic (Metcalf et al. 2003). Maize-derived LT-B has been evaluated for sequence homology to known allergens and does not appear to be homologous to any known allergens (Wolt et al. 2006). The protein is then subject to pepsin digestion assays determining how long the protein can resist degradation. Known allergens survive digestion for 8-60 minutes, whereas most non-allergens are degraded within 15 seconds (Metcalf et al. 2003). These tests have been carried out with maize-expressed LT-B; however the results are not straightforward. LT-B in maize resists degradation for greater than 15 minutes, which could indicate allergenic potential. Purified bacterial LT-B, however, is degraded within 5 minutes indicating that it is not necessarily allergenic (Chikwamba et al. 2003). LT-B in maize is likely able to resist degradation simply because of its encapsulation within the maize cells and not because of properties inherent to LT-B. Proteins that demonstrate allergenic potential based upon these assays are then subjected to in vitro and in vivo testing (Metcalf et al. 2003). Unfortunately, there are no known animal models that serve as good indicators of predicting allergy (Metcalf et al. 2003) and thus testing is conducted in humans. In vitro tests determine whether pooled serum IgE from individuals allergic to related proteins binds to the protein of interest (Metcalf et al. 2003). In vivo testing includes a skin prick test (SPT) and/or a double-blind placebo-controlled challenge in humans (Metcalf et al. 2003). SPT determines whether scratching the protein into the skin of individuals allergic to related proteins will result in an allergic response. The challenge is usually more informative, but is also much more dangerous with the rare possibility that challenge could lead to anaphylactic shock (Metcalf et al. 2003). None of the in vitro or in vivo tests have been carried out with maize-produced LT-B.
Testing for protein toxicity is typically conducted in animal models. This testing has been carried out for maize-produced LT-B and no toxicity was observed (Wolt et al. 2006). In addition, LT-B in plants has been tested for immunogenicity in both animals and humans with no toxicity reported (Chikwamba et al. 2002a; Moravec et al. 2007; Tacket et al. 1998; Tacket et al. 2004). However, testing for toxicity with LT-B is much different than other proteins due to its strong immunogenicity which could lead to indirect toxicity (Wolt et al. 2006). Until we completely understand the effects of LT-B on the immune system, we may not be aware of indirect toxic effects of LT-B. The research presented in the following chapters determines the effects of LT-B on the immune system to serve as baseline information for future studies on immunogenicity and possible toxicity. It includes identifying a minimum dose for which an immune response can be expected, evaluating the immune effects of daily consumption of maize transgenic for LT-B, and determining whether inadvertent exposure affects subsequent immunizations with maize-produce LT-B.

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Chapter 2: Low-dose exposure and immunogenicity of transgenic maize expressing the *Escherichia coli* heat labile toxin B subunit

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Abstract

**Background:** Transgenic maize, which produces the nontoxic B subunit of the *Escherichia coli* heat-labile toxin (LT-B) in seed, has proven to be an effective oral immunogen in mice. Currently, there is considerable concern over accidental consumption of transgenic maize expressing LT-B by humans and domestic animals. We have yet to define nonimmunogenic levels of transgenic LT-B when ingested.

**Objectives:** Our goal in this study was to determine the highest dose of LT-B orally administered in mice that does not result in a measurable immune response. We defined an immune response as specific serum or mucosal IgG or IgA significantly greater than background after three feedings (0.0002–20 μg) or a priming response induced by the intermittent feeding.
Methods: We fed transgenic maize pellets on days 0, 7, 21, and 49 and collected serum and fecal samples weekly. Serum was analyzed for LT-B–specific IgG and IgA, and feces was analyzed for LT-B–specific IgA.

Results: We observed a dose-dependent anti-LT-B antibody response with high specific antibody concentrations in groups fed high doses (0.2, 2, 20 µg) of LT-B maize. Mice fed 0.02 µg LT-B demonstrated immune priming in 62.5% of the animals. Mice that were fed ≤ 0.002 µg LT-B showed no increase in specific antibody nor did they demonstrate immune priming, indicating that 0.002 µg LT-B was the highest nonimmunogenic dose tested.

Conclusion: Our results demonstrate that LT-B derived from transgenic maize is immunogenic at nanogram levels when orally administered to mice.

Introduction

Many pathogens enter the body via mucosal surfaces. To prevent infection via this route, a protective secretory IgA response is required. Traditional parenteral vaccines primarily induce IgM and IgG responses, whereas mucosal vaccination, for example via the nasal or oral route, is able to elicit both an IgG and a secretory IgA response. Recently, expression of vaccine antigens in plants has been explored as an approach to facilitate oral vaccination (Kong et al. 2001; Ma et al. 2003; Tacket et al. 2000; Webster et al. 2002).

Plant-based oral vaccines have several advantages over traditional parenteral vaccines. Their production is more economical and their delivery is safer, as needles and animal products are not used (Lauterslager et al. 2001; Streatfield et al. 2001). Plant cells also provide protection to the antigen during long-term storage, transport at ambient temperature, and as it passes through the harsh environment of the stomach (Chikwamba et al. 2003). Most important, plants do not carry pathogens that are harmful to humans and animals (Streatfield et al. 2001).
Our work focuses on transgenic maize expressing the B subunit of the *Escherichia coli* heat-labile toxin (LT-B). Previous studies have shown that this maize is both immunogenic, eliciting the production of secretory IgA upon oral administration to mice, and protective against challenge with the holotoxin (LT) (Chikwamba et al. 2002). LT-B is expressed within the starch granules of the maize seed as demonstrated by Chikwamba et al. (2003). Because of this natural encapsulation, maize-expressed LT-B is protected against harsh environmental conditions including high temperatures and enzyme degradation, while purified recombinant LT-B is not (Chikwamba et al. 2003). In addition, the natural encapsulation in maize starch granules may provide better protection and stability than other plant-produced vaccines such as potato, tomato, or tobacco. This increased stability should lead to improved immunogenicity.

The enhanced stability of maize-expressed LT-B is highly desirable for oral vaccine production, storage, and effectiveness; however, LT-B and its stability are also a concern in regard to environmental contamination and/or accidental consumption by humans and domestic animals. Safety issues remain that must be addressed. The use of transgenic maize to produce a vaccine component opens the possibility for low-dose antigen exposure of workers involved in its production or of consumers should the product inadvertently occur in foods (Wolt et al. 2006). Low-dose exposures have implications for product efficacy, which we address in this article. Furthermore, as immunogenicity is highly sensitive, evidence of immunogenic effects at low doses helps to set the lower bound for subsequent considerations of dose-dependent allergenic potential. Thus, we must determine the minimum dose for which no immune stimulation occurs upon accidental consumption.

The work we present here focuses on determining the immunogenicity of maize transgenic for LT-B in an intermittent feeding schedule and identifying a maximum nonimmunostimulatory dose. We hypothesized that some low levels of LT-B could stimulate nondetectable levels of specific antibody but result in immune priming—an ability to induce
the production of memory B cells that can later respond to the same antigen and produce specific IgG or IgA. We defined immune priming as a 4-fold increase of antibody (IgG or IgA) over background after a 20-µg LT-B booster exposure (Tacket 2005; Tacket et al. 1998, 2004). Because of the high degree of specificity and sensitivity of the immune response to the highly immunogenic LT-B, doses of maize-derived LT-B that do not prime or stimulate the immune response should be considered an environmentally safe threshold.

Materials and Methods

Preparation of maize pellets. Production of maize transgenic for LT-B (seeds from 4th-generation transgenic plants) and preparation of the maize seed pellets were carried out as described previously (Chikwamba et al. 2002). The pellets were formed by combining appropriate amounts of ground transgenic maize seed with ground nontransgenic (nt) maize seed to ensure that each pellet was a consistent size and contained the amount of LT-B indicated for each group. For the second study, we prepared the lowest doses (0.0002–0.02 µg LT-B) by mixing the ground transgenic maize seed with ground nt maize seed 1:300 to make a lower LT-B level transgenic mix. The total weight of maize seed in each pellet was 0.811 g for the first study and 0.937 g for the second study. The ground maize seed for each pellet was mixed with 600–700 µL of phosphate-buffered saline (PBS) [1.9 mM NaH2PO4, 8.1 mM Na2HPO4, 0.15 M NaCl (pH 7.3)], formed into a pellet, and air-dried overnight. Ground nt maize seed was used to make pellets of similar weight to feed to the negative control groups.

To ensure that LT-B content in the maize remained constant during pellet formation, one extra pellet was formed per dose per feeding for analysis of LT-B content using a ganglioside-dependent ELISA. Three to four pellets of each type were frozen until ELISA analysis of LT-B content (four samples from each pellet). We detected no significant differences between the intended amount and the amount measured with ELISA for all
pellets except the 20-µg pellets. The 20-µg pellets had higher amounts of LT-B than intended. These amounts ranged from 26.5–40.8 µg LT-B per pellet instead of 20 µg (p < 0.0001; data not shown). Despite the range of measured LT-B in the 20-µg pellets, we will continue to refer to this dose as the nominal 20-µg dose and animals given that dose as the 20-µg group.

**Oral immunization of mice.** We obtained 4- to 6-week-old female BALB/c mice from Harlan Sprague Dawley (Indianapolis, IN, USA) and allowed them a 2-week acclimation period with a 12-hr reversed light/dark cycle before beginning the experiment with lights on at 2100 hr. The mice were housed four per cage in the Iowa State University (ISU) animal facility with food and water ad libitum. Animals were treated humanely, and all procedures were approved by the ISU Institutional Animal Care and Use Committee.

Before the maize seed pellets were fed to the mice, the mice were fasted overnight (during the light phase) with water ad libitum. While eating the pellets, mice were housed individually with water ad libitum and were allowed to eat the pellet for 4 hr or until the pellet was finished, typically a maximum of 6–8 hr for the slowest mice. Upon completion of the pellet, the mice were returned to group cages with mouse chow and water ad libitum.

In the first study, we divided mice in to six groups of four mice each. The experimental groups were fed 0.02, 0.2, 2, or 20 µg LT-B per pellet per feeding. The two control groups were fed nt maize pellets. The maize pellets were fed on days 0, 7, and 21. To test for immune priming, on day 49 all mice were fed a 20-µg LT-B pellet, including one group previously fed nt maize (nt + 20). In addition, one nt group was fed nt maize pellets on day 49.

In the second study, we used the same experimental design except that some mice were fed lower doses of LT-B. The groups consisted of mice fed 0.0002, 0.002, 0.02, and 20 µg LT-B per pellet per feeding and two groups fed nt maize. To test for immune priming, on day 49 the mice were all fed 20 µg LT-B, except one nt group. Thus, internal repeats of the
nt, nt + 20, 0.02-, and the 20-µg groups were included for a total of eight mice receiving each of these treatments over two experiments.

**Sample collection and preparation.** We collected serum and fecal pellets before the initial dosing and weekly throughout both studies to detect LT-B–specific serum IgG and IgA concentrations and fecal IgA concentrations. Blood was collected via the saphenous vein, using heparinized capillary tubes and centrifuged with serum separator gel (Vacutainer Plus plastic serum tube; Becton Dickinson, Franklin Lakes, NJ, USA) at 6,000  g in a bench top microcentrifuge (Jouan M14.11; Thermo Electron Corp., Waltham, MA, USA) for 10 min. The serum was collected in microcentrifuge tubes and stored at –20°C until analyzed for LT-B–specific IgG and IgA by ELISA as described below.

Fecal pellets were collected from each mouse, frozen, lyophilized, and stored at -20°C. We extracted lyophilized pellets by adding extraction buffer [0.05% NaN3, 10 µg/mL leupeptin (Sigma, St. Louis, MO, USA), 0.25 mM PefablocSC (Sigma) in PBS] at 10 µL/mg of lyophilized feces, vortexing, and extracting overnight at 4°C. Before analysis, the samples were placed on a shaker (Tekmar VXR-S10; Janke & Kunkel, Staufen, Germany) at 1,000 rpm for 90 min, then centrifuged at 6,000  g for 10 min with serum separator gel. The liquid extract was collected and analyzed by ELISA for LT-B–specific IgA as described below.

**Euthanization.** Mice were euthanized on day 57 for the first study; for the second, half the mice were euthanized on day 55 and the other half on day 56. For both studies, mice were euthanized with CO2.

**Lung lavage.** After mice were euthanized we performed lung lavages by exposing the trachea, inserting a TomCat catheter (Kendall Sovereign, Tyco Healthcare, Mansfield, MA, USA), and flushing the lungs twice with 0.8 mL sterile PBS. The samples were centrifuged at 6,000  g for 10 min to remove cells and stored at –20°C until analysis for LT-B–specific IgA by ELISA as described below.
**ELISAs.** We performed ELISAs for LT-B– and LT-B–specific antibodies as previously described (Chikwamba et al. 2002; Karaman et al. 2006) unless otherwise noted. All mouse samples, which were analyzed individually, and reagents were added 50 µL/well, and between each step wells were washed 4 times with 100 µL/well PBST [0.05% Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma) in PBS].

**Detection of LT-B–specific IgG and IgA.** Measurements of LT-B–specific IgG (serum samples) and IgA (serum, fecal, and lung lavage samples) were carried out as described previously (Karaman et al. 2006) with minor changes. Briefly, all fecal extracts were measured within 24 hr of extraction. Sample concentrations were determined by comparing to a standard curve consisting of wells coated with either purified mouse IgG (MOPC 21, Sigma) with a range of 1.37–1000 ng/mL or IgA (Bethyl Laboratories, Montgomery, TX, USA) with a range of 0.46–400 ng/mL. Samples were diluted appropriately to fall within the linear range of the curve. End point readings were taken at 405 nm using the EL 340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) and data collected using KC Junior software (version 1.17, Bio-Tek Instruments, Inc.) using a four-parameter fit standard curve. Samples reading below the standard curve were reported as one-half the value of the lowest detectable standard to permit log transformation of the data for statistical analysis.

**Detection of LT-B in maize.** We extracted LT-B from ground maize or crushed maize pellets equivalent to those used for feeding using a sodium phosphate extraction buffer [sodium phosphate buffer (pH 6.6) 25 mM; sodium chloride 100 mM; EDTA, 1 mM; Triton X-100, 0.1%] with protease inhibitors (leupeptin, 10 µg/mL; PefablocSC, 0.25 mM) at 10 µL/mg maize. Samples were extracted with buffer shaking at 1,000 rpm at 37°C for 2 hr. The extract was collected by centrifuging at 6,000 g for 15 min in a bench top microcentrifuge.

We determined LT-B content of maize extract using a ganglioside-dependent ELISA as described previously (Chikwamba et al. 2002) with minor changes. LT-B was detected by
incubating with rabbit anti-LT-B antibody (diluted 1:10,000; Immunology Consultants Laboratory, Inc., Newberg, OR, USA), then incubating with biotin-conjugated goat anti-rabbit IgG (diluted 1:5000; Sigma). The secondary antibodies were detected using streptavidin-horse radish peroxidase (diluted 1: 1000; Becton Dickinson), then incubating with ABTS (3-ethylbenzthiazoline-6-sulfonic acid; Sigma) substrate buffer [0.1 M citric acid, 0.55 mM ABTS (pH 4.25)]. Values were determined by comparing to a standard curve of purified bacterial LT-B (provided by J. Clements).

**Statistical analysis.** We analyzed the data with general analysis of variance, using LT-B dose and sample day as variables in the model. Because of unequal variance between the groups, the antibody data were log transformed before analysis (Kirk 1982). Each sample day that was significantly different (p < 0.05) from the prefeed date was further analyzed by between group contrasts for that day. Analysis was conducted using the statistical software Statistix (version 8; Analytical Software, Tallahassee, FL, USA). We used non-log-transformed data for graphs. Immune responses were divided into two types and defined as follows: An antibody response is demonstrated by antibody concentrations significantly higher (p < 0.05) than those of the nt-fed group, and immune priming is demonstrated by antibody concentrations 4-fold higher than those of the nt-fed group 5–6 days after a 20-µg LT-B dose (Tacket 2005; Tacket et al. 1998, 2004).

**Results**

**LT-B–induced dose-dependent response with immune priming.** In our first experiment we tested the immunogenicity of various doses of LT-B from transgenic maize. To identify a nonimmunostimulatory dose, we tested 10-fold differences of LT-B doses (ranging from 0.02 to 20 µg per feeding) using an intermittent feeding regimen previously shown to induce a robust antibody response in 28 days with pellets fed on days 0, 7, and 21 (Chikwamba et al. 2002).
**Serum IgG.** Intermittent feeding of LT-B induced a dose-dependent serum IgG response (Figure 1A). Mice fed 20 µg LT-B had low serum IgG levels (0.318 ± 0.061 µg/mL) but these levels were significantly higher than those of mice fed nt maize (0.169 ± 0.018 µg/mL) by day 6 (p = 0.0035) and were significantly higher than all other groups on days 13–48 (p < 0.05). Mice fed 2 and 0.2 µg LT-B had significantly higher concentrations of IgG by day 13 compared with the nt group (p < 0.0001 for both groups on days 13–48). The concentrations of specific IgG in the 0.02-µg group were low, but demonstrated a significant increase on day 27 (0.298 ± 0.039 µg/mL) compared with the nt group (0.159 ± 0.018 µg/mL) (p = 0.0038), with significance continuing through day 48 (p ≤ 0.0089).

The peak antibody concentration for all groups was reached on day 55, 6 days after the 20-µg booster as seen in Figure 1B. After the booster, LT-B–specific IgG levels were significantly increased for mice in the 20-, 2-, 0.2-, and 0.02-µg groups compared with the nt group (p ≤ 0.0065). All mice in the 20-, 2-, and 0.2-µg groups demonstrated antibody levels at least 4 times higher than nt mice on day 55. In the 0.02-µg group, two of four (50%) mice had antibody levels 4 times those of the nt control group, which is indicative of immune priming. There was no statistical difference between either of the nt groups for serum IgG throughout the study, even after feeding one group 20 µg LT-B on day 49 (p = 0.7085).

**Serum IgA.** As with serum IgG, feeding LT-B generally induced a dose-dependent response in serum IgA (Figure 2A). Significantly increased antibody levels compared with those in the nt group were observed in the 20-, 2-, and 0.2-µg LT-B groups beginning on day 13 and continuing to the end of the study (p ≤ 0.0071) (Figure 2A,B). The 0.2-µg group demonstrated higher but not significantly different levels of antibody compared with the 2- and 20-µg groups on days 13 and 20. Additionally, the antibody serum IgA levels of 0.2-µg group remained higher than those of the 2-µg group until the end of study. Although we are not sure why the lower dose resulted in higher antibody levels, we are certain that it was not a technical error, as the same serum samples demonstrated a dose-dependent IgG response.
We hypothesized that perhaps a lower dose, such as 0.2 µg LT-B, is optimal for stimulating an early IgA response. Also, the high average value was not just due to one outlier in the 0.2-µg group, as on day 13, three of four mice in the 0.2-µg group had higher antibody levels than the mice in the 2-µg group for serum IgA.

Because of the short half-life of IgA, obtaining a high level of IgA antibody significantly different from that in the nt group was not always observed with consecutive sample dates. This was demonstrated by the 0.02-µg group, which was marginally different from the nt group (p = 0.0785) on day 13 and significantly different on days 27, 34, 48, and 55 (p ≤ 0.0351). On day 55, three of four (75%) mice in the 0.02-µg LT-B group appeared to have responded to the 20-µg booster dose with LT-B–specific IgA levels 4 times higher than those of the nt control group (Figure 2B), suggesting that these three mice were immunologically primed. There was no significant difference between the nt and nt +20 groups throughout the study (p = 0.9481 on day 55) and none of the mice (zero of four) in the nt + 20 group had antibody levels 4 times higher than those of the nt group on day 55.

Because of a lack of samples, no data from day 6 of the study were obtained.

Fecal IgA. Mucosal IgA was measured in extracts of lyophilized fecal material and expressed as micrograms per gram of fecal material as shown in Figure 3. Mice fed 20 µg LT-B had significantly higher levels of IgA in fecal material than those of the nt group on day 13 (p < 0.0001) and again on day 27 throughout the rest of the study (p ≤ 0.0019). The 2-µg group had significantly higher levels than those of the nt group on days 13, 27, 34, 41, and 55 (p ≤ 0.0371). Similar to the serum IgA results, mice fed 0.2 µg LT-B had levels of fecal IgA that were significantly higher than those of the nt group by day 13 (p < 0.0001) and throughout the remaining sample dates (p ≤ 0.0384).

Fecal IgA levels were statistically higher in the 0.2 µg group than those in the 2 µg LT-B group on days 13, 20, and 55 (p < 0.05). Again, this was not because of an outlier in
the group, as on all those dates at least three of four mice in the 0.2-µg group had higher antibody levels than those of the mice in the 2-µg group.

Fecal antibody concentrations of mice fed 0.02 µg LT-B reached statistical significance on days 20, 27, 34, and 55 (p ≤ 0.0163) with antibody levels only marginally higher than the those of the nt group on days 41 and 48 (p = 0.0515 and 0.0712, respectively). On day 55, 1 week after the 20-µg booster, one mouse in the 0.02-µg group had 4 times the antibody concentration of that in the nt group (Figure 3B), suggesting that immune priming had occurred in one of four mice. The nt and nt + 20 groups were not statistically different throughout the study (p = 0.7278 on day 55).

**Confirmation of 0.02-µg threshold for immune priming.** During the second intermittent feeding study, antibody responses to low doses of LT-B administered orally in transgenic maize were measured. Because our first study demonstrated a significant increase in antibody concentrations and immune priming in some mice fed 0.02 µg of LT-B, we chose to repeat this dose and test two doses, each 10-fold lower in order to identify a nonimmunogenic dose. We also included a 20-µg LT-B dose as a positive control. Thus, two groups were repeated from the first experiment and two groups were new. As in the first experiment, we looked for production of serum and fecal antibody as well as immune priming.

**Serum IgG.** As seen in Figure 4A, mice fed 20 µg LT-B had significantly elevated levels of serum IgG (0.296 ± 0.035 µg/mL) by day 6 (p = 0.0046) compared with the nt group (0.201 ± 0.017 µg/mL) and had significantly higher levels throughout the study (p < 0.0001 for all remaining sample dates), reaching peak levels on day 54 that were similar to those observed in the first study (Figure 4B vs. Figure 1B). The 0.02-µg group had marginally higher levels than those of the nt group on days 27, 41, and 48 (p = 0.0535, 0.0594 and 0.0514, respectively) and significantly higher levels on days 34 and 54 (p = 0.0335 and 0.0257, respectively). No other groups had IgG concentrations significantly different from those of the nt group (Figure 4A,B). On day 54 for the 0.02-µg group, one of four (25%) animals
demonstrated an antibody level 4 times that of the nt group (4.2 µg/mL vs. 0.2 µg/mL), suggesting that immune priming had occurred in that animal (Figure 4B). Despite that one animal in the nt + 20 group demonstrated elevated antibody concentrations after the 20-µg LT-B booster, the increase may be the beginning of primary response because the level of antibody (1.4 µg/mL) is far below that of the primed mouse in the 0.02-µg group (4.2 µg/mL). Additionally, the nt + 20 group was not statistically different from the nt group.

**Serum IgA.** The serum IgA concentrations of the 20-µg LT-B group (0.5721 µg/mL) were significantly higher than those of the nt group (0.0607 µg/mL) by day 6 (p = 0.0088 on day 6 and p < 0.0001 for all remaining sample dates), as seen in Figure 5A. The antibody concentrations of the 0.02-µg group were marginally different from those of the nt group on day 27 (p = 0.0548) and significantly different on day 34 and all subsequent days (p ≤ 0.0294). No other group had antibody concentrations significantly different from those of the nt group throughout the study. Once again the 0.02-µg LT-B group had two of four (50%) mice that appeared to be primed (Figure 5B). To calculate the level of antibody 4 times higher than those of the nt group for this data set, we used the data from the nt + 20 group, as all mice in the nt group had no detectable antibody on day 54. No other group (0.0002 or 0.002 µg) had responders by this standard.

**Fecal IgA.** The 20 µg LT-B group had antibody concentrations marginally higher than those of the nt group by day 13 (p = 0.0574) and significantly higher by day 20 (p = 0.0046) and throughout the remainder of the study (p ≤ 0.0202), as seen in Figure 6A. The 0.02-µg LT-B group did not have a significant increase in antibody concentrations compared with those of the nt group; however, after the 20-µg booster, one of four (25%) mice responded with 4 times the antibody concentrations of those of the nt group, suggesting immune priming (Figure 6B). No animals fed lower doses of LT-B responded by either measure.

**IgA from lung lavages.** Mucosal IgA was measured in lung lavage fluid of euthanized mice (Figure 7). The 20- and 0.02-µg LT-B groups had significantly elevated levels of IgA
compared with those of the nt group and the negative control group, which was never fasted, handled, or fed maize pellets (p < 0.0001 and p = 0.0029, respectively).

**Frequency of immune priming with 0.02 µg LT-B.** Although the fecal IgA measurements in the second study for the 0.02-µg group were not significantly higher than those of the nt group, this dose resulted in significant levels of antibody in all other measures for both studies at least at one sample day before the 20-µg booster as well as by day 54 or 55. Additionally, approximately half the mice in both studies were primed, based upon antibody levels at least 4-fold higher than those of the nt group. One mouse in each study demonstrated priming using all three measures (serum IgG, serum IgA, and fecal IgA), whereas all other mice responded in only one or two of the measures (Table 1). The rates of immune priming varied between different measures (serum IgG vs. serum IgA vs. fecal IgA), indicating that some are more sensitive than others. We found serum IgA to be the most sensitive, which is likely because oral exposure to an antigen primarily elicits an IgA response as opposed to IgG. Also, fecal IgA is difficult to measure accurately because of the high rate of antibody breakdown in the feces. Using our most sensitive measure, serum IgA, we observed that the overall rate of immune priming in mice fed 0.02 µg LT-B intermittently is 62.5%.

**Effect of maize consumption on mouse weight.** Upon euthanization, the weight of each mouse was recorded. For both studies, we found no significant difference between mice fed transgenic maize compared with mice fed nt maize (p ≥ 0.14). In addition, for the second study, no significant difference was detected between mice that were handled, fasted, and fed transgenic or nt maize compared with mice that were never handled, fasted, or fed maize (p ≥ 0.11), thereby demonstrating that the nt and transgenic maize at the doses administered had no toxic effects on the mice (data not shown).
Discussion

In this study we addressed the immunologic effects in mice of accidental consumption of low doses of maize transgenic for LT-B. The doses fed to the mice included 20 µg LT-B, a dose protective in vaccine studies, as well as five additional doses, with each 10-fold lower than the previous one (2, 0.2, 0.02, 0.002, and 0.0002 µg LT-B). The 20-µg dose was included in both experiments as a positive control. Chikwamba et al. (2002) and Mason et al. (1998) previously demonstrated that 10–50 µg LT-B fed 3 times intermittently to mice results in a protective level of antibody. Here we demonstrate that the 20-µg functional dose also results in a robust memory response that elicited 2-fold increases in serum IgG and 10-fold increases in serum and fecal IgA. Serum IgA represents a monomeric nonsecreted form of IgA. Although the biological function of serum IgA is unclear, differences in the concentration of serum IgA between animals correlates with differences in mucus-secreted IgA.

We have extended previous studies (Chikwamba et al. 2003; Karaman et al. 2006) by testing doses of 0.0002, 0.002, 0.02, 0.2, 2, and 20 µg LT-B for serum IgG and IgA as well as fecal IgA and we have observed a dose-dependent antibody response. Only mice that were fed ≥ 0.02 µg LT-B elicited significantly elevated levels of antibody. It should be noted that although the antibody response in the 0.02 µg group was low, it was significantly higher than that of the nt group in six of seven measures. The biological relevance of the low antibody concentration is underscored by the fact that approximately half the mice in the 0.02-µg group demonstrated a 4-fold increase in antibody when fed a 20-µg dose. No lower dose resulted in an antibody response or immune priming. From this study, we found that serum IgA was the most sensitive measure for detecting mice that had been immunologically primed. In addition, we identified 0.002 µg as the highest nonimmunostimulatory dose, one that resulted in neither an antibody response nor immune priming when fed intermittently.

Although we considered 0.02 µg LT-B a dose that results in immune priming, not all the mice in these groups resulted in a priming response. Some animals had fair antibody
responses while others appeared to have no response at all on days 54 or 55. One explanation for this is that there is some variability even between genetically inbred BALB/c mice that becomes obvious when feeding a dose that is borderline for inducing a response. Alternatively, the amount of LT-B in the maize fed to each mouse may not have been uniform. Our group has observed that the finer the transgenic maize kernels were ground, the more LT-B could be extracted and detected in ELISA (Chikwamba et al. 2002). Although the extra pellets that we prepared and assayed were all very close to containing 0.02 µg LT-B (0.0226, 0.0184, and 0.0195 µg), perfect homogenous mixes are not practical using ground maize and it is not inconceivable that one or more of the experimental pellets may have been more variable in LT-B content. This is more of a problem with borderline doses than with the high, immunogenic doses used as a functional vaccine.

Although there are many advantages to producing LT-B in maize, there are also concerns associated with genetically modified organisms (Schmidt 2005). Because of the high immunogenicity of LT-B, even at low levels, intermittent accidental consumption of maize transgenic for LT-B could result in an antibody response, immune priming, or both depending on the dose ingested. Whether immune priming has a positive or negative effect on future vaccine administrations has yet to be determined. We can hypothesize that it would boost the immune response to a future vaccination meant to protect animals or humans from the LT holotoxin; however, if LT-B were to be used as an adjuvant in a vaccine against a heterologous antigen, the response to that vaccine may be altered. Additionally, further research is needed to ensure that LT-B administered orally will not promote the development of tolerance to vaccines or allergies to co-administered food proteins.

This study provides data that are the first of their kind to begin assessing the consequences of accidental consumption of LT-B in maize. Risk assessment for noncancerous, nontoxic transgenic plants is evolving and includes the use of uncertainty factors to extrapolate from animals to humans (Kodell and Gaylor 1999). A recent risk
assessment by Wolt et al. (2006) of human exposure to LT-B in transgenic maize indicates that a dose 200-fold lower than a functional dose in humans is small enough to consider that maize unadulterated from a toxicologic perspective. However, in our mouse study, a dose 1,000-fold lower than the functional 20-µg LT-B dose, 0.02 µg LT-B was immunogenic and caused immune priming in approximately half the mice. The highest nonimmunostimulatory dose tested was 10,000-fold lower than the functional 20-µg LT-B dose. Using this information, we can estimate a safe, nonimmunogenic dose for humans. Tacket et al. (2004) fed maize transgenic for LT-B to humans. The functional dose in this study was 1.1 mg LT-B per 70-kg adult. A dose 10,000-fold lower than this functional dose is equivalent to 0.11 µg LT-B, which is our best estimate for a nonimmunogenic dose in humans.

Consuming low immunostimulatory doses of LT-B does not result in adverse toxic effects, as indicated by no changes in animal body weight or overall health; however, the low dose may be enough to alter immune responses. An immune response to LT-B may not be harmful on its own, but if accidental consumption were to alter the way in which a person or animal would later respond to an oral vaccine containing LT-B in transgenic maize, it could render the vaccine less useful. For example, daily exposure to LT-B may induce oral tolerance. If accidental consumption of transgenic LT-B maize were to occur daily (as opposed to the intermittent feeding schedule used in this study), this could lead to vaccine inefficiency as well as a reduced ability of the immune system to eliminate an ETEC infection.

The data presented here apply to the intermittent feeding schedule used. We understand that mice exposed to the same doses of LT-B at differing intervals may have different immune responses. Further studies are needed to compare intermittent and continuous feeding regimens for transgenic maize.
References


Table 1. Frequency of immune priming in mice fed 0.02 µg LT-B three times intermittently.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum IgG</td>
<td>2/4&lt;sup&gt;a&lt;/sup&gt; (50%)</td>
<td>1/4 (25%)</td>
<td>3/8 (37.5%)</td>
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<tr>
<td>Serum IgA</td>
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<td>2/4 (50%)</td>
<td>5/8 (62.5%)</td>
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<tr>
<td>Fecal IgA</td>
<td>1/4 (25%)</td>
<td>1/4 (25%)</td>
<td>2/8 (25%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of primed mice/total number of mice tested  
<sup>b</sup>Percentage of primed mice out of total mice tested

Figure Legends

Figure 1. Study #1 serum IgG concentrations in response to three intermittent feedings of LT-B (A) or a booster dose of 20 µg of LT-B given on day 49 (B). (A) LT-B feeding days are indicated by the arrows. Antibody concentrations are presented as mean ± SEM. (B) Group means are represented as bars (---). For both (A) and (B), n=4 mice per group, except for the non-transgenic group (nt) which had an n=8 on days -3 to 48.

Figure 2. Study #1 serum IgA concentrations in response to three intermittent feedings of LT-B (A) or a booster dose of 20 µg of LT-B given on day 49 (B). (A) LT-B feeding days are indicated by the arrows. Antibody concentrations are presented as mean ± SEM. (B) Group means are represented as bars (---). For both (A) and (B), n=4 mice per group, except for the non-transgenic group (nt) which had an n=8 on days -3 to 48.

Figure 3. Study #1 fecal IgA concentrations in response to three intermittent feedings of LT-B (A) or a booster dose of 20 µg of LT-B given on day 49 (B). (A) LT-B feeding days are indicated by the arrows. Antibody concentrations are presented as mean ± SEM. (B) Group means are represented by bars (---). For both (A) and (B), n=4 mice per group, except for the non-transgenic group (nt) which had an n=8 on days -3 to 48.
Figure 4. Study #2 serum IgG concentrations in response to three intermittent feedings of LT-B (A) or a booster dose of 20 µg of LT-B given on day 49 (B). (A) LT-B feeding days are indicated by the arrows. Antibody concentrations are presented as mean ± SEM. (B) Group means are represented by bars (---). For both (A) and (B), n=4 mice per group, except for the non-transgenic group (nt) which had an n=8 on days -3 to 48.

Figure 5. Study #2 serum IgA concentrations in response to three intermittent feedings of LT-B (A) or a booster dose of 20 µg of LT-B given on day 49 (B). (A) LT-B feeding days are indicated by the arrows. Antibody concentrations are presented as mean ± SEM. (B) Group means are represented by bars (---). For both (A) and (B), n=4 mice per group, except for the non-transgenic group (nt) which had an n=8 on days -3 to 48.

Figure 6. Study #2 fecal IgA concentrations in response to three intermittent feedings of LT-B (A) or a booster dose of 20 µg of LT-B given on day 49 (B). (A) LT-B feeding days are indicated by the arrows. Antibody concentrations are presented as mean ± SEM. (B) Group means are represented by bars (---). For both (A) and (B), n=4 mice per group, except for the non-transgenic group (nt) which had an n=8 on days -3 to 48.

Figure 7. Study #2 lung lavage IgA concentrations on day 54 in response to a booster dose of 20 µg LT-B given on day 49. Antibody concentrations are presented as mean ± SEM with n=4 mice per group. The groups are labeled according to the amount of transgenic maize fed on days 0, 7 and 21 in µg. The group nt + 20 is the non-transgenic group fed 20 µg on day 49. The group nt is the non-transgenic group fed all non-transgenic maize. The group neg ctrl is the negative control group never handled, fasted or fed maize.
Figure 1.

(A) Serum IgG in μg/ml over time with different treatment groups.

(B) Scatter plot showing Serum IgG in μg/ml for Day 48 and Day 55 across different treatment groups.
Figure 2.

(A) Serum IgA in μg/ml over time for different treatment groups.

(B) Serum IgA in μg/ml at Day 48 and Day 55 for different treatment groups.
Figure 3.
Figure 4.

(A) Serum IgG in μg/ml over the course of 54 days for different treatment groups. 

(B) Serum IgG in μg/ml for specific treatment groups at days 48 and 54.
Figure 5.

(A) Serum IgA in μg/ml

Day 0
Day 7
Day 21

(B) Serum IgA in μg/ml

Day 48
Day 54

Treatment Group

- 20 μg
- 0.02 μg
- 0.002 μg
- 0.0002 μg
- nt

Day of Sample

-3 6 13 20 27 34 41 48
Figure 6.

(A) Fecal IgA in μg/gram over time.

(B) Fecal IgA in μg/gram across different treatment groups at Day 48 and Day 54.
Figure 7.

Lung Lavage IgA in ng/ml

Treatment Group

20 0.02 0.002 0.0002 nt+20 nt neg ctrl
Chapter 3. Immunogenicity of daily and intermittent oral administration of transgenic maize expressing the *Escherichia coli* heat-labile toxin B subunit

A paper to be submitted to *Infection and Immunity*

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\(^6\)Provided guidance in writing the manuscript
\(^7\)Provided guidance in writing the manuscript and author for correspondence

Abstract

Transgenic maize expressing the non-toxic B subunit of the *Escherichia coli* heat-labile toxin (LT-B) in seed has proven to be an effective oral immunogen in mice. Currently, there is considerable concern over accidental consumption of transgenic maize expressing LT-B by humans and domestic animals. While consuming maize-expressed LT-B appears to have no toxic side effects, we have yet to determine if accidental exposure to LT-B could affect a later response to a vaccine containing LT-B as either an antigen or a carrier.

Our goal was to determine whether previous exposure to LT-B in maize, either daily or intermittent, would affect the immune response to oral vaccine-like boosters of LT-B in maize. We fed mice intermittently (days 0, 7 and 21) with maize pellets containing 0.2 – 20 µg LT-B per dose, or daily for 28 days with transgenic maize incorporated into their diet for daily consumption of 0.2 – 20 µg LT-B to resemble two distinct inadvertent exposure
scenarios. We then boosted both treatment groups with high, vaccine-level transgenic maize pellets (20 µg LT-B per dose) on days 56 and 77. Serum and fecal pellets were collected weekly to determine immune responses by measurement of LT-B-specific antibodies.

We observed dose-dependent antibody responses to the priming doses that were similar in both treatment groups. In addition, all animals that had been previously exposed to LT-B by both intermittent and daily feeding responded strongly to the vaccine-like oral booster doses. Mice that were exposed to maize-derived LT-B, whether daily or intermittently, generated dose-dependent antibody responses to LT-B. When these primed mice were subsequently boosted with a vaccine dose of LT-B, strong LT-B-specific antibodies were detected. This result indicates that mice frequently exposed to LT-B do not develop oral tolerance to LT-B, implying that inadvertent exposure to LT-B should not negatively impact future vaccinations containing LT-B as either an antigen or carrier.

Introduction

Recently, expression of vaccine antigens in plants has been explored as an approach to facilitate oral vaccination (10, 12, 18, 21). Unlike the parenteral route, oral delivery of vaccine antigens is able to elicit both a serum IgG and a secretory IgA response, the latter being essential for protecting the location by which most pathogens enter: mucosal surfaces. Plant delivery of vaccine antigens has many important advantages including economical production, safe delivery, and low risk of carrying mammalian pathogens (11, 17).

Our work focuses on transgenic maize expressing the B subunit of the *Escherichia coli* heat-labile toxin (LT-B). Previous studies have shown that this transgenic maize is both immunogenic and partially protective in a mouse model (2). Because of the natural encapsulation of the LT-B within the maize seed, maize-expressed LT-B is protected against harsh environmental conditions including high temperatures and enzyme degradation (3). This increased stability should lead to improved immunogenicity.
The enhanced stability of maize-expressed LT-B is highly desirable for oral vaccine production, storage and effectiveness; however, it also poses a concern in the case of environmental contamination and/or accidental consumption by humans and domestic animals. Safety issues remain that must be addressed. The use of transgenic maize to produce a vaccine component opens the possibility for low dose antigen exposure of workers involved in its production or of consumers, should the product inadvertently occur in foods (23).

As opposed to the systemic immune system which responds to nearly all foreign antigens, the mucosal immune system is designed to selectively respond to foreign antigens (13). This makes food consumption an activity that generally does not result in an immune response. For example, repeated oral exposure to an egg protein such as ovalbumin will induce a state of immune non-responsiveness often referred to as oral tolerance (13, 22). However, unlike most protein antigens, LT-B is highly immunogenic when administered orally even at low doses (1). Because of its immunogenicity, LT-B is not a typical protein and whether or not oral tolerance can be induced with daily exposure is still in question. While oral tolerance is typically tested by parenteral delivery following oral exposure, maize transgenic for LT-B was designed for an oral vaccine, therefore in this work we test for oral tolerance using oral exposure followed by oral boosters.

The possibility of inadvertent exposure to maize transgenic for LT-B leads to the question of whether previous inadvertent exposure will affect the ability of a person or animal to respond later to a vaccine containing LT-B either as an antigen or as a carrier. In this study, we address two potential exposure scenarios in mice, daily and intermittent exposure. We subsequently booster animals from these two exposure paradigms using a vaccine-like booster strategy in order to observe whether the earlier exposure affects the vaccine response. Our results indicate that the immune system of mice responds to maize-derived LT-B in a dose-dependent manner rather than a frequency-dependent manner. In
addition, all mice responded strongly to the booster dose of LT-B whether they had been previously exposed daily or intermittently.

Materials and Methods

Preparation of maize pellets. Production of maize transgenic for LT-B (4th generation transgenic seeds) and preparation of the maize seed pellets used as intermittent and booster doses were carried out as described previously with the following changes (1, 2). Briefly, the total weight of ground maize seed in each pellet was 0.767 grams and the ground seed was mixed with 550-600 µl of phosphate buffered saline (PBS) (1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 0.15 M NaCl, pH 7.3). Two extra pellets were formed per dose for analysis and verification of LT-B content. Pellets of each type were refrigerated until ELISA analysis of LT-B content (four samples from each pellet).

Preparation of transgenic and non-transgenic diet. Mouse chow in powdered form was obtained from Harlan Teklad (Madison, WI, USA). For the mice that were fed LT-B daily, the diet was mixed with 0.237 grams transgenic (tg) and/or non-transgenic (nt) maize per gram of powdered custom diet specifically formulated to account for the maize added so that the final diet would closely resemble the Global Laboratory Rodent Diet® 2018 (2018 diet). A basal diet made from powdered Global Laboratory Rodent Diet® 2018 was used in feeding all mice during the acclimation period, the intermittently fed mice from days 0-28, and all mice from days 28-84.

To prepare the diets, the powdered components (diet with or without ground maize) were combined and mixed thoroughly before adding 70 ml nanopure water per 100 grams diet. The diet was mixed into dough and formed into a patty about 8 mm thick. The patty was scored, placed on a vented surface and allowed to dry for 24 hours. The patties were broken into ~4 cm squares along the scored lines, flipped over, and allowed to dry for an additional
24 hours. The diet squares were stored at 4°C until use. Samples from extra tg and nt diets were assayed by ELISA for verification of LT-B content (eight samples per diet type).

**Oral immunization of mice.** Five-week-old female, BALB/c mice were obtained from Harlan Sprague Dawley, (Indianapolis, IN, USA) and were allowed a two week acclimation period prior to beginning the experiment with a 12-hour reversed light/dark cycle (lights on at 9 pm). The mice were housed one per cage in the Iowa State University (ISU) animal facility with food and water *ad libitum*. All procedures were approved by the ISU Institutional Animal Care and Use Committee.

Mice were divided into two main treatment groups: daily feeding and intermittent feeding. Within each treatment, mice were divided into four groups of four mice based upon the dose of LT-B they were consuming either daily or intermittently (20, 2, 0.2 µg LT-B or nt maize).

Mice in the daily treatment consumed their appropriate dose of LT-B or nt diet daily for 28 days (days 0 – 28). During this time, mice in the intermittent treatment consumed basal diet and were given pellets of the proper dose on days 0, 7 and 21. After day 28, all mice were allowed a washout period and were only fed basal diet (contains no added tg or nt maize). This basal diet continued from day 28 to the end of the study. On days 56 and 77, all mice were given a booster dose in the form of a tg maize pellet containing 20 µg LT-B except for two of the nt mice from each treatment (daily and intermittent feeding). Mice previously in the nt group that were given 20 µg pellets on days 56 and 77 formed the nt + 20 group. Mice not receiving 20 µg LT-B pellets received nt pellets (nt group) of equal weight. Prior to feeding maize seed pellets, the mouse chow was removed overnight (during the light phase). Mice were allowed to eat the pellet for 2-4 hours with water *ad libitum*. Upon completion of the pellet, the mice were returned to clean cages with mouse chow and water *ad libitum*.
Analysis of extra pellets and diet indicated that the 2 and 0.2 µg LT-B pellets and diet contained amounts of LT-B very similar to the target amount. For these groups, the target amount of LT-B fell within the 95% confidence interval of the sample measurements. However, for the 20 µg pellets and diet, the values measured were higher than anticipated and the target value fell outside the 95% confidence interval. For the 20 µg pellets, the measured values ranged from 19.36 – 40.67 µg LT-B per pellet. For the 20 µg daily diet, the target value was 6.175 µg LT-B/gram which would give about 20 µg LT-B per day with total food consumption of 3.239 g/day as determined prior to the feeding study and monitored during the study. The measured values for this group ranged from 7.89 – 14.03 µg LT-B/gram. No LT-B was detected in either the nt pellets or commercially prepared diet (data not shown). Despite the differences in the target values and the measured values, this dose will be referred to as the nominal 20 µg dose and the groups receiving this dose will be referred to as the Intermittent and Daily 20 µg groups, respectively.

**Mouse weight and food consumption.** Mouse weights were collected before as well as weekly throughout the study in order to determine the effects of fasting and tg maize consumption on general health of the animals. In addition, food consumption was measured at weekly intervals from day 0 – 28. This data was used to confirm the total amount of LT-B that was consumed by mice fed LT-B daily.

**Sample collection, preparation and mouse euthanization.** Blood samples and fecal pellets were collected before the initial dosing as well as weekly throughout the study. Serum and fecal pellets were prepared and stored and fecal pellets were extracted as described previously (1). Mice were euthanized with CO₂ on day 84 with subsequent lung lavages performed at necropsy as described previously (1).

**Quantification of LT-B and LT-B-specific IgG and IgA.** ELISAs for the quantification of LT-B and LT-B-specific IgG and IgA were performed as previously described (1, 2, 8).
Quantification of LT-B-specific IgG1 and IgG2a. Measurements of LT-B-specific IgG1 and IgG2a in serum samples were carried out in a similar manner as measurements of LT-B-specific IgG and IgA (described above) with modifications. Briefly, LT-B-specific antibodies were detected using biotin-conjugated rat anti-mouse IgG1 (diluted 1:2000; BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) or biotin-conjugated rat anti-mouse IgG2a (diluted 1:500; BD Biosciences Pharmingen). Sample concentrations were determined by comparing to a standard curve consisting of wells coated with either purified mouse IgG1 (MOPC 21, Sigma, St. Louis, MO, USA) with a range of 0.78 – 100 ng/ml or IgG2a (UPC 10, Sigma) with a range of 1.95-250 ng/ml.

Statistical analysis. We analyzed antibody and mouse weight data with general analysis of variance, using treatment (intermittent or daily feeding schedule), LT-B dose, and sample day as variables in the model. Each day that was significantly different (p < 0.05) from the pre-feed date was further analyzed by between group contrasts. All antibody data were log transformed prior to analysis to correct for unequal variance (9). We used non-log transformed data for graphs. Pearson’s correlation values were obtained by using raw data for determination of correlation between antibody data sets. Analyses were conducted using the statistical software Statistix 8 (Analytical Software, Tallahassee, Florida). 95% confidence intervals were calculated in order to confirm that the measured amounts of LT-B in the pellets and diets were within the expected range. This method was also used to confirm that the actual amounts of diet consumed by mice fed LT-B approximated the intended amount.

Results

Effect of maize consumption on mouse weight and total food consumption. Mouse weights were recorded before beginning the experiment as well as weekly throughout the experiment. No differences were detected between treatments or doses of LT-B (data not shown). In addition, fasting prior to feeding pellets did not affect mouse weight. The
average mouse weight at the beginning of the study was 18.2 \((\pm 0.17)\) g and at the end of the study was 20.8 \((\pm 0.19)\) g.

Food consumption was recorded over the first five weeks of the experiment during the priming period as well as twice during the booster period. No difference was detected between mice that had been fasted (intermittent groups) and those that had not (daily groups). When analyzing whether LT-B consumption affected total food consumption, no difference was detected between those that had been given tg maize and those that received only nt maize (data not shown). This data supports the claim that ingesting maize transgenic for LT-B had no negative effects on food consumption and growth of mice.

The daily diets were formulated so that the mice would consume the proper dose of LT-B if they ate 3.239 g/day. All groups, except for the Daily 20 group consumed the expected amount of diet each day as indicated by the target value falling within the 95\% confidence interval for the measured values for each group. The Daily 20 group consumed less food than intended; however, because we found the diet for this group actually contained more LT-B than expected, all mice still consumed more than 20 µg LT-B/day with an actual range of 26.8 – 31.8 µg LT-B per day. This amount was quite similar to the Intermittent 20 µg pellets which on average contained 33.9 µg LT-B.

**Serum IgG.** From days -2 to 55 (the priming period), a dose-dependent antibody response was observed as evidenced by a significant linear effect of dose \((p < 0.0001)\) (figure 1A). While there was a statistical difference between intermittent and daily treatments \((p = 0.0092)\), this difference was only observed on days 48 and 55 with all doses (20, 2 and 0.2 µg LT-B) either significantly or marginally higher for daily feeding than for intermittent feeding on these days \((p \leq 0.0899)\). From days 62-83 (the booster period; figure 1B), a dose-dependent response based on the priming dose was observed (linear effect: \(p < 0.0001\)) although it is not as visually obvious because most groups responded strongly to the 20 µg LT-B booster doses despite the fact that they were primed with differing amounts of LT-B. In
general, animals fed lower doses of LT-B during the priming period demonstrated a greater fold increase in antibody level from day 55 – 83.

We further compared serum IgG concentrations between groups for days 34 and 83 which were peak days for most groups following priming and boosting, respectively. Moreover, day 34 was not significantly different from days 41, 48 and 55. As seen in figure 2A, on day 34 we found that groups fed similar doses of LT-B (ie. Intermittent 20 vs Daily 20) were not significantly different from one another. Another way to compare data is to compare responses for groups receiving similar total amounts of LT-B during the priming period. For example, the Intermittent 20 µg group received a total of 60 µg across three pellet feedings and the Daily 2 µg group received a total of 56 µg across 28 days of feedings. When we compare the groups fed similar total amounts of LT-B during the priming feedings, those fed a total dose of about 60 µg were not significantly different from each other, but those fed a total dose of about 6 µg were significantly different. This may indicate that 2 µg LT-B daily was sufficient for a strong priming response; however, 0.2 µg daily resulted in only a weak priming response. On day 83, we found that any group previously primed with LT-B, either daily or intermittently, responded strongly (5- to 105-fold) to the two 20 µg LT-B boosters (figure 2B). We define a strong response as a ≥ 5-fold increase in antibody concentration from day 55 to day 83 for IgG and IgA. There was no difference between groups fed similar doses but with different feeding treatments (ie. Intermittent 20 vs. Daily 20), or between groups fed similar total amounts of LT-B during the priming period.

Although the Daily 0.2 µg group did not respond as strongly to boosting as the other groups, it was not significantly different from most groups fed LT-B during priming and serum IgG increased over 17-fold from day 55 to day 83.

**Serum IgA.** A dose-dependent response was observed with the priming data set for serum IgA concentrations induced by LT-B as indicated by a significant linear effect in dose (p < 0.0001) as shown in figure 3A. Over time there was no difference between the intermittent
and daily treatments. A dose response was observed for the booster data set based upon the
priming dose as indicated by a significant linear effect (p < 0.0001) (figure 3B).

We further compared differences between groups on days 27 and 83. Day 27 was
significantly different from days 34 – 55; however, as it demonstrated peak antibody levels
for all groups, we chose to analyze this data set. Day 83, although not the peak day,
demonstrated the same group comparison trends as the peak day (day 62), and also
demonstrated the level of antibody in all groups following two booster doses. As seen in
figure 4A, for day 27 serum IgA, groups fed similar doses of LT-B whether daily or
intermittently were not significantly different from one another. However, groups fed similar
total amounts of LT-B (60 and 56 or 6 and 5.6 µg LT-B) during the priming feedings were
significantly different from each other on day 27 indicating that intermittent feeding of larger
doses is better able to stimulate an IgA antibody response than small doses daily. On day 83,
we found that any group previously primed with LT-B, either daily or intermittently,
responded strongly (~8- to 34-fold increase from day 55-83) to the 20 µg LT-B boosters
(figure 4B). While antibody concentrations were much higher on day 62 than any other day,
this is due in part to the short half-life of IgA. By day 83, there was no difference between
groups fed similar doses whether daily or intermittently, or between groups fed similar total
amounts of LT-B during the priming period. Despite the weaker response of the Daily 0.2 µg
group, it was not significantly different from the Intermittent 2 and 0.2 µg groups and it
demonstrated a nearly 17-fold increase from day 55 to day 83. Additionally, the nt + 20
group clearly responded to the 20 µg booster as demonstrated by the high levels of antibody
by day 83. The nt + 20 group on day 83 (four weeks after the first exposure to LT-B) had 1.5
µg/ml serum IgA which was similar to the highest concentrations obtained by the daily and
intermittent groups on day 27 (Daily 20 µg group = 1.3 µg/ml serum IgA; Intermittent 20 µg
group = 1.4 µg/ml serum IgA).
**Fecal IgA.** Because of the high rate of antibody breakdown in feces, a dose-dependent response to LT-B was not as obvious as in the serum antibody measurements; however, a dose-dependent response was present in the priming data set as evidenced by a significant linear effect ($p < 0.0001$) as shown in figure 5A. Over time there was no significant difference between intermittent and daily treatments. A dose-dependent response was observed ($p < 0.0001$) for fecal IgA in the booster data set based upon the priming dose of LT-B although it was not visually obvious as most mice responded strongly to the booster doses despite being primed with different doses of LT-B (figure 5B).

We further compared groups on days 48 and 83. Day 48 was significantly different from days 27-41 and 55; however, it demonstrated peak antibody levels for most groups. As seen in figure 6A, on day 48 we found that groups fed similar doses of LT-B whether daily or intermittently were not significantly different from one another. However, we had mixed results when comparing groups that were primed with similar total amounts of LT-B. Groups fed a total amount of about 60 µg LT-B were significantly different from one another, suggesting that intermittent feeding is the most efficient means of immunizing. However, the groups fed a total amount of about 6 µg LT-B were not significantly different from one another with both groups demonstrating relatively weak responses. On day 83, we found that any group previously primed with LT-B, either daily or intermittently, responded strongly (~7- to 35-fold increase from day 55 – 88) to the 20 µg LT-B boosters (figure 6B). By day 83, there was no significant difference between groups fed similar doses during priming whether daily or intermittently, or between groups fed similar total amounts of LT-B during the priming period. Additionally, the nt + 20 group was clearly responding to the 20 µg boosters as demonstrated by the high levels of antibody (mean 0.7 µg/gram) by day 83.

**Lung lavage IgA.** Upon euthanization, lung lavages were performed to measure LT-B-specific IgA in the lungs. As shown in figure 7, all groups receiving LT-B either daily or intermittently during priming demonstrated significantly higher levels of LT-B-specific IgA
than the nt control group ($p \leq 0.0161$) except for the Intermittent 0.2 µg group which was only marginally higher than the nt group ($p = 0.0658$). While the nt + 20 group was not significantly higher than the nt group, the nt + 20 data did demonstrate elevated levels of IgA that were not significantly different from either of the 0.2 µg groups. There were no differences between groups fed similar doses but different treatments, or between groups fed similar total amounts of LT-B during the priming period.

**Correlation of data.** Serum IgG and serum, fecal, and lung lavage IgA measurements from day 83/84 were analyzed for correlation between data sets. We found that serum IgG levels did not correlate with any of the IgA data sets; however, all three of the IgA data sets were significantly correlated ($p \leq 0.0001$), with the highest correlation found between serum and fecal IgA.

**IgG1:IgG2a ratios.** To determine whether oral administration of maize transgenic for LT-B will lead to a Th1- or a Th2-type antibody response, LT-B-specific IgG1 and IgG2a were measured in serum samples from days 34 and 83. Ratios of IgG1:IgG2a > 1 are considered indicative of a Th2-type response, while ratios < 1 are considered indicative of a Th1-type response (5). On both days, all mice that received LT-B, either daily or intermittently demonstrated the presence of a strong Th2 response as indicated by high levels of IgG1 in comparison to IgG2a levels (Table 1).

**Discussion**

In this study, we address the immunologic effects of inadvertent exposure to maize transgenic for LT-B using laboratory mice as a model. Mice were given two exposure scenarios: daily and intermittent. We then administered LT-B to these animals in a vaccine-like fashion to determine the effects of the daily and intermittent exposure on a future vaccine response. We found that both intermittent and daily exposures to maize transgenic for LT-B are able to elicit dose-dependent antibody responses with no consistent difference
between treatments. Further, we found that animals previously exposed to LT-B, whether daily or intermittently, responded strongly to the booster doses given on days 56 and 77. This clearly indicates that daily exposure to LT-B does not induce immune non-responsiveness commonly referred to as oral tolerance. While daily exposure to LT-B induced strong antibody responses, especially in the 20 and 2 µg groups, it did not induce responses that were consistently higher than mice receiving intermittent doses of LT-B despite the fact that the daily fed mice were exposed to a total quantity of LT-B that was much higher than those fed the same dose intermittently. Thus, clearly indicating that for an optimal vaccine antibody response, only a few well-spaced doses are necessary and more frequent administration would simply be a waste of product.

The data from the nt + 20 group provides another example of how dose spacing affects antibody responses. This group received no LT-B until the 20 µg LT-B booster doses on days 56 and 77. Unlike the Intermittent 20 µg group whose doses were spaced one and two weeks apart (days 0, 7 and 21), the nt + 20 group received only two doses three weeks apart. Of interest is that four weeks after the I 20 and nt + 20 groups received their first dose, the serum IgG levels were quite different. The Intermittent 20 µg group, after three doses, had a serum IgG level of 18.8 µg/ml as compared to the nt + 20 group which, after two doses, had a serum IgG level of 43.5 µg/ml which is more than 2-fold higher. This suggests that increased spacing between doses enhances IgG responses. Interestingly, the serum and fecal IgA levels were not affected in the same way as serum IgG, as both groups demonstrated similar antibody levels. While it is possible that the age of the mice could have affected the resulting IgG response, it is not likely as the mice were only 8 weeks apart in age and all were adults at first exposure to LT-B.

While fecal IgA measurements for the priming data set (figure 5A) demonstrate a similar dose response for both treatments on days 34 – 55, the earlier days show more inconsistencies. This is especially true for the 20 µg groups where it appears as though daily
mice did not develop an antibody response until after the mice stop receiving daily doses of LT-B. While this may be due to difficulty in measuring antibody concentrations in the feces due to antibody breakdown, we have been able to measure fecal IgA in intermittently fed mice and their fecal IgA correlates with serum IgA, thus we suspect that another problem may be that the LT-B that is in the gut at all times for daily fed mice may be binding the LT-B-specific IgA. Because our ELISA depends on the IgA binding to LT-B in the wells, any antibody already bound to LT-B would not be detected. This would not have been a problem with mice fed intermittently since feedings were carried out one day after serum is collected and the LT-B should pass through the gut long before the next collection 6 days later.

During the booster period (shown in figures 1B, 3B, 5B), it is clear that the response to the first booster dose is much stronger than the response to the second booster dose for all measurements, but especially for serum IgA. This strong response is indicated by a steeper slope from days 55 to 62 than from days 76 to 83. While this may be partially due to previously present antibodies binding the LT-B from the second booster before it can bind to the gut and generate an immune response, it is more likely due to antibody-mediated feedback regulation (see Hjelm et al. (7) for a recent review). Because higher levels of antibodies are present on day 77, the antibodies compete with LT-B-specific B cells and prevent clonal expansion (5). It is possible that if we had given the second booster after antibody levels had waned, we may have seen a more robust response to the second booster.

Our findings concerning IgA at multiple locations also demonstrate the potential of LT-B in preventing or alleviating disease. First, the correlation of serum, fecal and lung IgA data sets demonstrates the range of the common mucosal immune system and emphasizes the potential of oral immunization with LT-B as a carrier. Not only could oral immunization protect against gastrointestinal pathogens, but also respiratory pathogens as indicated by the presence of specific IgA in the lung. Because the mice consumed the LT-B willingly and were not gavaged, it is unlikely that the IgA in the lung is due to aspiration. While vaginal
IgA was not measured, the common mucosal immune system may make it possible to orally induce IgA in the vagina to protect against sexually transmitted diseases (19). Another way in which LT-B may be therapeutic is by modulating T helper cell responses. Our data demonstrating high IgG1 and low IgG2a levels supports previous findings that orally administered LT-B leads to a Th2 response (4, 15, 16). Th1 responses are generally pro-inflammatory while Th2 responses tend towards anti-inflammatory (6, 14). This would make LT-B a good candidate to serve as a carrier in therapies for diseases in which the inflammatory process is the primary cause of disease such as type I diabetes, multiple sclerosis and rheumatoid arthritis (6, 16, 20). LT-B conjugated to a target antigen could potentially direct or shift the response towards anti-inflammatory in order to prevent or relieve disease symptoms. One downfall to this Th2 response is that it also leaves the potential for development of allergies to linked proteins as Th2 cytokines can induce class-switching to IgE in some ill-defined situations (14). Further studies of LT-B, conjugated or unconjugated, are necessary to determine whether LT-B as a carrier for other antigens is both safe and therapeutic.

The data presented here show that consumption of maize transgenic for LT-B does not result in adverse toxic effects as confirmed by no changes in animal body weight or overall health. In addition, we found that inadvertent daily exposure is not likely to negatively impact the immune response to a vaccine or therapeutic agent that may contain LT-B. While further safety studies are needed to ensure that consumption of LT-B is not likely to induce allergies to co-administered food antigens, observations thus far indicate that consumption of LT-B is safe.
References


Table 1. Serum IgG1:IgG2a Ratios

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG1 (µg/ml)</th>
<th>IgG2a (µg/ml)</th>
<th>IgG1:IgG2a</th>
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<tr>
<td>I 20</td>
<td>11.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04</td>
<td>319.0</td>
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<tr>
<td>I 2</td>
<td>7.5</td>
<td>0.10</td>
<td>73.8</td>
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<tr>
<td>I 0.2</td>
<td>0.9</td>
<td>0.02&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>D 20</td>
<td>19.7</td>
<td>0.08</td>
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</table>

Day 83

<table>
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<tr>
<th>Group</th>
<th>IgG1 (µg/ml)</th>
<th>IgG2a (µg/ml)</th>
<th>IgG1:IgG2a</th>
</tr>
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<tr>
<td>I 20</td>
<td>67.9</td>
<td>0.43</td>
<td>157.1</td>
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<tr>
<td>I 2</td>
<td>48.4</td>
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<tr>
<td>I 0.2</td>
<td>56.8</td>
<td>1.81</td>
<td>31.4</td>
</tr>
<tr>
<td>D 20</td>
<td>57.0</td>
<td>2.04</td>
<td>28.0</td>
</tr>
<tr>
<td>D 2</td>
<td>56.4</td>
<td>0.11</td>
<td>520.8</td>
</tr>
<tr>
<td>D 0.2</td>
<td>21.6</td>
<td>0.30</td>
<td>71.0</td>
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<td>nt + 20</td>
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<tr>
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<td>0.03</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>n = at least 3-4 except where noted  
<sup>b</sup>n= 2 due to lack of sample after running other ELISAs  
<sup>c</sup>n=1 due to lack of sample after running other ELISAs  
<sup>d</sup>Intermittent and daily nt groups combined for day 34, n= 5-7 due to lack of sample after running other ELISAs
Figure Legends

Figure 1. Serum IgG concentrations during the priming period (A) or the boosting period (B).
(A) Arrows indicate feedings for intermittent LT-B groups and the bar represents the period during which daily LT-B groups were given diet containing LT-B ad libitum. (B) Arrows indicate 20 µg booster doses on days 56 and 77 given to all mice except the nt group. Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during priming and a number representing the dose of LT-B given in µg or nt representing the non-transgenic control group during the priming period. For (B), nt + 20 represents a previously unprimed group that was administered 20 µg LT-B on days 56 & 77. Antibody concentrations are presented as mean ± SE and n = 4 per group.

Figure 2. Serum IgG concentrations on days 34 (A) and 83 (B). Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during the priming period and a number representing the dose of LT-B given in µg or nt representing the non-transgenic control group. For (B), nt + 20 represents the previously unprimed group that was administered 20 µg LT-B on days 56 & 77. Total LT-B refers to the total amount of LT-B the mice were exposed to during the priming period in µg. Groups significantly different from one another are indicated by different letters beneath each group. Antibody concentrations are presented as mean ± SE and n = 4 per group.

Figure 3. Serum IgA concentrations during the priming period (A) or the boosting period (B).
(A) Arrows indicate feedings for intermittent LT-B groups and the bar represents the period during which daily LT-B groups were given diet containing LT-B ad libitum. (B) Arrows indicate 20 µg booster doses on days 56 and 77 given to all mice except the nt group. Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during priming and a number representing the dose of LT-B given in µg or nt representing the non-transgenic control group during the priming period. For (B), nt + 20
represents a previously unprimed group that was administered 20 µg LT-B on days 56 & 77. Antibody concentrations are presented as mean ± SE and n = 4 per group.

Figure 4. Serum IgA concentrations on days 27 (A) and 83 (B). Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during the priming period and a number representing the dose of LT-B given in µg or nt representing the non-transgenic control group. For (B), nt + 20 represents the previously unprimed group that was administered 20 µg LT-B on days 56 & 77. Total LT-B refers to the total amount of LT-B the mice were exposed to during the priming period in µg. Groups significantly different from one another are indicated by different letters beneath each group. Antibody concentrations are presented as mean ± SE and n = 4 per group.

Figure 5. Fecal IgA concentrations during the priming period (A) or the boosting period (B). (A) Arrows indicate feedings for intermittent LT-B groups and the bar represents the period during which daily LT-B groups were given diet containing LT-B *ad libitum*. (B) Arrows indicate 20 µg booster doses on days 56 and 77 given to all mice except the nt group. Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during priming and a number representing the dose of LT-B given in µg or nt representing the non-transgenic control group during the priming period. For (B), nt + 20 represents a previously unprimed group that was administered 20 µg LT-B on days 56 & 77. Antibody concentrations are presented as mean ± SE and n = 4 per group.

Figure 6. Fecal IgA concentrations on days 48 (A) and 83 (B). Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during the priming period and a number representing the dose of LT-B given in µg or nt representing the non-transgenic control group. For (B), nt + 20 represents the previously unprimed group that was administered 20 µg LT-B on days 56 & 77. Total LT-B refers to the total amount of LT-B the mice were exposed to during the priming period in µg. Groups significantly
different from one another are indicated by different letters beneath each group. Antibody concentrations are presented as mean ± SE and n = 4 per group.

Figure 7. Lung lavage IgA concentrations on day 84. Group abbreviations consist of a letter indicating intermittent (I) or daily (D) feeding during the priming period and a number representing the dose of LT-B given in µg. The nt + 20 group represents a previously unprimed group that was boosted with 20 µg and the nt group represents the non-transgenic control group. Total LT-B refers to the total amount of LT-B the mice were exposed to during the priming period in µg. Groups significantly different from one another are indicated by different letters beneath each group. Antibody concentrations are presented as mean ± SE with n = 4 per group.
Figure 1

A

Day of Sample

Serum IgG (µg/ml)

-2 6 13 20 27 34 41 48 55

B

Day of Sample

Serum IgG (µg/ml)

55 62 69 76 83
Figure 2

A

Day 34

Serum IgG (µg/ml)

Total LT-B  60  6  0.6  560  56  5.6
AB  B  C  A  AB  C  E

B

Day 83

Serum IgG (µg/ml)

Total LT-B  60  6  0.6  560  56  5.6
AB  AB  AB  A  BC  C  D

nt  nt + 20
Figure 3

A

Day of Sample

Serum IgA (µg/ml)

Day of Sample

B

Day of Sample

Serum IgA (µg/ml)
Figure 4

Day 27

Serum IgA (µg/ml)

Day 83

Serum IgA (µg/ml)
Figure 5

A

Day of Sample

Fecal IgA (µg/gram)

B

Day of Sample

Fecal IgA (µg/gram)
Figure 6

A

Day 48

Fecal IgA (µg/gram)

Day 83
Figure 7

Lung lavage IgA (ng/ml)

Total LT-B

120 60 12 6 1.0 0.6 D 20 560 D 2 56 D 0.2 5.6 nt+20 nt

A A ABC A A AB BC C
Chapter 4. Discussion

Maize-expressed LT-B has much potential for use in oral vaccines, whether as an antigen or as a carrier due to its high oral immunogenicity (Fingerut et al. 2006; Guidry et al. 1997). However, because the use of plants as production and delivery systems for oral vaccines is a relatively new area of research, many questions concerning the efficacy and safety of these vaccines remain to be addressed (Wolt et al. 2006). We attempted to determine baseline safety information for use in future safety studies of LT-B as an oral vaccine component.

In the first study, we set out to determine at what level LT-B is nonimmunogenic when ingested. This was to identify the immunological consequences of inadvertent exposure to LT-B in maize should it escape confinement, assuming that immunogenicity is one of the most sensitive measures of whether an ingested protein has an effect on the body. We found that LT-B in maize results in dose-dependent antibody responses upon oral administration. In addition, we found that 0.02 µg LT-B, when consumed three times intermittently, results in immune priming even though it does not elicit measurable antibody responses. This indicates that consuming low doses of LT-B may result in immunological or other outcomes that are not detectable, even with highly sensitive assays. Furthermore, we found that 0.002 µg LT-B was the highest dose tested that did not result in a measurable antibody response or immune priming. The fact that LT-B is immunogenic in mice at levels as low as 0.02 µg, a dose 1000-fold lower than a vaccine dose, underscores the extraordinary immunogenic potential of LT-B for use as a vaccine component.

The second study evaluated the immunological effects of intermittent and daily administration of LT-B in maize in order to resemble periodic or near continuous inadvertent exposure to LT-B. We found that antibody responses were LT-B dose-dependent more so than frequency-dependent with mice fed similar doses but at differing intervals generating
very similar antibody responses. In addition, all mice, whether primed intermittently or
daily, responded strongly to the subsequent booster doses of LT-B. Because mice previously
fed daily responded as strongly to the booster doses as mice previously fed intermittently,
and daily oral exposure elicited LT-B-specific serum IgG, we can conclude that daily
exposure to LT-B does not result in oral tolerance. The ability of LT-B to avoid oral
tolerance induction, even when administered at low doses daily, demonstrates its outstanding
potential as an oral vaccine component.

Together, these two studies begin to assess the immunologic effects of oral exposure
to transgenic maize expressing LT-B. Wolt et al. (2006) suggest that two primary categories
that must be addressed in regard to safety evaluation of plant-derived pharmaceuticals are
exposure and hazard assessment. Our first study addresses exposure assessment by
identifying a level at which oral exposure should not have an effect on the body. Both
studies address hazard assessment in regard to measurement of mouse weights throughout the
study and finding no difference between mice orally administered LT-B and those
administered non-transgenic maize. The second study further evaluates the hazard of
inadvertent exposure in a practical use setting by assessing whether previous exposure would
have a negative impact on a vaccine response. Taken together, the results from these two
studies indicate that oral exposure to LT-B in transgenic maize is not hazardous.

Safety issues that remain to be addressed for maize-expressed LT-B include
allergenicity studies. No indication of allergy development due to oral exposure to LT-B in
maize has been reported in previous studies (as reviewed by Wolt et al. 2006) or observed in
our animal studies; however, because we found that LT-B induces antibody responses that
are strongly Th2 biased, the potential for allergy development exists (Moss et al. 2004).
These studies should first evaluate whether allergy to LT-B develops. Subsequent studies
focusing on the development of allergies to conjugated or co-administered protein antigens
would also be beneficial. This would indicate whether using LT-B as an adjuvant or carrier
is likely to induce allergy to the antigen of interest. It would also be beneficial to examine whether LT-B in transgenic maize elicits an allergenic response to maize proteins which would cause food allergy.

LT-B as a model antigen for safety evaluation of pharmaceutical products produced in plants is an extreme example due to its high immunogenicity (Fingerut et al. 2006; Guidry et al. 1997). Most other proteins administered orally will likely not result in strong immune responses as those demonstrated by LT-B. However, LT-B is a good model antigen because it demonstrates the furthest extent to which a vaccine component is likely to be orally immunogenic and provides a baseline for further safety evaluation of LT-B, whether as a vaccine antigen or carrier, and other plant-produced vaccine antigens.

References


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