2009

Allergy models and related assays to test the allergic qualities of Escherichia coli heat labile toxin subunit B

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by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Microbiology

Program of Study Committee:
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Iowa State University

Ames, Iowa

2009

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Abstract

Transgenic maize containing *Escherichia coli* heat labile toxin subunit B (LT-B) has proven to be a strong mucosal immunogen in animal models and stimulates an IgG1 and IgA antibody response when administered orally. There is concern the T-helper cell 2 response stimulated by LT-B could sensitize the immune system to produce an allergic response. This concern is further compounded by the approximate 80% homology which exists between *E. coli* heat labile toxin and cholera toxin (CT), a known allergy inducer. Thus, the research was based on ascertaining what possible allergic stimulation, if any, LT-B could have on animal models.

The first experiment was to reproduce an established allergy model as a positive control, using peanuts as the allergen and CT as the adjuvant. The animals were administered 10 µg CT and 5 mg of peanut extract via intragastric gavage per dose and received 4 sensitizing doses. A number of assays were also established to measure various aspects of an allergic response. Mice treated with CT and peanut extract showed higher concentrations of total and peanut specific IgE, peanut and CT specific IgG1, and higher visual scores of allergy symptoms. A second experiment was designed in which animals were fed food pellets containing LT-B (100 µg or 20 µg) with peanuts. One control group was fed CT and peanuts. Neither LT-B fed animal group produced an allergic response to peanut; however, the animals fed CT and peanuts also did not produce an allergic response. A third experiment was designed to optimize the parameters of the CT and peanut extract positive control, with CT and peanuts both being gavaged or fed at various concentrations (CT = 20, 10, or 0 µg; peanut = 20, 10, 5, or 0 mg). The route of delivery was the most important factor. Mice treated with CT and peanut both by intragastric gavage showed

significant increases of total and peanut specific IgE, and peanut specific and CT IgG1 from the naïve mice. Mice fed CT and peanuts did not show a significant difference from the naïve mice in any measured antibody concentrations.

These experiments demonstrated LT-B does not induce an allergic response to co-fed novel proteins. Cholera toxin needs to be administered via intragastric gavage to induce allergies. There is no difference between 10 μg and 20 μg concentration doses of intragastric gavage CT. When administered via fed pellet, CT does not elicit an immune response. As the CT is not excreted in the feces, the toxin is probably broken down before it reaches the intestines.

Peanut is only capable of eliciting an allergic response when both it and the adjuvant CT is administered via intragastric gavage, and the peanut can be gavaged after CT and still elicit an allergic response. There is no difference between 10 mg and 5 mg concentrations of intragastric gavaged peanut. If peanut is gavaged without CT, it will elicit an IgG1 immune response, which is not an indicator of an allergic reaction.

Chapter 1: Literature Review

I. Allergic Responses

A. Introduction. Food allergies are a significant health concern in the twenty first century, affecting roughly 2-8% of infants and young children, as well as 1-2% of adults (Helm and Burks 2000). Peanut allergies in particular affect roughly 1.5 million Americans (Li et al. 2000). The prevalence of people with allergies is increasing in the population (Kimber and Dearman 2002). Food allergies are a major healthcare issue, which means vaccines that stimulate the mucosal immune system must be examined to ensure a lack of an allergic reaction. In order to study the likelihood of an allergic reaction, a basic understanding of the immune response which causes an allergic reaction is needed.

B. Immune response of Allergies (T-helper 2 responses). Two of the most easily quantified factors for measuring an allergic response are concentrations of IgE and histamine (Helm and Burks 2000; Kimber and Dearman 2002). In fact, allergic responses are defined by the presence of an IgE-mediated response (Dearman and Kimber 2001; Helm and Burks 2000). Both of these responses are induced, either directly or indirectly, by a T-helper cell 2 (Th2) mediated response.

The Th2 response begins when an antigen has entered the body, is processed, and is presented to a naïve CD4 T-cell by an antigen presenting cell. Dendritic cells are the most potent activator of naïve T-cells. Antigen presentation can occur in either a local lymph node or the spleen; however, antigen presentation which results in an ingested allergen allergic response usually takes place in a mesenteric lymph node (Davies and O'Hehir 2008). When the naïve CD4 T-cell comes into contact with its antigen, it begins the process of differentiation which leads the T-cell to become either a T-helper 1 (Th1) or a Th2 cell. This

process is controlled by the cytokine environment, transpires in three stages as described by Gilmour and Lavender (2008), and briefly is summarized below.

The first stage is the commitment phase. The initial differentiation decision is dictated by cytokines, present in the local environment, which bind to receptors on the naïve CD4 cells. These cytokines are primarily produced by macrophages and dendritic cells, but also by other surrounding T-cells and eventually, the differentiating T-cell itself. Interleukin-4 (IL-4) and interleukin-13 (IL-13) are the most important cytokines to induce the Th2 differentiation. IL-4 will bind to type I (IL-4 Rα/γ_c) receptor complexes while both IL-4 and IL-13 will bind to type II (IL-4 Rα /IL-13Rα 1) receptor complexes.

During the second stage, the reinforcement phase, the naïve T-cell begins to produce transcription factors which amplify different specific gene loci, producing proteins that stimulate the T-cell's production of IL-4, IL-5, and IL-13, as well as the transcription factors GATA3 and c-Maf. GATA3 stimulates genes which encode a variety of receptor chains on the T-cell used for various cellular interactions and proliferation. GATA3 has been found in low concentrations in naïve and Th1 cells, but in high concentrations in Th2 cells. It is necessary for the initial stages of Th2 cell differentiation, but does not affect mature Th2 cells. After the Th2 cell becomes mature, it requires c-Maf to regulate the production of IL-4 and IL-5. The transcription factor c-Maf, however, is not able to stimulate cytokine production initially and only begins to have an impact during the later portion of the reinforcement phase.

The third and final stage is the maturation phase. During this phase, newly produced transcription factors such as activating protein 1 (AP-1) and nuclear factor-κB (NF-κB) are constant or of sufficient concentration to allow the Th2 cell to fully regulate its own cytokine

production, so the Th2 cell no longer needs to depend on surrounding cells for cytokines. At this point, the fully differentiated Th2 cell in turn provides T cell help to antigen stimulated B-cells and begins the process of B-cell activation. This occurs in the paracortex, primary follicles, and germinal centers of a lymph node.

An activated B-cell begins to produce antibodies. By undergoing a process called isotype switching, the B-cell can produce a variety of different antibodies, including IgM, IgD, many subclasses of IgG, IgA, and IgE. If the B-cell is stimulated by a Th2 cell, it first manufactures IgM, then, via isotype switching, can produce one of three subclasses of IgG, IgG1, IgG3, or IgG4, and then switch again to either the isotype IgE or IgA. The B-cell can also go straight from producing IgM to IgE (Takhar et al. 2005). Not every B-cell undergoes isotype switching until it produces IgE. Some B-cells remain at IgM, some stop isotype switching at an IgG subtype, and some isotype switch to IgA. Only 0.4 to 2 B cells per every one hundred thousand produce IgE (Davies and O'Hehir 2008). IgE has a serum half life of 60 hours, thus it will take 60 hours for one half of the concentration of IgE in serum to attach to Fcε RI receptors, which are present on antigen presenting cells such as monocytes and dendritic cells, as well as basophils, eosinophils, and mast cells (Kubota et al. 2006; Murphy et al. 2007). IgE binding to Fcε RI receptors has a very high affinity binding (Ka = 10^{-10} M), nearly a hundred times stronger than IgG binding to Fcγ RI receptors (Ka = 10^{-8} M) (Davies and O'Hehir 2008). Once IgE has bound to the Fcε RI receptor, it remains there until the IgE antibody binds to its allergen or the cellular membrane turns over.

When an allergen binds to multiple antibody receptors complexes on the mast cell, they become cross-linked, or clustered. Cross linking of the IgE Fcε receptors will trigger the mast cell to degranulate, causing the release of histamine into the blood stream.

Histamine is a potent mediator of vasodilation, and sets off a series of physiological responses, including vascular permeability, resulting in an increase of mucin secretion, and smooth muscle constriction, which leads to difficulty breathing, hypotension, and a decrease in body temperature (Bischoff and Kramer 2007; Ohtsu 2008). Histamine is one of the most commonly measured mediators in allergic reactions and is used as a marker of allergic response in many studies (Kimber and Dearman 2002). Mast cells located in the intestines, however, also release a number of other mediators. These mediators include proteases and heparin, which are preformed mediators of protein modification and anticoagulants, or tumor necrosis factor and leukotriene B4, which mediate innate immune responses. Other allergy mediators released by activated mast cells include eicosanoids and interleukin 5 (IL-5): the former is a proinflammatory hormone and the latter is a cytokine which recruits eosinophils (Bischoff and Kramer 2007).

Eosinophils are granulocytes that are involved in the allergic responses, although their involvement does not begin until several hours after exposure to an allergen. Eosinophils release several granule mediators, including eosinophil peroxidase, major basic protein, eosinophil cationic protein, and eosinophil derived neurotoxin (Minai-Fleminger and Levi-Schaffer 2009). These mediators are toxic to epithelial cells and alter smooth muscle responses resulting in difficulty breathing and changes in blood pressure. The presence of eosinophils in the lungs is of particular research interest, since chronic exposure to an allergen induces tissue remodeling caused by the eosinophil released mediators, which can result in scar tissue and thicker airway walls (Minai-Fleminger and Levi-Schaffer 2009).

To summarize, the allergic response is typically measured by concentrations of IgE and histamine found in serum. During the development of an allergic state, a Th2 response

occurs, which leads to the stimulation of B-cell antibody production and, after isotype switching, may result in the production of IgE. IgE receptor complexes on mast cells can lead to the release of histamine, which is not related to a Th2 response but is an indication of an allergic response. By understanding from where the measurements for an allergy come, and the mechanics behind allergies, the process of assessing animal allergy models becomes more straightforward.

II. Animal Allergy Models

A *Introduction.* Animal models have been very important in understanding mechanisms of allergy induction. There are no naturally occurring animal models of allergy used in research. Most models systems need both an antigen and an adjuvant. There are currently a variety of animal allergy models present in the literature. These models have a number of delivery methods for allergy models, including intraperitoneal injection, intranasal delivery, and intragastric gavage administration. There are also a number of possible allergens for induction of an immune response, including cow's milk, ovalbumin, eggs, and peanuts. There have been several different types of rodents used for these allergy models, including BALB/c mice, AKR/J mice and C3H mice, and Brown Norwegian rats. While there are other adjuvants available to increase the immune response to an allergen, they typically must be administered by injection. The only orally administered mucosal adjuvant used to elicit an allergic response is cholera toxin (CT). As the purpose was to study orally induced allergies, this discussion will focus on studies using CT as adjuvant unless otherwise indicated.

Allergy studies are performed in two phases. During the first phase, referred to as the sensitization phase, the animal is exposed to an allergen, with or without an adjuvant. After

the animal has rested for a period of time, the second phase, referred to as the challenge phase, is performed, during which the animal is exposed to the allergen again, and different allergic indicators are measured.

B Forms of Delivery. As with any other type of animal model, there are a number of ways to administer the allergen/adjuvant and induce an allergic response. When dealing with the induction of a food allergy, the three most commonly used methods are intraperitoneal injection (i.p.), intranasal delivery (i.n.), and intragastric gavage administration (i.g.).

Many of early attempts to produce an allergy model used i.p. delivery. This method consists of injecting the mouse in the lower abdomen with both the allergen and the adjuvant, delivering them directly to the i.p. cavity. One study by Adel-Patient and colleagues (2005) compared the methods of i.p. delivery to i.g. delivery. Mice were sensitized against peanut via i.p. injection, which contained both the allergen peanut and the adjuvant CT, while another group received an identical treatment dose via i.g. administration. Sera was collected at multiple time points and tested for concentrations of peanut specific IgE and IgG1, cytokines IL-4, IL-5, and interferon gamma (IFN-γ), and histamine. Mice receiving peanut via i.p. injection had much higher peanut specific IgE and IgG1 concentrations than i.g. administered mice. Since antibodies for either peanut specific IgE or IgG1 were not detected in naïve mice, however, both i.p. and i.g. methods were positive for allergy induction. In addition, mouse spleens were harvested, cultured in the presence of peanut extract, and the resulting supernatants were tested for the cytokines IL-4, IL-5, and IFN-γ. As previously noted, the cytokines IL-4 and IL-5 stimulate a Th2 response, while IFN-γ stimulates a Th1 response. All three cytokines were significantly higher in the spleen supernatants from i.p. and i.g. administered mice than the naive mice. Fecal pellets were collected and tested for

histamine concentrations, and the i.p. and i.g. administered mice were significantly higher than the naive mice on three of the four tested days.

Dearman and colleagues (2001) also compared i.p. injection to i.g. administration of the allergen peanut in BALB/c mice. Mice receiving i.p. injection showed an immune response, measured by the presence of allergen specific IgE and IgG. Intragastric gavage administration of the allergen resulted in lower concentrations of these antibodies; however, the response was still significantly higher than the naïve mice.

The i.p. injection method of allergen induction demonstrates an ability to elicit an allergic response in mice and has been used as a positive control during studies to develop allergy models with other delivery methods. This administration method, however, bypasses a large portion of the digestive system, and thus does not truly reflect the possible sensitization caused by an ingested protein (Dearman and Kimber 2001).

Exposure to an allergen via intranasal (i.n.) delivery will also cause an immune reaction at the mucosal surface. This route consists of applying a small volume of treatment solution (~10 μl) into a mouse's nasal passage (Takeda et al. 2004). Based on conclusions from Constant and colleagues, administering the antigen via the i.n. route induces Th2 cell activation. This is indicated by their studies in which mice genetically predisposed towards a Th1 immune response produced a Th2 response after receiving treatments via i.n. delivery. The allergic response resulted in an increase in IL-4 and IL-5 production, as well as a decrease in IFN-γ (Constant et al. 2000).

A paper by Takeda and colleagues (2004) reported i.n. sensitization with the *Blomia tropicalis* (Bt) antigen found on house dust mites. Only mice receiving both Bt and CT by i.n. delivery produced Bt specific IgE, as well as an increase in total IgE. Exposure to the

allergen Bt only or the adjuvant CT only by i.n. delivery is not sufficient to cause an allergic response.

Intranasal delivery has been compared to other delivery methods in several studies. Fischer and colleagues (2005) treated mice with CT and peanuts by either i.n. delivery or i.g. administration. Mice treated by i.g. administration had a much higher concentration of peanut specific IgE and a lower concentration of peanut specific IgG than i.n. mice. Interestingly, there was a lower IgG1 to IgG2a ratio associated with i.n. delivery mice (1.3 ± 0.1) than i.g. mice (1.8 ± 0.3). After an i.n. challenge with peanut, lung tissue was analyzed and mice sensitized by i.g. administration had IL-4 concentrations twice as high as i.n. delivery, while mice sensitized by i.n. delivery had higher concentrations of IL-17, a cytokine associated with a Th1 response. These responses may show a stimulation of both the Th2 and Th1 immune response during i.n. delivery.

Intranasal delivery is used in several allergy models which do not use a single delivery method. These models tend to include i.p. or intramuscular injection in conjunction to i.n delivery. In some cases, the mice are challenged by i.n. delivery but sensitized with other delivery methods (Bodinier et al. 2009; McCaskill et al. 1984). In other cases, mice are sensitized by i.n. in conjunction with another method, typically an injection, administered at the same time (Bublin et al. 2007).

While intranasal delivery of the allergen results in the production of IgE, the immune response is not entirely due to Th2 activation. Other studies demonstrate an increase in IgG2a, as well as cytokines IL-2, IL-12, and IFN-γ. These results indicate Th1 cellular stimulation in conjunction with a Th2 response to the allergen when administered via i.n. delivery (Bitsaktsis et al. 2009; Jones et al. 2001). Although i.n. sensitization can be used to

induce food derived allergies, i.n. mouse models are much more suitable for use in respiratory allergy studies, not food allergy studies.

The allergen delivery model which most closely resembles allergen ingestion, the route by which food allergies naturally develop, is intragastric gavage (i.g). This method consists of using a feeding needle to deliver a liquid combination of allergen and adjuvant or allergen alone to the mouse's stomach. Many studies have shown it is possible to elicit an IgG1 and an IgE response from rodents when the proper adjuvant and allergen are used. For the sensitization portion, many investigators use weekly treatments over the course of two to eight weeks (Fischer et al. 2005; Ganeshan et al. 2009; Li et al. 2000; Marinaro et al. 1995; Morafo et al. 2003), though other groups have induced allergic responses with other sensitization schedules, varying from daily gavages to once every three weeks (Adel-Patient et al. 2005; Atkinson et al. 1996; Knippels et al. 1998; Snider et al. 1994; van Wijk et al. 2004).

Knippels and colleagues (1998) did a thorough investigation of different sensitization schedules. Brown Norway rats were administered the allergen ovalbumin (OVA). No adjuvant was used in this study, which may have affected the type and intensity of the immune response. In this study, rats were exposed to OVA for six weeks either *ad libitum* via the drinking water, or were gavaged daily, twice a week, once a week, or once every two weeks. When OVA was administered via the drinking water, the rats produced a significant amount of OVA specific IgG, but no OVA specific IgE. Based on this data, the authors concluded that *ad libitum* oral administration induces a Th1 response. An antigen specific IgE response was observed in the rats gavaged with OVA on a daily basis, indicating a Th2 response (Knippels et al. 1998). The other gavage groups, however, did not exhibit an OVA

specific antibody response to either IgG or IgE. These results show when the allergen alone is used via i.g. administration during the sensitization phase of the model, the schedule is very important to the development of an allergen specific IgE response. A number of studies with other i.g. models using adjuvants have been able to produce allergic responses on sensitization schedules when the Knippel group did not. These successes indicate the presence of an adjuvant is a key component to animal allergy models (Fischer et al. 2005; Ganeshan et al. 2009; Li et al. 2000; Marinaro et al. 1995; Morafo et al. 2003). Later studies performed with mice given OVA in the drinking water without adjuvant found the induction of oral tolerance to OVA (De-Gennaro et al. 2009).

Intragastric gavage treatment is accompanied by the issue of oral tolerance. Oral tolerance is a systemic hyporesponsive or non-responsive immune reaction to an injected antigen due to prior oral exposure of the same antigen (De-Gennaro et al. 2009; Weiner 1994). The T-cells which reacted to the oral antigen are no longer able to proliferate, thus preventing the immune system from reacting to the specific antigen. Oral tolerance induction is typically tested by introducing the antigen repeatedly to the animal via its digestive tract, then immunizing the animal to the antigen via an injection, typically i.p. or subcutaneous injection (Bowman and Selgrade 2008; De-Gennaro et al. 2009; Rask et al. 2000). Antigen specific lymphocyte responsiveness or antigen specific antibody production from these animals is then compared to other animals which were injected but not gavaged with the antigen. If oral tolerance has occurred, the animals exposed to the antigen orally will have significantly lower antigen specific lymphocyte and antigen specific antibody responses. When utilizing oral allergy models, oral tolerance is a possibility. This problem can be limited by the use of mucosal adjuvants and choice of allergens.

C Types of Allergens. There are a number of foods which induce allergies, including eggs (ovalbumin), wheat, fish, shellfish, tree nuts, soy, cow's milk, corn, and peanuts (Adel-Patient et al. 2005; Kimber et al. 2003; Morafo et al. 2003). When the purpose of the study is not to examine a specific allergen, there are a number of factors involved in selecting the proper allergen for a study, including the amount of information in the literature regarding the allergen, likelihood of an animal being previously exposed to the allergen, delivery method of the allergen, and the allergenic qualities of the allergen.

Peanuts are responsible for roughly 25% of the food allergies in the United States and Europe, which gives researchers in both the medical field and the food industry cause to study peanut allergies (Murphy et al. 2007). Unlike other food allergies such as cow's milk and egg, peanut allergies are very persistent and only outgrown in 10 – 20 % of all cases, which causes the allergy to affect both adult and child populations (Adel-Patient et al. 2005; Clark et al. 2009; Li et al. 2000; Strid et al. 2004; van Wijk et al. 2004). In all age groups, peanut allergies account for the majority of the near fatal and fatal anaphylactic reactions (Strid et al. 2004). A number of mouse models to induce allergies to peanuts have been developed with varying timetables and animals, adding a considerable amount of information to scientific literature. Also, mice bred in Jackson laboratories, as well as mice maintained in the animal care facilities at Iowa State University, are fed peanut-free diets (Harlan 2009; Jackson 2009). These diets are not free of other food allergens, such as corn, cow's milk, wheat, and soy, which are common components of normal mouse chow diets (Harlan 2009; Jackson 2009). Since the mice have not been fed products containing peanut, this eliminates the possibility of the mouse's immune system being primed or tolerant to that particular allergen. Prior exposure to the allergen in the animal or its antecedents can affect the

animal's ability to produce an antibody response to subsequent treatments, resulting in oral tolerance (Dearman et al. 2001). One way to circumvent this is to work with animals that are naïve to the allergen and born from animals which are also naïve to the allergen. Food allergies can develop *in utero* or through exposure to the allergen in breast milk (Strid et al. 2004).

While administering whole peanut can elicit an allergic response, a number of proteins in peanuts have been identified as being particularly allergenic. These proteins have been designated Ara h 1 – 8, although there are a number of other peanut proteins against which people have produced IgE (Lee and Burks 2009; van Wijk et al. 2004). Of these proteins, Ara h 1 and Ara h 2 cause an allergic response in 90 - 95% of humans afflicted with peanut allergies (Li et al. 2000; van Wijk et al. 2004). Ara h 3 is also a major allergen and causes a response in 45% of humans with peanut allergies (van Wijk et al. 2004). Each of these three proteins contains at least ten different epitopes for IgE binding and Ara h 2 alone accounts for roughly 6% of the entire peanut structure (Germolec et al. 2003; van Wijk et al. 2004). These proteins were identified through electrophoresis and immunoblotting (Burks et al. 1992). Serum was collected from human patients with peanut allergies. The chromatographically separated IgE was then used to identify specific peanut protein bands via immunoblotting (Burks et al. 1992). Further study into these Ara h proteins could yield insight into the specific interactions between these proteins and the Th2 response. With this understanding, it will be possible to invent new and better treatments for peanut allergies, design more succinct methods to induce oral tolerance in patients with peanut allergies, prevent the development of peanut allergies, genetically engineer new peanuts that lack allergens, or avoid using genes which encode for peanut allergens in transgenic plants.

Peanut allergens have been compared to other allergens in mouse studies. Other allergens studied include cow's milk, ovalbumin (OVA), and potato acid phosphate (Adel-Patient et al. 2005; Dearman and Kimber 2001; Ganeshan et al. 2009). Adel-Patient and colleagues (2005) compared the allergic response to cow's milk to the allergic response to peanuts. Intragastric gavage treatments of peanuts or cow's milk and the adjuvant CT were administered to mice. Mice treated with peanuts and CT or cow's milk and CT demonstrated allergen-specific IgG1 and IgE responses after the fourth treatment. Mice administered peanut produced a more intense antibody response and had higher concentrations of IL-5 and histamine than mice administered cow's milk. Similar results have been shown in previous studies comparing peanut and cow's milk allergic responses. Morafo and colleagues (2003) used sensitization protocols which began at different ages for each allergy model. Mice exposed to cow's milk and CT began their treatments at three weeks of age, whereas mice exposed to peanut and CT began their treatments at five weeks of age. In both models mice were sensitized five times, once per week, but the mice in the peanut model were given two additional "boosting treatments" which also contained peanut and CT. The first boosting dose was administered two weeks after the fifth sensitization dose, and the second was administered four weeks after the fifth sensitization dose. The mice administered peanut had higher allergen specific IgE concentrations, higher plasma histamine concentrations, and higher serum IL-10 concentrations. The serum IL-4 concentrations, however, were more than 10 fold higher in mice administered cow's milk. The authors concluded peanut is a more potent allergen, since it is able to sensitize mice at an older age. Similar to mice, children are more susceptible to allergy development, especially if they are exposed to the allergen before they reach two years of age. This may be due to the higher gut permeability

and an inability of the immature immune system to develop oral tolerance (Germolec et al. 2003).

Allergy models using peanuts have also been compared to eggs/ovalbumin (OVA) in a series of studies. Ganeshan and colleagues (2009) administered peanut or OVA to mice once a week for a period of eight weeks. Mice administered peanut had higher concentrations of eosinophils than mice administered OVA (Ganeshan et al. 2009). Another study compared mice administered peanut, OVA, or potato protein extract (PPE) daily via intragastric gavage (Dearman et al. 2001). Mice receiving peanut began producing detectable concentrations of allergen specific IgG within seven days and attained their highest concentrations by day twenty one, while mice receiving OVA produced detectable concentrations by day fourteen and attained their highest concentrations by day twenty eight. It should be noted the highest concentration of allergen specific IgG achieved by mice administered peanuts was substantially higher with titers of approximately 1600, while mice administered OVA attained titers of approximately 250. Potato protein extract (PPE) showed an intermediate response compared to peanut allergens and cow's milk allergens; PPE produced detectable concentration of allergen specific IgG by day 14 and by day 42 the titer was approximately 1600. The presence of IgG, however, does not indicate the specific presence of a Th1 or Th2 response. Allergen specific IgE was also tested, and only mice exposed to peanut and PPE produced allergen specific IgE. Peanut exposed mice had positive IgE titers of 32, while PPE had a titer of one, i.e. the sample was run undiluted.

D Animal Species Used. Numerous strains of mice have been bred over the years, each exhibiting different susceptibilities to an allergic response. A number of stains have been tested for an allergy animal model. Mouse strains included BALB/c, AKR/J, C3H/HeSn,

and C3H/HeJ; rats have also been used, specifically the Brown Norway (BN) rat (Dearman et al. 2001; Li et al. 1999; Morafo et al. 2003). Several comparative studies have been done showing the differences between these animal models. The principle mouse strains tested were the BALB/c and the C3H.

One study compared BALB/c mice to BN rats using peanut and OVA as the allergens and showed that while BN rats produce a high IgE response when treated via i.p. injection, they do not produce a detectable amount of IgE treated via i.g. administration. BALB/c mice exhibit an IgE response to both delivery methods, although they have a more intense response to i.p. injection (Dearman et al. 2001). When BALB/c mice are compared to AKR/J and C3H/HeSn mice with peanuts as the allergen, only C3H/HeSn had an anaphylactic reaction to the peanut challenge. C3H/HeSn mice produced larger concentrations of allergen specific IgG1 (~4000 ng/ml), compared to the other mouse strains. All three mouse strains produced an allergen specific IgG2 response with the strain AKR/J having a significantly higher response (Li et al. 1999). BALB/c mice have been compared to C3H/HeJ mice as well. C3H/HeJ mice are very similar to C3H/HeSn mice; however, C3H/HeJ mice carry a mutation in the gene for toll-like receptor four (TLR-4). This mutation makes C3H/HeJ mice more resistant to immune responses towards endotoxin, primarily LPS. Due to the lack of immune response, C3H/HeJ mice are more susceptible to infection by Gram-negative bacteria (Jackson 2009). C3H/HeJ mice respond to a peanut challenge with anaphylactic shock if sensitized with peanut, whereas BALB/c mice do not. Also, C3H/HeJ mice produce higher concentrations of IL-4, and IL-10, peanut specific IgE, and histamine, while producing lower concentrations of IFN-γ when compared to BALB/c mice. This combination of cytokines and antibodies indicates a stronger Th2 response in the

C3H/HeJ mice than the BALB/c mice (Morafo et al. 2003). This data shows the C3H mouse strain is likely to be the most sensitive in studying a rodent allergy model.

In summary, there are a variety of factors to consider when designing an animal allergy model. These factors include the form of delivery for the allergen and adjuvant, the allergen, the animal strain, and the adjuvant. The allergen and adjuvant can be delivered by i.p. injection, i.n. delivery, or i.g. administration. Intraperitoneal injection produces the strongest allergic response but does not reflect factors involved in allergic sensitization caused by the ingestion of a protein. Intranasal delivery elicits an IgE response, but it does not activate an entirely Th2 cellular response or resemble the route of exposure for an ingested protein. Intragastric gavage produces an allergic response and closely resembles the path an ingested protein takes. From the numerous allergens that can be used, peanuts are a major health concern, are not commonly a component of rodent diets, prove to be a more potent allergen when compared to other allergens, and do not induce oral tolerance. C3H mice showed stronger allergic responses than BALB/c mice, and BALB/c mice produce better allergic responses than the other animals discussed. As previously mentioned, administration of allergen alone does not usually result in the induction of an allergic response. A potent mucosal adjuvant is needed. The mucosal adjuvant CT is commonly used in allergy models and primarily used in mucosal allergy models.

III. Cholera Toxin and *Escherichia coli* Heat Labile Toxin

A. Introduction. Cholera toxin is an enterotoxin produced by *Vibrio cholerae* that is used as a mucosal adjuvant. A mucosal adjuvant stimulates an immune response at a mucosal surface to both itself and any other foreign proteins present. Cholera toxin is a well studied mucosal adjuvant used in numerous animal models to elicit allergies, making it useful

as a positive control. Cholera toxin is approximately 80% homologous to *Escherichia coli* heat labile toxin (LT), and CT and LT are two of the most potent known mucosal adjuvants (Pizza et al. 2001). LT has been used in a number of experimental mucosal vaccine studies over the years; however, not all aspects of its immunogenicity have been studied. Due to the structural similarity between the two toxins, it would be advantageous to have a basic understanding of both toxins when using either of them as immunogens.

B) Cholera Toxin. Cholera toxin has been of interest to vaccine research due to its strong immunological properties as a mucosal adjuvant. While there are over one hundred and forty serogroups of *V. cholerae*, only a handful produce CT, and of those two serogroups, serogroup 01 and serogroup 0139, are responsible for the bulk of the medical cases of cholera (Sanchez and Holmgren 2008). CT is an A-B subunit toxin, comprised of an A subunit surrounded by the pentameric B subunit (Holmgren et al. 2005). The B subunit binds specifically to a ganglioside membrane receptor referred to as GM1 (Guidry et al. 1997). This receptor is present on a number of different cells, including cells which line the intestines (Guidry et al. 1997). The B subunit uses this receptor to deliver the entire toxin through the cellular membrane and into the cytosol. There the A subunit dissociates from the pentameric B subunit and interferes with the formation of adenosine triphosphate (ATP) (Spangler 1992). The A subunit is an ADP-ribosyltransferase and catalyzes the adenosine diphosphate ribosylation process upon entering the cytosol. Nicotinamide adenine dinucleotide (NAD+) binds to the active site on the A subunit. The NAD+ is unable to remove the phosphate group from the guanosine triphosphate (GTP), which leads to adenylate cyclase (AC) remaining active. The active AC increases the production of cAMP, which results in changes in the ion transporters. This greatly reduces or stops the intake of

sodium and increases the excretion of chloride. By decreasing the salt concentrations in the epithelial cells and increasing those concentrations in the intestine, fluid in the intestine can no longer be taken up by the epithelial cells, and fluid present in the cell leaves to enter the intestine (Sanchez and Holmgren 2008; Spangler 1992). This increased fluid results in a loss of 500 to 1000 mls per hour in humans, causing diarrhea and dehydration (Sanchez and Holmgren 2008). Cholera toxin has an LD 50 in mice of 250 µg when administered via intravenous delivery but an LD 50 in mice at 33.3 ± 7.3 µg when administered via i.p. delivery (Dragunsky et al. 1992; Gill 1982).

Cholera toxin's potency as a mucosal adjuvant is due to a variety of attributes. It causes an increased permeability in epithelial cell layers, particularly the mucosal layer in the intestine, which leads to an increased uptake of any co-administered antigens (Holmgren et al. 2005). Cholera toxin promotes the production of antibodies with the isotype IgG1, IgA, and IgE by causing the immune system to increase the production of IL-4, IL-5, IL-6, and IL-10, decrease the production of IL-12, TNF-α, and INFγ, and inhibit the expression of the β1 and β2 chains of the IL-12 receptor on human T cells (Braun et al. 1999; Lavelle et al. 2003). While the antibody IgG1 provides general protection for the body, and IgE is only observed in allergic reactions, IgA is secreted at mucosal surfaces such as the respiratory tract and the intestinal tract, allowing the body to protect itself where many pathogens first enter the body. Cholera toxin also increases the proliferation of antigen presenting cells (APCs) and enhances the maturation of dendritic cells and B-cells, indicated by the increased expression of the receptors MHC II, CD40, CD80, and CD86 (George-Chandy et al. 2001). Cholera toxin induced maturation is due to GM1 receptors present on APCs, which give CT access to the cytosol of the cell. The enhanced presentation by APCs of specific antigens will

increase the amount of T-cell exposure the antigen receives and stimulate a stronger response to the vaccine (George-Chandy et al. 2001). Cholera toxin also has a number of physical properties which make it very stable in the face of degrading factors in the stomach and intestines, including proteases and bile salts, making CT useful as a fed adjuvant (Sanchez and Holmgren 2008). These properties include a number of polar bonds and hydrophobic interactions both within the individual subunits and the toxin as a whole. Degradation of the toxin can only occur at pHs below 3, or in boiling water (Sanchez and Holmgren 2008). The stomach pH can range from 3 to1, which may allow it to degrade CT, but when suspended in liquid and administered to an empty stomach, CT passes quickly into the small intestine.

C) Escherichia coli *Heat Labile Toxin used in Vaccines.* *E. coli* heat labile toxin (LT) is an enterotoxin produced by Enterotoxigenic *Escherichia coli* (ETEC). Enterotoxigenic *E. coli* can also produce a number of heat stable toxins, including STa, STb, and EAST1, although for the purposes of this literature review, only LT will be discussed (Dubreuil 2008). LT is an A-B toxin composed a single A subunit surrounded by the pentameric B subunit. The toxin uses the same molecular interactions as CT to gain entry into the cell. The ability of the B subunit to deliver both itself and whatever is attached to it into cells makes it a very useful tool in the development of oral vaccines. An oral vaccine is taken by mouth and travels the digestive tract until it reaches the intestines, where it interacts with the mucosal surface. Oral vaccines require a mucosal immunogen, which is any immunogen that elicits an immune response when exposed to a mucosal surface (Pizza et al. 2001). A number of researchers have experimented with vaccines which use LT or LT-B as an immunogen in conjunction with another antigen. Additionally LT-B has been used alone in experimental vaccines against ETEC-caused diarrhea. Since LT and LT-B are strong

mucosal immunogens, they are typically administered by i.n. delivery, or i.g. administration (either orally or fed). Several different LT mutants have been produced in *E. coli* and used in these models.

LT mutants are necessary when working with the whole toxin and consist of the unaltered pentameric B subunit and a modified A subunit to reduce toxicity. Exposure to LT that still contains the active A subunit under normal conditions results in diarrhea which can cause severe dehydration and electrolyte loss. Just as with cholera, fluid in the intestine causes diarrhea, and the inability of the intestine to absorb fluid causes dehydration. Diarrhea and dehydration are classic symptoms of an ETEC infection. However, if LT has been genetically mutated to produce a subunit A which is either partially or completely inactivated, LT can be safely used to stimulate the immune system.

Single amino acid changes can drastically change the toxicity of LT. For example, LT-K63 is a mutant which has no ADP-ribosylating activity with a single amino acid change at site 63 from serine to lysine. LT-R72 has greatly reduced ADP-ribosylating activity, less than one percent of the activity found in wild type LT with a single amino acid change at site 72 from alanine to arginine (Barackman et al. 1999; Giuliani et al. 1998; Pizza et al. 2001). LT-R192G has a single amino acid changed from Arg to Gly at site 192, which reduces the toxicity for that mutant (Maier et al. 2005). LT-H44A has a single animo acid changed from His to Ala at site 44, which reduced ADP-ribosylating activity to less than 0.0021 percent (Hagiwar et al. 2001). More than fifty different LT mutants have been recombinantly or genetically produced (Pizza et al. 2001). Use of the four mutants listed above as adjuvants will be reviewed below and summarized in Table 1.

A number of experimental vaccines trials have used LT or a mutant of LT as an adjuvant. In intranasal (i.n.) vaccination systems, which used the mutant forms LT-K63 and LT-R72, the experimental vaccine protecting against *Bordetella pertussis* conferred the same amount of protection against colonization as the intramuscular vaccine currently in use, which uses alum as its adjuvant. It also stimulates the production of a higher concentration of IgA than the alum vaccine (Ryan et al. 1999). Another experimental i.n. vaccine which used the influenza virus hemagglutinin HA as the antigen stimulated high titers of the viral neutralizing antibodies IgG and IgA (Barackman et al. 1999). In the case of both vaccines, however, IgG1 and IgG2a were produced in equal amounts, indicating the immune response may be due to both Th2 and Th1 responses (Barackman et al. 1999; Ryan et al. 1999). In transcutaneous vaccination systems, the forms of LT used were wild type LT or the mutant forms LT-K63 or LT-R72, and an immune response was shown by the formation of antibodies against both LT and the co-administered antigen, as well as an increase in antigen specific lymphocyte proliferation. The transcutaneous systems showed a Th2 response, indicated by high concentrations of antigen specific IgG1 compared to IgG2 (Beignon et al. 2001; Tierney et al. 2003). Both the intranasal and transcutaneous systems showed a Th2 response as indicated by high concentrations of IgA and an increased survival rate when injected i.p. with double the lethal dose of LT. Both systems also induce an immune response against the co-administered antigens (Barackman et al. 1999; Beignon et al. 2001; Ryan et al. 1999; Tierney et al. 2003). Some of the problems surrounding the use of bacterial LT mutants in vaccines include mutations that restore the LT to its original, wild type strength. Also, there is concern that the mutants, which are considered safe, but have partially active A subunits, could cause illness in the very old, infirm, or children.

As LT-B does not have a toxic effect on the cells, other experimental vaccines have been designed and tested using LT-B alone. Vaccines which use LT-B are discussed below, and summarized in Table 2. Since ETEC-caused diarrhea is a serious health problem in many areas of the world, experimental vaccines use the immunogenic effects of LT-B to protect subjects against the diarrhea, thus combining the antigen and adjuvant into a single element (Mason et al. 1998; Rosales-Mendoza et al. 2008; Tacket et al. 1998). In many of these experimental vaccines, the genes encoding for the B subunit of LT have been placed into a number of different transgenic plants, which are administered to the animals such as mice and chickens (Beyer et al. 2007; Chikwamba et al. 2002; Mason et al. 1998; Tacket et al. 1998). In these fed LT-B only experimental vaccines, partial if not complete protection against whole toxin LT was achieved in all of the studies, and one study showed the animals had developed immunity to CT as well (Chikwamba et al. 2002). Another study showed fed LT-B would elicit an immune response in animals receiving treatments as low as 0.02 µg of LT-B per dose would develop an immune response to LT-B. All treatments showed increases in IgA and IgG.

LT-B only experimental vaccines have been administered by i.g. administration as well (Table 2). One vaccine produced an LT-B specific IgG and IgA response and gave the animals partial protection during challenges with whole toxin LT (Rosales-Mendoza et al. 2008). The other vaccine also produced LT-B specific IgG and IgA, although the mice were not challenged with LT (Haq et al. 1995).

Two experimental oral LT-B vaccines have been genetically engineered in plants with the antigens fused to the LT-B. These vaccines have produced mixed results (Table 2). The experimental vaccine against *Mycobacterium tuberculosis* did not protect animals during

an *M. tuberculosis* challenge, although the vaccine did produced high concentrations of IL-10 and increased the proliferation of antigen specific T helper cells in the mesenteric lymph nodes, indicating a Th2 response (Rigano et al. 2006). The experimental vaccine against *Chlamypdophila psittaci*, however, protected 8 out of 15 animals during the challenge and elicited an antigen specific IgG and IgA antibody response (Zhang et al. 2009).

LT-B was also fused to viral protein 2 (VP2) from infectious bursal disease and administered i.g (Table 2). It conferred full protection during the challenge with infectious bursal disease. Interestingly, while the animals showed high VP2 specific antibody titers, the animals did not exhibit a specific antibody response to LT-B (Fingerut et al. 2005).

D) Similarities and Differences Between Cholera Toxin and E. coli *Heat Labile Toxin.*

While there are a number of similarities between CT and LT, it is important to keep the differences between the toxins in mind when designing vaccines and animal allergy models. Among the similarities between the two toxins, CT has an approximately 80% homology to LT on the primary structural level, as well at a nearly identical tertiary structure (Pizza et al. 2001). Both are A-B enterotoxins with one A subunit surrounded by the pentameric B subunit. The B subunit will use the GM1 receptor to enter cells and deliver attached proteins (Mason et al. 1998; Spangler 1992). Both have been used in a number of experimental vaccines as an adjuvant that will induce a Th2 response in during trials. Both whole toxins CT and LT can be used as adjuvants, stimulating an immune reaction to novel antigens which are present in the same physical location as the toxin, whereas CT-B and LT-B need to have a physical link between it and the antigen in order to stimulate a reaction to the antigen (Giuliani et al. 1998; Li et al. 2009; Maier et al. 2005; Negri et al. 2009).

Despite these similarities, there are several major differences between the two toxins. Cholera Toxin subunit B will bind primarily with GM1, but LT-B will bind to other glycosphingolipids and glycoprotein receptors as well (Pizza et al. 2001). Cholera toxin is a common adjuvant used in allergy studies. It is very harmful and responsible for the diarrhea and dehydration associated with the disease cholera. Exposure to LT will also cause diarrhea and dehydration, although LT induced illness is not as severe as CT induced diarrhea (Beignon et al. 2001). A number of forms of LT used in experimental vaccines include LT with A subunit mutated to reduce toxicity, while other LT forms have the A subunit removed altogether, leaving the B subunit (LT-B). These experimental vaccines have shown a high degree of success in trials. Most importantly, although CT acts as a mucosal adjuvant and is capable of eliciting an allergic reaction, there is no information available in the literature which would indicate whether that type of sensitization will take place with LT or LT-B.

In conclusion, food allergies are an important health concern. When experimenting with a mucosal immunogen like LT-B, which induces a Th2 response and is similar to CT, the possibility of stimulating an allergic response needs to be examined closely. Several indicators of allergic or possibly allergic reactions should be measured. These indicators include concentrations of allergen specific IgG1, allergen specific and total IgE, and histamine. Also, a number of aspects for animal allergy models must be considered. These aspects include the form of allergen and adjuvant administration such as i.g., type of allergen such as peanuts, species/strain of animal such as C3H/HeJ mice, and the type of adjuvant. One adjuvant in particular, CT, has been used consistently used for oral animal allergy models. This is because it is an adjuvant capable of inducing an immune response at the gut mucosal level. Cholera toxin is very similar to LT, with a few significant differences. One

difference is that CT is a whole toxin which is often used for allergy studies, while there is no information regarding whether LT or LT-B will induce an allergic response when fed. In order to research this aspect of LT-B, a fed allergy model will need to be designed. The designing and optimization of this model will be discussed in the following chapter.

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Table 1: Summary of some of the previous experiments with LT and LT mutants, listed first by antigen present, if any, then by delivery method and year.

Antigen	**Adjuvant**	**Delivery Method**	**Species/ Strain**	**Outcome**	**Paper**
Heat-killed *B. pertussis*	Bacterial mutant LT-K63 and LT-R72	Intranasal	Murine - BALB/c	Conferred protection against *B. pertussis*	(Ryan et al. 1999)
Influenza virus hemagglutinin	Bacterial mutant, LT-R72	Intranasal	Murine - BALB/c	Produced high IgG and IgA conc.	(Barackman et al. 1999)
Influenza virus hemagglutinin	Bacterial LT	trans-cutaneous	Murine - BALB/c	Produced high IgG and IgA conc.	(Beignon et al. 2001)
Tetanus Toxid	Bacterial mutant, LT-K63 and LT-R72	trans-cutaneous	Murine - BALB/c	Conferred protection against Tetanus Toxid	(Tierney et al. 2003)

Table 2: Summary of some of the previous experiments with LT-B, listed first by antigen, then by delivery method and year.

Antigen	**Source of antigen**	**Delivery Method**	**Species/ Strain**	**Outcome**	**Paper**
Cp. psittaci antigen (MOMP) fused with LT-B	Transgenic rice	Fed	Murine - BALB/c	Conferred protection against *Cp. psittaci*	(Zhang et al. 2009)
LT-B	Transgenic maize	Fed	Murine - BALB/c	Conferred protection against LT	(Beyer et al. 2007)
LT-B	Transgenic maize	Fed	Murine - BALB/c	Conferred protection against LT and CT	(Chikwamba et al. 2002)
LT-B	Transgenic potato	Fed	Humans	Conferred protection against LT	(Tacket et al. 1998)
LT-B	Transgenic potato	Fed	Murine - BALB/c	Conferred protection against LT	(Mason et al. 1998)
LT-B	Transgenic carrot	Intragastric gavage	Murine - BALB/c	Conferred protection against LT	(Rosales-Mendoza et al. 2008)
LT-B	Transgenic tobacco	Intragastric gavage	Murine - BALB/c	Produced high IgG and IgA conc.	(Haq et al. 1995)
M. tuberculosis antigen ESAT-6 fused with LT-B	Transgenic *Arabidopsis thaliana* plants	Fed	Murine - C57BL/6	No protection against *M. tuberculosis* high conc. of IL-10 and T-cell proliferation	(Rigano et al. 2006)
Viral protein 2 of infectious bursal disease fused with LT-B	Transgenic yeast *Pichia pastoris*	Intra muscular injection, intragastric gavage	Avian - chicken	Conferred protection against infectious bursal disease	(Fingerut et al. 2005)

Chapter 2: Cholera Toxin or *Escherichia Coli* Heat Labile Toxin Subunit B and Mouse Peanut Allergy Model

I. Abstract

As transgenic plants become more common, humans are experiencing increased contact to proteins in a novel context, proteins which may have the potential to be allergenic or allergy inducing. The gene for *Escherichia coli* heat-labile toxin subunit B (LT-B) has been introduced into transgenic maize for use as an oral vaccine. There is concern that LT-B will sensitize the immune system to produce an allergic response, causing people to develop an allergy to co-fed proteins. To examine the possibility of a transgenic plant inducing an allergic response, an animal model in which the animal acquires an allergy by eating the immunogen and the allergen needs to be developed. The need to design an animal allergy model such as this resulted in three separate research objectives.

Experiment one reproduced an intragastric gavage animal allergy model previously described in the literature, which used cholera toxin (CT) as an adjuvant and co-administered peanuts as an allergen. In addition, assays for measuring the allergic response were established. In experiment two, the model was modified so the CT and peanut treatment was administered via maize food pellet in the same manner as the LT-B maize. The LT-B maize, mixed with peanut or alone, was administered via food pellet to measure the allergy eliciting qualities. During experiment three, multiple concentrations of CT and peanut extract were tested to find the optimum concentrations for inducing allergies. Administration methods of intragastric gavage or fed via food pellet were compared.

The results showed both concentrations of LT-B (20 µg and 100 µg) and peanuts resulted in IgE antibody concentrations which are not significantly different between experimental mice and naïve mice. LT-B did not elicit an immune response (IgE or IgG1) to peanut. Experiment three showed the method used to administer CT and peanut was vital to the development of an allergic response, while the dose concentrations of CT and peanut were not. CT and peanuts had to be administered via intragastric gavage to induce an allergic response, though both portions do not need to be gavaged at the same time. A non-allergic immune response, as indicated by increased IgG1, can be elicited by administering the antigen peanut alone by intragastric gavage.

II. Introduction

Food allergies are an increasing concern in health care. In 2007, the National Center for Health Statistics reported four out of every one hundred children have been diagnosed with some form of food allergy (Branum 2008). Whenever a novel transgenic-plant derived product such as a food or vaccine is produced, allergy induction in atopic individuals should be considered.

There are two ways the transgenic product could induce allergies in atopic individuals. One concern is the transgenic product, which before genetic manipulation was non-allergenic, could now contain the DNA for an allergen and produce it. Thirteen years ago, a company produced a transgenic soy plant which contained genes from the Brazil nut, a tree nut known for its allergenic qualities (Nordlee et al. 1996). The Brazil nut gene was inserted to increase methionine concentrations in soy. Methionine is an amino acid that is difficult to include in vegetarian based diets (Nordlee et al. 1996). A protein, 2S albumin, was produced in the transgenic soy. The 2S albumin protein proved to be one of the major

allergens in the Brazil nut (Nordlee et al. 1996). People with Brazil nut allergies had IgE antibodies which reacted strongly to the transgenic soybeans as well, showing that non-allergenic plants which contain genes from an allergenic plant can cause an allergic response.

A second concern is the transgenic plant contains an adjuvant which, when co-fed with other proteins, could induce allergies. When CT is administered with peanuts, it will induce an allergic response in subjects which do not have a previous history of peanut allergies (Adel-Patient et al. 2005; Fischer et al. 2005; Li et al. 2000; Morafo et al. 2003; van Wijk et al. 2004). This response is of special interest when developing a transgenic plant based vaccine which will prime the immune system.

Heat labile toxin subunit B produced in transgenic maize can elicit a robust immune response with increased concentrations of both serum IgG1 and mucosal IgA antibodies to LT-B (Beyer et al. 2007; Chikwamba et al. 2002). These antibody responses indicate a Th2 immune response. The Th2 response is also associated with B-cell class switching to IgE (Gizzarelli et al. 2006). In parallel with the vaccine response studies, it would be beneficial to validate the safety of transgenic maize containing LT-B in regard to allergy induction. To accomplish this, the research should demonstrate the Th2 response invoked by LT-B will not induce any mediators of an allergic response.

As a positive control for allergy induction it was necessary to validate an induced allergy model in mice using CT with peanut extract administered by intragastric gavage (i.g.) (Adel-Patient et al. 2005; Fischer et al. 2005; Li et al. 2000; Morafo et al. 2003; van Wijk et al. 2004). Once the model had been reproduced, the primary goal was to extend it to a fed model, wherein mice would eat maize food pellets containing CT and peanut extract, mimicking the route of LT-B exposure. Mice receiving CT with peanut extract via food

pellets were expected to demonstrate significantly higher concentrations of total IgE, peanut specific IgE, peanut specific IgG1, CT specific IgG1, histamine, and signs of anaphylaxis than naïve mice, similar to mice gavaged with CT and peanut in experiment one. Mice receiving LT-B with peanut extract via food pellets were not expected to be significantly different from the naïve mice by any measurement. Later, the allergy model was optimized by comparing different doses of CT and peanut extract and different delivery methods. Mice receiving higher doses of CT and peanut extract were expected to have increased allergy responses. Also, while i.g. administered mice would have allergic responses that were greater than pellet fed mice, both would have significantly higher immune responses than naïve mice.

III. Materials and Methods

Animals

Four to five week old CH3/HeJ female mice were obtained from Jackson Laboratories (Bar Harbor ME) and fed peanut-free diets and water *ad libitum*. Mice placed on a twelve hour reverse light/dark cycle with the light phase beginning at 2200 hours and acclimated to the Iowa State University animal facility two weeks before beginning the experiment. Animals were treated in a humane manner and all procedures were approved by the ISU Institutional Animal Care and Use Committee.

Peanut Extracts

Peanut extract was prepared from a procedure adapted from Adel-Patient and colleagues (2005). Approximately one hundred grams of raw peanuts (Wheatsfield Cooperative, Ames IA) were finely ground, then diluted one to five by peanut weight to volume into nanopure water (pH 9) and incubated 18 hours at 4°C while stirring. Peanut

extract was separated by unit gravity overnight at 4°C. Supernatant was collected and frozen at -20°C. Protein concentration of the final solution was measured using the Micro BCA Protein Assay Kit (Pierce, cat #23235 Rockford, IL). Nanopure water was used to dilute the peanut extract to the final concentration in the first and second experiment. Bicarbonate water (1.5%) was used in the third experiment.

Maize Pellet Preparation

Maize pellet preparation for feeding was performed as previously described (Chikwamba et al. 2002). Each pellet type was prepared separately to prevent toxin or peanut cross-contamination. Two sets of pellets were used. One set was used for experiment two and had a final weight of 3.35 grams. Pellets containing LT-B were formed using appropriate amounts of ground transgenic maize and ground non-transgenic maize. Pellets not containing LT-B were formed using ground non-transgenic maize only. Solutions of peanut extract and/or CT were added to maize, and nanopure water was added as needed. A second set of pellets were used for experiment three and had a final weight of 0.719 gm. All pellets were formed using ground non-transgenic maize. Peanut extract and/or CT were added in solution, and nanopure water was added as needed.

Toxins

Cholera toxin was obtained from Sigma-Aldrich, St. Louis MO. For experiments one and two, CT was reconstituted to 1 mg/ml with nanopure water. For experiment three, CT was reconstituted to 1 mg/ml with ten-fold concentrated phosphate buffered saline (PBS) to increase CT stability (McClosky; personal communication). Ground transgenic maize containing LT-B was grown and provided by Iowa State University Plant Transformation

Facility, ground, and stored at -20°C. Purified bacterial LT-B was a generous gift from J. Clements of Tulane University Medical Center, New Orleans, LA.

Mouse Treatments

Experiment One – Allergic responses induced by cholera toxin and peanuts administered by intragastric gavage:

Mice were randomly assigned to four groups (n = 3 - 5). Treatment groups are summarized in Table 1. Mice were treated via intragastric gavage (i.g.) with 10 μg of cholera toxin and 5 mg of peanut extract (CT and PE), 5 mg of peanut (PE), or 10 μg of cholera toxin (CT) in nanowater, or an equal volume (200 μl) of nanopure water (Naïve) (Li et al. 2000). Twelve hours before their treatments, mice were fasted but allowed free access to water during their light cycle. Mice received gavage treatments once per week for four weeks and were challenged at 2.5 and 4.5 weeks after the last sensitization (Adel-Patient et al. 2005; Morafo et al. 2003). The timeline for treatments is summarized in Figure 1. For challenge days mice from all treatment groups were gavaged with 5 mg of peanut extract (Adel-Patient et al. 2005; Li et al. 2000; Morafo et al. 2003). Visual scoring was performed during both challenges. Mice were monitored for signs of anaphylaxis on the visual scoring system adapted from Li and colleagues (2000). Symptoms and visual scoring are listed in Table 2. Mice were monitored for two hours after the first challenge for visually observable allergic reactions. After the second challenge, mice were monitored for one hour for visually observable allergic reactions, at which point they were euthanized by CO_2 inhalation.

During experiment one, blood was collected from the saphenous vein of each mouse six times. See Figure 1. Blood was collected with heparin coated capillary tubes (#22-362-566 Fisher Scientific, Pittsburgh, PA). Plasma was separated and stored at -20°C. Upon

euthanasia blood was collected with ethylenediaminetetraacetic acid (ETDA; 7.5%) coated syringes via cardiac punctures. Plasma was separated by centrifugation and stored at -80°C.

Experiment Two - Allergic responses induced by cholera toxin and peanuts or LT-B and peanuts administered by pellet:

Mice were randomly assigned into eight treatment groups (n = 4). Treatment groups are summarized in Table 3. Experimental mice were fed pellets containing 100 μg or 20 μg of LT-B and 5 mg of peanut extract (LTB 100 PE or LTB 20 PE). Control mice were fed 100 μg or 20 μg of LT-B only (LTB 100 only or LTB 20 only), 10 μg of cholera toxin with 5 mg of peanut extract (CT and PE), peanut extract only (PE only), cholera toxin only (CT only), or non-transgenic maize only (Naïve). Mice were fasted but allowed free access to water twelve hours before their treatments, during their light cycle. During the treatments mice were placed in individual cages with a 3.35 g maize treatment pellet and allowed 24 hours to consume it. Mice received pellets once per week for four weeks and were challenged at 2.5 and 4.5 weeks after the last sensitization. The timeline is summarized in Figure 2. For challenge days mice from all treatment groups were gavaged with 5 mg of peanut extract. Visual scoring was performed during both challenges as described in experiment one except mice were monitored for one hour after the first challenge.

Blood was collected as previously described for experiment one. See Figure 2.

Experiment Three – Allergic responses induced by cholera toxin and peanuts administered by gavage, food pellet or a mixture of the two:

Mice were randomly assigned to thirteen treatment groups, summarized in Table 4. Experimental groups received either 20 or 10 μg cholera toxin by gavage and 10 or 5 mg peanut extract by gavage [20 μg CT(G)/ 10 mg PE(G) or 10 μg CT(G)/ 5 mg PE(G)]; or 20

μg cholera toxin by gavage and 20 mg peanut extract fed [20 μg CT(G)/ 20 mg PE(F)]; or 20 or 10 μg cholera toxin fed and 20 or 10 mg peanut extract fed [20 μg CT(F)/ 20 mg PE(F) or 10 μg CT(F)/ 10 mg PE(F)]. Control groups received 20 or 10 μg cholera toxin only by gavage [20 μg CT(G) Only or 10 μg CT(G) Only]; 20 μg cholera toxin only fed [20 μg CT(F) Only]; 10 mg peanut extract only by gavage [10 mg PE(F) Only]; 20 or 10 mg peanut extract fed only fed [20 mg PE(F) Only or 10 mg PE(F) Only]; or pellets containing nanopure water [Naïve].

Due to the increased amount of CT present in the treatments, some mice receiving 20 μg of CT by i.g. suffered from dehydration. Cholera toxin treated mice were monitored for 48 hours after treatment and given oral rehydration solution by i.g. or saline by intraperitoneal injection (i.p.) as needed. After the first treatment, groups receiving 20 μg CT by i.g. were administered 15 μg CT on subsequent treatment days to reduce morbidity and mortality. All fed groups remained the same. Treatment groups will be referred to as 20 μg CT for the remainder of this paper, as that was the highest dosage to which the mice were exposed.

Mice were fasted twelve hours before their treatments during their light cycle but allowed free access to water. During treatments, mice were placed into individual cages. Mice in a gavage group received 250 μl treatments and then were fasted for half an hour before being given their maize pellet. Mice not receiving a gavage treatment were immediately given their maize food pellet treatments. All maize pellets were 0.719 g and the mice were allowed 36 hours to consume it. They were monitored every 4-8 hours, and once a mouse consumed the pellet, it was returned to its cage with food *ad libitum*. Maize pellets were dyed green and green fecal pellets were collected during the 36 hour time period and

tested for CT. Mice received treatments once per week for four weeks and were challenged at 2 and 4.5 weeks after the last sensitization. The timeline is summarized in Figure 3. For challenge days, mice from all treatment groups were gavaged with 10 mg of peanut extract and after thirty minutes received a second 10 mg dose of peanut extract. Mice were monitored for an additional thirty minutes for anaphylactic reactions, then euthanized and blood was collected via cardiac punctures (Li et al. 2000). Visual scoring was performed during both challenges. See Table 2. Each mouse was monitored for thirty seconds every ten minutes, beginning five minutes after the first gavage, until they were euthanized.

Blood was collected as previously described for experiment one. See Figure 3.

Fecal samples were collected and stored at -20°C, then placed at -80°C for 24 hours prior to lyophilization. Fecal pellets were lyophilized for a 48 hour period in a Virtis Model 3.5L DBTZL Benchtop FreezeDryer lyophilizer (Gardiner, NY).

Assays

Total IgE

Total serum IgE was measured by ELISA. Briefly, rat anti-mouse IgE antibody (#553413, BD Biosciences, Franklin Lakes, NJ) diluted to 1.5 μg/ml in coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; and 3 mM NaN_2; pH 9.6) was used to coat all wells in high-binding ELISA microtiter plates (#3590 Costar, Pittsburgh, PA) and incubated overnight at 4°C. Plates were blocked with 5% reconstituted milk (Nestle USA Inc, Solen OH) for one hour at room temperature. Mouse IgE Standard curve (500 ng/ml – 3.91 ng/ml) was generated by adding mouse IgE (#557079, BD Biosciences) to appropriate wells while diluted sample serum was added to other wells in duplicate and incubated overnight at 4°C. Biotinylated rat anti-mouse IgE (#553419 BD Biosciences), diluted to 2.5 μg/ml in 1%

reconstituted milk, was added for two hours at room temperature. Streptavidin-HRP (#554006, BD Biosciences), diluted 1:1000 in reconstituted milk, was added and incubated for thirty minutes at room temperature. The substrate [0.55 mM ABTS (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis) in 0.1 M citric acid buffer (pH 4.25)], activated with hydrogen peroxide, was added to the wells and incubated for thirty minutes at room temperature before reading at 405 nm using a EL 340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Data was collected using KC Junior software (version 1.17, Bio-Tek Instruments, Inc.) and calculated using a four-parameter fit standard curve. Samples below the standard curve which did not read out were reported as one-half the value of the lowest detectable standard.

Peanut Specific Serum IgE

Peanut Specific serum IgE was measured by ELISA. Briefly, peanut extract diluted to 10 μg/ml in coating buffer (pH 9.6) was used to coat sample wells in high-binding ELISA microtiter plates (Costar) while a mouse IgE (BD Biosciences) standard curve (250 ng/ml – 0.98 ng/ml) was generated in coating buffer and used to coat the standard wells. Plates were incubated overnight at 4°C and blocked as described for total IgE. Diluted sample serum was added to sample wells in duplicate and 1% reconstituted milk was added to standard wells. Plates were incubated overnight at 4°C. Bound mouse IgE was detected as described for total IgE.

Peanut Specific Serum IgG1

Peanut Specific serum IgG1 was measured by ELISA. Briefly, peanut extract diluted to 10 μg/ml in coating buffer (pH 9.6) was used to coat sample wells in high-binding ELISA microtiter plates (Costar) while a mouse IgG1 (#M9269, Sigma) standard curve (50 ng/ml –

0.78 ng/ml) was generated by coating the standard wells. Plates were incubated and blocked as described for total IgE. Diluted sample serum was added to sample wells in duplicate and 1% reconstituted milk was added to standard wells. Plates were incubated overnight at 4°C. Biotinylated rat anti-mouse IgG1 (#553441, BD Biosciences), diluted 1:2000 in 1% reconstituted milk, was added and incubated for two hours at room temperature. Visualization was carried out as previously described for total IgE using Streptavidin-HRP (BD Bioscience) and ABTS (Sigma).

Cholera Toxin Specific Serum IgG1

Cholera toxin specific serum IgG1 was measured by ELISA. Briefly, ganglioside GM1 (Alexis Biochemicals, San Diego, CA), diluted to 10 μg/ml in coating buffer (pH 9.6), was used to coat sample wells in high-binding ELISA microtiter plates (Costar) while a mouse IgG1 (Sigma) standard curve (50 ng/ml – 0.78 ng/ml) was generated by coating the standard wells. Plates were incubated and blocked as described for total IgE. Cholera toxin (Sigma), diluted to 20 ng/ml in 1% reconstituted milk, was added to the sample wells and 1% reconstituted milk was added to the standard wells, then incubated for two hours at room temperature. Diluted sample serum was added to sample wells in duplicate and 1% reconstituted milk was added to standard wells and incubated overnight at 4°C. Bound IgG1 was detected as previously described for peanut specific IgG1.

Heat Labile Toxin subunit B Specific Serum IgG1

Heat labile toxin subunit B specific serum IgG1 was measured by ELISA. Briefly, ganglioside GM1 (Alexis Biochemicals, San Diego, CA), diluted to 10 μg/ml in coating buffer (pH 9.6), was used to coat sample wells in high-binding ELISA microtiter plates (Costar) while a mouse IgG1 (Sigma) standard curve (50 ng/ml – 0.78 ng/ml) was generated

by coating the standard wells. Plates were incubated and blocked as described for total IgE. Heat labile toxin subunit B (Clements), diluted to 20 ng/ml in 1% reconstituted milk, was added to the sample wells and 1% reconstituted milk was added to the standard wells, then incubated for two hours at room temperature. Diluted sample serum was added to sample wells in duplicate and 1% reconstituted milk was added to standard wells and incubated overnight at 4°C. Bound IgG1 was detected as previously described for peanut specific IgG1.

Histamine

Serum histamine concentrations were measured using an enzyme immunoassay kit (Catalog #IM2015 Beckman Coulter, Fullerton, CA), according to the manufacturer's instructions.

Toxin Extraction from Maize Pellets

During preparation of food pellets, at least one extra pellet from each treatment was prepared and stored up to six months at -20°C for testing. Pellets were ground into powder and three samples (~ 50 mg) were taken from each pellet. Extraction buffer [0.05% NaN3, 21.03 μM leupeptin (Sigma), 0.25 mM PefablocSC (Sigma) in PBS] was added (10 μl per mg of sample), and the samples were shaken vigorously for two hours at 37°C. Supernatants were collected and measured for CT or LT-B using the procedures outlined below.

Cholera Toxin in Feces

Lyophilized fecal samples were weighed, and extraction buffer was added (10 μl per mg). Samples were vortexed, incubated overnight at 4°C, and then vigorously shaken for an hour at 37°C. Supernatants were collected and measured for CT concentrations, using the CT ELISA procedure outlined below.

Heat Labile Toxin Subunit B (LT-B)

Concentrations of LT-B were measured by ELISA as previously described (Beyer et al. 2007).

C*holera Toxin*

Cholera toxin concentrations were measured by ELISA. Briefly, ganglioside GM1 (Alexis Biochemicals) diluted to 10 μg/ml in coating buffer (9.6) was used to coat all wells in high-binding ELISA microtiter plates (Costar) and were incubated for two hours at room temperature and blocked as previously described for total IgE. Diluted sample was added to the sample wells in duplicate and a CT (Sigma) standard curve (20 ng/ml – 0.3125 ng/ml) was added to the standard wells. Plates were incubated overnight at 4°C. Rabbit anti CT (#C-3062, Sigma), diluted 1:5000 in 1% reconstituted milk, was added and incubated for one hour at 37°C. Biotinylated goat anti rabbit IgG (#B7389, Sigma), diluted 1:2500 in 1% reconstituted milk, was added and incubated for one hour at 37°C. Visualization was carried out as previously described for total IgE.

Statistical analysis

The statistical analysis was performed using the software Statistix (version 8; Analytical Software, Tallahassee FL, USA). Due to unequal variance in all three experiments, data was log transformed before analysis as necessary. The data for any given assay was gathered from all time points and analyzed using a repeated measures ANOVA to detected significant changes between different treatments over the course of the entire experiment. If a significant day by treatment interaction was found, the data from each day was analyzed using a general ANOVA. If a significant or a marginally significant interaction between treatments was found, contrasts between groups were performed using an F-test.

Significant differences in data had a value of ($p \leq 0.05$). Marginally significant data had a value of ($0.05 \leq p \leq 0.09$).

IV. Results

Experiment One - Allergic responses induced by cholera toxin and peanuts administered by gavage:

The purpose of experiment one was to establish an allergy model using CT and peanut extract. The allergic response was quantified by measuring the presence of total and peanut specific serum IgE, peanut specific and CT specific serum IgG1, serum histamine, and visually evident physiologic responses.

Visual Score

Visually evident physiological responses were scored for two hours during challenge I and one hour during challenge II. The results are shown in Figure 4. The treatment groups show a significant difference in the visual scoring ($p = 0.0020$). On both challenge days, the CT and PE group showed a significantly elevated visual score compared to the other three groups ($p \leq 0.0000$). On Day 40, the remaining three groups were not significantly different with each other. On Day 53, the CT Only group showed a significant difference from the PE Only and Naïve groups ($P = 0.0010$).

Total IgE

Total IgE was measured before the treatments began, weekly for four weeks, four days post challenge I, and on challenge day II as indicated on bleed dates in Figure 1. The results are shown in Figure 5. The treatment groups show a significant day by treatment interaction for total IgE production during the experiment ($p = 0.0156$). On days 12 to 26, the treatments were significantly different or marginally different compared to each other (p

values $\leq$ 0.0661). Statistical contrasts of data for these three days show the CT and PE group had significantly elevated concentrations of IgE in comparison to the other three groups (p values < 0.013). Overall, the mice fed CT and PE showed up to a four and a half fold increase in total IgE concentrations (Day 19 mean = 5777 ng/ml IgE) over the other groups (Day 19 mean = 1279 ng/ml IgE) during the sensitization phase, but during the challenge phase the remaining three groups showed more variability. This indicates mice administered both the adjuvant and the allergen produce a stronger allergic response than mice receiving only one or nothing at all.

Peanut specific IgE

Peanut specific IgE was measured on bleed dates indicated by Figure 1. The results are shown in Figure 6. The treatment groups showed a significant treatment by day interaction for peanut specific IgE production (p = 0.0160). On days 12 to 44, the treatment groups were either marginally or significantly different from each other (p values $\leq$ 0.0779). The CT and PE group had significantly higher concentrations of peanut specific IgE than the other three groups (p values $\leq$ 0.014). On day 54, the treatments were not significantly different from each other; however, given the low n in this study (n = 3), and the unequal variance present in the samples, all four groups were contrasted using an F-test to see if there was a significant or marginal difference among the groups. There was a significant increase in IgE for the CT and PE group compared to the other three groups (p = 0.0345). Overall, the mice administered CT and PE showed up to a five fold increase in peanut specific IgE concentrations (Day 26 mean = 68 ng/ml IgE) over the other groups (Day 26 mean = 9 ng/ml IgE) during the sensitization phase, indicating that mice administered both the adjuvant and the allergen produce a stronger allergic response than mice receiving only one or nothing.

Peanut Specific IgG1

While IgG1 is not an indicator of an allergic response, the presence of IgG1 indicates a Th2 response, thus serum was tested for peanut specific IgG1. Since IgG1 has a 28 day half life, the presence of peanut specific IgG1 can be found after much longer time periods than IgE. Only samples from day prebleed, Day 5, Day 26, and Day 54 were tested. The results are shown in Figure 7.

The repeated measures ANOVA demonstrated a significant treatment by day interaction ($p = 0.000$). On days 26 and 54, the treatment groups were significantly different from each other (p values ≤ 0.0045). On both days the PE only group was significantly higher than the CT only & naïve groups (p values ≤ 0.0205). On Day 26, there was no significant difference between the CT and PE group and the peanut only group; however, on Day 54, the CT and PE group was significantly higher than the PE only group ($p = 0.0152$). These results indicated that mice administered both the adjuvant and the antigen produced a stronger Th2 immune response than mice receiving only the allergen, while mice administered the allergen only produce a stronger response than mice administered nothing.

Cholera toxin specific IgG1

While CT is used as an adjuvant to induce a reaction to peanuts in this study, it also can cause an antibody mediated immune response to itself. The serum from Day 54 was tested for CT specific IgG1 and the results are shown in Figure 8. The treatment groups show a marginal difference between each other ($p = 0.0658$). There was no significant difference, surprisingly, between the CT only group and the PE only & naïve groups. The CT and PE group was significantly higher than the other groups ($p = 0.0361$).

Histamine

The serum from Day 54 was tested for histamine and the results are shown in Figure 9. The treatment groups do not show a significant difference in histamine concentrations.

Experiment Two - Allergic responses induced by cholera toxin and peanuts or LT-B and peanuts administered by food pellet:

The purpose of experiment two was to test for the induction of an allergic immune response when LT-B is introduced to the body by food pellet. The presence of an allergic response was tested by measuring total and peanut specific serum IgE, peanut specific and LT-B specific serum IgG1, and visually observed allergic responses.

Toxin Concentrations in Food Pellets

Food pellets were assayed to verify the concentrations of LT-B and CT toxins. One pellet per treatment group per week was tested using ELISAs for both CT and LT-B. Due to the 80% structural similarity between the two toxins, the ELISA for one type of toxin showed a degree of detection in pellets containing the other type of toxin (Chikwamba et al. 2002). Results are summarized in Figures 10 and 11. Toxin concentrations measured in the pellets were similar to targeted concentrations. Antibodies against CT detected approximately fifty percent of the LT-B present, while antibodies against LT-B detected approximately ten percent of the CT present.

Visual Score

Visually evident physiological responses were scored during challenge I and challenge II. The results are shown in Figure 12. The treatment groups did not show a significant difference in the visual scoring between each other on either challenge day.

Total IgE

Total IgE was measured on bleed dates indicated by Figure 2. The results are shown in Figure 13. After running a repeated measures ANOVA, the treatment groups did not show a significant treatment by day interaction during the experiment. In experiment two the total serum IgE concentration for CT and PE mice (Day 18 mean = 2052 ng/ml IgE) is about a third of the IgE concentrations from experiment one (Day 19 mean = 5777 ng/ml IgE).

Peanut Specific IgE

Peanut Specific IgE was measured on bleed dates as indicated in Figure 2. The results are shown in Figure 14. After running a repeated measures ANOVA, the treatment groups did not show a significant treatment by day interaction during the experiment. In experiment two, the peanut specific serum IgE concentration for CT and PE mice (Day 25 mean = 18 ng/ml IgE) are about one quarter of the concentrations from experiment one (Day 26 = 68 ng/ml IgE).

Peanut Specific IgG1

While IgG1 is not an indicator of an allergic response, peanut is an antigen presented in a novel fashion which could theoretically induce a Th2 immune response. Samples collected from prebleed, Day 25, and Day 54/55 were tested for peanut specific IgG1. The results are displayed in Figure 15.

After running a repeated measures ANOVA, the treatment groups did not show a significant treatment by day interaction during the experiment. In experiment two the peanut specific serum IgG1 concentrations for peanut treated mice (Day 25 mean = 0.29 μg/ml) are about two hundred fold less than the peanut specific serum IgG1 concentrations from experiment one (Day 26 mean = 55.4 μg/ml).

Heat Labile Toxin Subunit B Specific IgG1

While LT-B is not considered an allergen, it is an immunogen. Thus, the serum from Day 25 and Challenge II was tested for LT-B specific IgG1. The results are shown in Figure 16. After running a repeated measures ANOVA, the treatment groups did not show a significant difference in treatment by day during the experiment. In experiment two the LT-B specific serum IgG1 concentrations for LT-B treated mice (Challenge II mean = 92.62 ng/ml IgG1) are about thirty eight fold less than the CT specific serum IgG1 concentrations for CT treated mice from experiment one (Challenge II mean = 3508.40 ng/ml IgG1).

Experiment Three – Allergic responses induced by cholera toxin and peanuts administered by gavage, food pellet, or a mixture of the two:

The purpose of experiment three was to compare different concentrations and delivery methods of the adjuvant (CT) and the allergen (peanut extract), and measure which combination would produce the strongest allergic reaction. The allergic reactions were measured by concentrations of total and peanut specific serum IgE, peanut specific and CT specific serum IgG1, serum histamine, and visually observed allergic responses.

Toxin Concentrations in Food Pellets

Assays were run to verify the concentration of CT in the food treatment pellets. At least one pellet per treatment group was saved and assayed for CT content. Due to the storage time (6 months) of the sample food pellets, new pellets were made using the same formulas and assayed in parallel with the original test pellets to verify the long term stability of CT. Results are summarized in Figure 17. Cholera toxin concentrations measured in the pellets reserved from the experiment were at least 70% of the target values, while the new pellets were at least 85% of the target values.

Toxin Concentration in Fecal Pellets

Fecal pellets were collected during and at the end of each sensitization treatment and tested for CT. Since the highest average was 0.0093 μg CT per gram of fecal matter compared to the minimum of 13.9 μg CT per gram of food pellet administered to the mice, it is apparent whole CT is not excreted. Also, mice receiving CT via any administration method were not significantly different from the naïve mice.

Visual Score

Visually evident physiological responses were scored during challenge I and challenge II. The results are shown in Figure 18. The treatment groups did not show a significant difference between each other during either challenge.

Total IgE

Total IgE was measured on bleed dates as indicated by Figure 3. The results are shown in Figure 19. After running a repeated measures ANOVA, the treatment groups showed a significant treatment by day interaction during the experiment ($p = 0.0000$).

The form of CT delivery, gavage [CT(G)], fed [CT(F)], or no CT [Naïve], showed a significant difference on days 12-53 (p values ≤ 0.0051). The gavage form induced a significantly higher concentration of total IgE from the other groups on days 12-53 (p values ≤ 0.0013). The data from groups gavaged with CT was analyzed for the effect of the CT dose concentration. They were grouped as 20 μg, 10 μg, or 0 μg of CT and the groups analyzed were [20 μg CT(G)/ 10 mg PE(G)]; [20 μg CT(G)/ 10 mg PE(F)]; [20 μg CT(G) Only] vs. [10 μg CT(G)/ 5 mg PE(G)]; [10 μg CT(G) Only] vs. [Naïve]. On days 19 to 39, CT gavage treatment groups were marginally or significantly different from each other (p values ≤ 0.0903). On days 19 and 26, treatments with 20 μg and 10 μg of CT were not

significantly different from each other, but they were either marginally or significantly higher than the 0 μg mice (p values $\leq$ 0.0690). On Day 39, 10 μg CT groups were significantly higher than both 20 μg and 0 μg CT groups (p = 0.0030).

Peanut Specific IgE

Peanut specific IgE was measured on bleed dates as indicated by Figure 3. The results are shown in Figure 20. After running a repeated measures ANOVA, the treatment groups show a significant treatment by day interaction during the experiment (p = 0.0000). On days 12-39 the individual treatments were significantly different compared to each other (p values $\leq$ 0.0016). On days 12-26 treatments [20 μg CT(G)/10 mg PE(G)] and [10 μg CT(G)/5 mg PE(G)] were significantly higher from all the other groups (p values $\leq$ 0.000) On Day 39, treatments [20 μg CT(G)/10 mg PE(G)] and [10 μg CT(G)/5 mg PE(G)] were significantly higher from all other groups, and treatment [20 μg CT Only] was significantly higher than the remaining groups (p values $\leq$ 0.0395). Although the effect of [20 μg CT Only] appears to be an anomaly for anti-peanut IgE antibodies, this group was previously challenged with peanut on day 35/36.

The form of CT delivery among treatment groups showed a significant difference on days 12-39 (p values $\leq$ 0.0066). Gavage delivery was significantly higher on days 12-39 (p values $\leq$ 0.0020).

The form of PE delivery also showed a significant difference on days 12-39 (p values $\leq$ 0.0032). The gavage group was significantly higher than the other two groups on days 12-39 (p values $\leq$ 0.000). Further analysis was done using only mice who received CT by i.g. and peanuts by i.g. or food pellet. This statistical analysis examined whether mice would have increased concentrations of peanut specific IgE as the treatment dose of peanut

increases, and whether mice would have an allergic reaction if they were administered the adjuvant and the allergen by different administration routes. The treatment groups included were [20 μg CT(G)/ 10 mg PE(G)] vs. [10 μg CT(G)/ 5 mg PE(G)] vs. [20 μg CT(G)/ 20 mg PE(F)] vs. [Naïve]. On days 12 to 39, peanut treatment groups were significantly different from each other (p values $\leq$ 0. 0195). The two groups [CT(G)] with 10 mg and 5 mg PE gavaged were significantly higher (p values $\leq$ 0. 0043) than the groups [CT(G)] with 20 mg PE fed and naive.

Peanut Specific IgG1

While IgG1 is not an indicator of an allergic response, the presence of IgG1 indicates a Th2 response. Peanut specific IgG1 was measured before the treatments began, four days after the fourth sensitization treatment, and on challenge II. The results are shown in Figure 21. After running a repeated measures ANOVA, the treatment groups show a significant treatment by day interaction during the experiment ($p = 0.0000$). Analysis of all treatment groups for each day show on Day 26 treatments [20 μg CT(G)/10 mg PE(G)] and [10 μg CT(G)/5 mg PE(G)] were significantly higher from all the other treatment groups (p values $\leq$ 0.0082). Also, the treatment group [10 mg PE(G) Only] was significantly lower than the previously mentioned two groups ($p = 0.0082$), but significantly higher from all the other groups ($p = 0.0000$). On Day 53/54 [20 μg CT(G)/10 mg PE(G)]; [10 μg CT(G)/5 mg PE(G)]; and [10 mg PE(G) Only] were significantly higher than the other groups ($p = 0.0000$). Overall, only mice that received peanut by gavage produced peanut specific IgG1. Mice administered peanut in food pellets were not significantly different than the naïve mice.

Cholera Toxin Specific IgG1

Cholera toxin is an adjuvant with a tendency to stimulate a Th2 response, thus the serum collected from the mice before the treatments began, four days post the fourth sensitization treatments, and on challenge II was tested for CT specific IgG1. The results are shown in Figure 22. After running a repeated measures ANOVA, the groups show a significant treatment by day interaction during the experiment ($p = 0.0000$).

Statistical analysis of the form of CT delivery showed gavage delivery was significantly higher than fed or naïve groups on days 26 and 53/54 (p values ≤ 0.0000). Further analysis was done using mice who received CT by gavage [CT(G)]. This statistical analysis examined the differences between dose concentrations (20, 10 or 0 μg CT) administered to mice. The treatment groups included were [20 μg CT(G)/ 10 mg PE(G)]; [20 μg CT(G) Only]; [20 μg CT(G)/ 20 mg PE(F)] vs. [10 μg CT(G)/ 5 mg PE(G)]; [10 μg CT(G) Only] vs. [Naïve]. On days 26 and 53/54, all treatment groups receiving CT by gavage [20 μg CT(G)/ 10 mg PE(G)]; [20 μg CT(G) Only]; [20 μg CT(G)/ 20 mg PE(F)]; [10 μg CT(G)/ 5 mg PE(G)]; and [10 μg CT(G) Only] were significantly higher than the naïve mice ($p = 0. 0000$).

Histamine

The serum from Day 53 was tested for histamine concentrations and the results are shown in Figure 23. The treatment groups do not show a significant difference in histamine concentrations between the groups.

V. Discussion:

Three experiments were run, the first of which replicated a previously established gavage allergy model with a variety of measurements for allergy and Th2 responses. Total and peanut specific IgE, peanut specific IgG1 and CT specific IgG1 were easily and consistently measured. Mice administered CT and peanut extract were significantly or marginally higher than control mice. In the literature, previously established models showed peak concentrations of peanut specific IgE (~ 100 ng/ml to 3300 ng/ml), peanut specific IgG1 (~ 100,000 ng/ml) and visual scores (0 to 5) (Adel-Patient et al. 2005; Li et al. 2000; Morafo et al. 2003). This model showed peak concentrations of peanut specific IgE (68 ng/ml), peanut specific IgG1 (197,000 ng/ml) and visual scores (2 to 3). While the peanut specific IgE antibody concentration was slightly lower than the range present in the literature, these measurements and measurements in experiment one of total IgE (2052 ng/ml) and CT specific IgG1 (3508 ng/ml) were all significantly higher in the experimental group than the control groups.

Total IgE concentrations in control groups during experiment one showed increased variability towards the end of the experiment, especially on days 40 and 54. This may be due to the nature of the mouse strain. C3H/HeJ mice are known for easily inducing strong allergic responses (Li et al. 1999; Morafo et al. 2003). Also, due to the variation within each treatment group, there was no significant difference between any of the groups. And, with the short half life of IgE, and 2.5 to 4.5 week time period between CT exposure and blood collection, IgE concentrations in the mice may not be an accurate reflection of the experiment. It should be noted that in experiment three, total IgE concentrations dropped for days 35/36 and 53/54 across all the groups.

Interestingly, during challenge II visual scoring, the CT Only group average score increased from 0 to 1.67, indicated the adjuvant CT, which was administered two weeks previously, may have continued to affect the intestine and permitted sensitization to peanuts during challenge I. However, a concurrent increase in peanut specific IgG1 or IgE was not observed. This may be due to the timing of sample collection. Blood is collected four days and twenty three days post challenge I peanut exposure and on the same day as challenge II peanut exposure. With the brief half life of IgE (60 hours) and IgG1 (21 days), samples may not have been collected on peak days.

With subsequent experiments, some measurements did not prove reliable. Visual scoring, though consistent during experiment one, was more variable for subsequent experiments. This could be due to the increased total number of mice in the experiments (14 mice vs. 43 mice). This increase made it more difficult to accurately observe all mice. Scanning sampling was implemented in experiment three but did not resolve the variability. The subjectivity of the observer was also an issue, especially since a score of "1" and normal mouse grooming behavior are very similar to each other. Visual scoring was not a reliable measurement for these studies.

In experiment one, serum histamine concentrations were not significantly different in any of the experimental groups, although the CT and PE treatment group had a significantly greater visual score, and the histamine concentration was highest in this group. This could be due to timing issues present in collecting serum samples for histamine. Serum was collected one hour after the peanut gavage, yet histamine release is a rapid process that occurs in minutes (Minai-Fleminger and Levi-Schaffer 2009). In experiment three, the peanut challenge was administered as 2 gavaged doses 30 minutes apart (Li et al. 2000) and was

hypothesized to reduce the time between the histamine release and serum collection to 30 minutes. Since histamine concentrations were not significant in experiment three, splitting the challenge dose did not increase the histamine concentration. In both experiments it appears the peak histamine concentration was missed. An earlier collection time after allergen exposure is needed to gather peak concentration samples. Histamine was not a reliable measurement for these experiments.

Experiment two was run to measure the allergenic qualities, or lack thereof, for LT-B. Instead of administering the immunogens LT-B or whole CT via gavage, they were administered by food pellet, along with peanut extract, in the same manner as previous tests of oral LT-B vaccines (Beyer et al. 2007; Chikwamba et al. 2002; Karaman et al. 2006). The LT-B was tested at the therapeutic concentration of 20 μg per dose, and a five-fold increase, 100 μg per dose. The excessive LT-B dose demonstrated the lack of allergic qualities in the immunogen; if it was possible for LT-B to induce an allergic response to co-fed proteins, the mice needed to be exposed to the largest dose possible, and a food pellet containing 100 μg of LT-B in 3.35 g maize is the average amount a mouse can eat in a twenty four hour period, as determined by previous studies (Beyer et al. 2007). The mice which received 20 μg or 100 μg of LT-B and peanut extract did not show a significant difference from the naïve mice in any of the allergy measurements. Since these mice did not exhibit a significant increase in any allergy indicator, this is a promising first test demonstrating the lack of allergy inducing capabilities of LT-B. The lack of response shows LT-B, as part of a maize food matrix, is not likely to induce an allergic response.

The mice which received 10 μg of CT and 5 mg of peanut extract in food pellets also did not exhibit a significant difference from the naïve mice. This lack of response in

comparison to experiment one could be caused by a number of things, including the form of delivery (gavaged vs. food pellet) or the related time difference in ingesting the treatment doses (a few seconds for i.g. delivery vs. 24 hours to consume the food pellet). Other factors could include the age of the mice (five weeks vs. seven weeks), and the additional components delivered with the treatment (nanopure water vs. a maize food matrix). These aspects may indicate a need to increase the dosage of the adjuvant component (CT), or the allergen component (peanut extract).

LT-B specific antibodies were also present in extremely low concentrations and not significantly different from the antibody responses in naïve mice. LT-B has previously shown an immune response when administered daily (unpublished observation) or with an intermittent dosing schedule of treatment on days 0, 7, 21, 49; days 0, 3, 7, 21; weekly for three weeks; and 5 times a week for 7 weeks (Beyer et al. 2007; Chikwamba et al. 2002; Mason et al. 1998; Tacket et al. 1998). The mouse strain may have affected the experiment. BALB/c is the strain used for most LT and LT-B experiments in the literature; they are high responders to this toxin, while the C3H/HeN strain has been shown as a low responder to LT (Takahashi et al. 1996); however, the C3H/HeJ strain used for this series of experiments has a mutation which increases their response to CT (Morafo et al. 2003). It was thought this mutation would increase the response to LT-B, however the LT-B specific IgG1 antibody concentrations were similar to the concentrations previously reported in C3H/HeN mice (Takahashi et al. 1996). This stain did demonstrate the ability to response to CT administered by i.g. gavage in experiments one and three, although they did not respond to CT administered by food pellet in experiments two and three. Other factors could contribute to the lack of response in this experiment, including the timing of dose administration during

this experiment, once per week for four weeks, which may not be optimal for producing a LT-B specific antibody response. Also, the size of the pellet prolonged the time for the ingestion of the LT-B doses, making it longer for this experiment (24 hours) than others in the literature (max 16 hours).

The third experiment was designed to test whether increased concentration doses of CT and peanut in a food pellet could overcome the lack of allergy induction observed in experiment two. Several additional changes were made to optimize the allergic response to fed pellet delivery. The food pellets were smaller (0.719 g), approximately 22% the size of the 3.35 g pellets used in experiment two. Since CT tends to break down at a pH of 3, and stomach acid can range from pHs of 3 to 1 during digestion, the increased acidity can break structural bonds, causing the CT subunits to separate or lose tertiary structure with increased retention in the stomach (Untersmayr and Jensen-Jarolim 2008). The mice ate the smaller pellets faster so the CT and PE was administered over a shorter period of time, spending less time in the acidic stomach. It was theorized that the shorter period of time for food pellet consumption would cause stronger immune responses than the reactions seen in experiment two. Meanwhile, gavaged mice received their treatments in bicarbonate water (1.5%) to help increase the pH in the mouse stomachs, slowing the breakdown of CT.

For this model allergy system, the form of delivery for CT seemed to be the vital aspect to sensitize the immune system, not the amount of CT administered. In all serum antibody measurements, only mice who received CT by gavage showed a significant increase from the mice who received CT by fed food pellet or no CT. Evidence suggests increasing the CT dose from 10 μg to 20 μg per treatment does not increase the effectiveness, only the morbidity of the mice.

Mice which received CT in food pellets did not become ill or have an immune response to CT or peanuts. The lack of response is probably due to increased break down of CT prior to reaching the intestines since there was no CT detected in any fecal pellet treatment groups. Given the route of delivery is so important to the allergy model, fed CT may be exposed to stomach acid long enough to begin breaking down. Bicarbonate was not added to food pellets in experiments two and three; however, in the future it may need to be added to inhibit CT breakdown. Further testing could be done to define the exposure time to stomach acid needed to begin the CT breakdown process and define possible methods to inhibit the acidic environment.

When testing for peanut specific antibodies, the delivery method also dictated the allergic reaction. Groups which received peanut extract and CT both by gavage showed allergic responses; as opposed to mice which received peanut extract and CT by any other delivery method or combination of delivery methods, which were not significantly different from naïve mice. This allergic response did not change based on the concentration of the peanut dose administered. These findings indicate that with the allergen peanuts, the form of delivery was an important factor, but the co-administered presence and route of delivery for the adjuvant CT was also essential for allergy induction. Peanuts alone delivered by i.g. administration induced a significant increase in peanut specific IgG1 production, although this increase was not as high as mice receiving both PE and CT by gavage. IgG1 is not an indication of an allergic reaction, however, only a Th2 response. Without the adjuvant, peanuts administered by gavage were unable to produce an allergenic reaction. When peanuts were fed as part of a food matrix, they were unable to stimulate any detectable antibody response. Interestingly, the mice receiving CT alone by gavage had a significant

increase in production of peanut specific IgE compared to naïve mice after peanut exposure in challenge I. This indicated the mucosal adjuvant CT affected the intestinal cells for over two weeks, long enough for the i.g. challenge peanut dose to elicit an allergic response. This suggests a possible future model for allergy induction where CT administration by gavage is followed by novel, transgenic foods or peanuts by gavage.

The findings from experiment three indicate if the intragastric gavage allergy model is going to be used in the future, the mice should receive 10 µg of CT and 5 mg of PE. Since the concentrations of CT and peanut do not matter provided the doses are gavaged, using the lower concentrations will keep morbidity and mortality to a minimum.

Overall, antibody concentrations from all the assays in experiment three were higher than experiment one. This could be due to several technical changes made between the two experiments. The CT from experiment three was reconstituted in concentrated 10x PBS instead of water. Concentrated PBS slowed the CT degradation process, giving it more potency throughout the experiment (McClosky; personal communication). The gavaged mice received treatments diluted in bicarbonate water (1.5%) during experiment three instead of nanopure water. This increased the pH of the mouse stomachs, which would give the CT more potency (Untersmayr and Jensen-Jarolim 2008) and may be needed in food treatment pellets as well.

Further adaptations of this research model for evaluation of transgenic maize may include administering CT and PE by i.g., and co-administering LT-B by food pellet. Unlike peanut extract, which travels passively through the intestines, LT-B will attach to the GM1 receptors present and bring itself into the body, giving it more exposure to components of the immune system and the localized Th2 cytokine environment CT induces (Pizza et al. 2001).

Combining oral administration of CT with LT-B also could reduce the allergenic effect of the CT and PE gavage model by competing with CT binding to GM1 receptors and either lower the concentrations of antibody produced or produce a cytokine environment which prevents isotype switching to IgE. Conversely, if LT-B is present in the CT and PE gavage model, mice may develop an allergic response to LT-B as it represents a novel food. Also, it would be informative to examine the response induced if CT and PE are administered via i.g. as a priming dose, and subsequent sensitization treatments administered LT-B and PE via food pellet. By using a strong adjuvant to trigger the initial immune response, later doses with a weaker adjuvant may induce isotype switching to IgE (Takeda et al. 2004). The ability to shift the isotype antibody response could affect portions of the population which currently have an IgG immune response to various allergens (Kimber and Dearman 2002) or genotypes indicative of atopy (Dean et al. 2007). One other issue to address would be if LT-B could induce or inhibit an allergic immune response when the allergen is not co-fed, but physically linked to LT-B. While mice did not induce an allergic response to co-fed proteins in the presence of LT-B, novel co-fed peanut proteins did not induce an IgG1 response either, indicating there was no immune response. Linked proteins, which can induce an immune response, may be capable of eliciting an allergic response. Conversely, studies using the B subunit of CT linked to a known allergen have shown that an allergic response will not be induced, and in cases where the allergy is already present in mice, the allergic response is suppressed (Rask et al. 2000). LT-B linked proteins could also behave in this manner and be used to actively suppress allergies in humans.

Acknowledgements

We thank J. Clements for supplying bacterial LT-B, the Iowa State University Plant Transformation Facility for supplying LT-B maize, and L. Moeller for LT-B transgenic maize technical consultation. Funding was provided by the Iowa State University Plant Sciences Institute through its Biopharmaceuticals Research Initiative. The authors declare they have no competing financial interests.

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Table 1: Treatment groups and the abbreviations used in experiment one.

Treatment Administered (gavage)	**Group Abbreviation**
10 μg of Cholera Toxin and 5 mg of Peanut Extract	CT and PE
5 mg of Peanut Extract	PE
10 μg of Cholera Toxin	CT
Nanopure Water	Naïve

Table 2: Visual Scoring Table depicts the six different categories for the visually observable allergic reactions seen in mice during challenge I and challenge II. Adapted from Li and colleagues (2000).

Visual Score	**Symptoms**
0	No symptoms
1	Scratching and rubbing around the nose, head, and feet
2	Puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate
3	Wheezing, labored respiration, and cyanosis around the mouth and tail
4	No activity after prodding or tremor and convulsion
5	Death

Table 3: Treatment groups and the abbreviations used in experiment two.

Treatment Administered (fed pellet)	**Group Abbreviation**
100 μg LT-B and 5 mg Peanut Extract	LTB 100 PE
20 μg LT-B and 5 mg Peanut Extract	LTB 20 PE
100 μg LT-B only	LTB 100 only
20 μg LT-B only	LTB 20 only
10 μg of Cholera Toxin and 5 mg of Peanut Extract	CT and PE
Peanut Extract only	PE only
Cholera Toxin only	CT only
Non-transgenic maize Only	Naïve

Table 4: Treatment groups and the abbreviations used in experiment three.

Treatment Administered (gavage or fed pellet)	**Group Abbreviation**
20 μg Cholera Toxin by gavage and 10 mg Peanut Extract by gavage	20 μg CT(G)/ 10 mg PE(G)
10 μg Cholera Toxin by gavage and 5 mg Peanut Extract by gavage	10 μg CT(G)/ 5 mg PE(G)
20 μg Cholera Toxin by gavage and 20 mg Peanut Extract fed	20 μg CT(G)/ 20 mg PE(F)
20 μg Cholera Toxin fed and 20 mg Peanut Extract fed	20 μg CT(F)/ 20 mg PE(F)
10 μg Cholera Toxin fed and 10 mg Peanut Extract fed	10 μg CT(F)/ 10 mg PE(F)
20 μg Cholera Toxin only by gavage	20 μg CT(G) Only
10 μg Cholera Toxin only by gavage	10 μg CT(G) Only
20 μg Cholera Toxin only fed	20 μg CT(F) Only
10 mg Peanut Extract only by gavage	10 mg PE(G) Only
20 mg Peanut Extract fed only fed	20 mg PE(F) Only
10 mg Peanut Extract fed only fed	10 mg PE(F) Only
Non-transgenic maize Only	Naïve

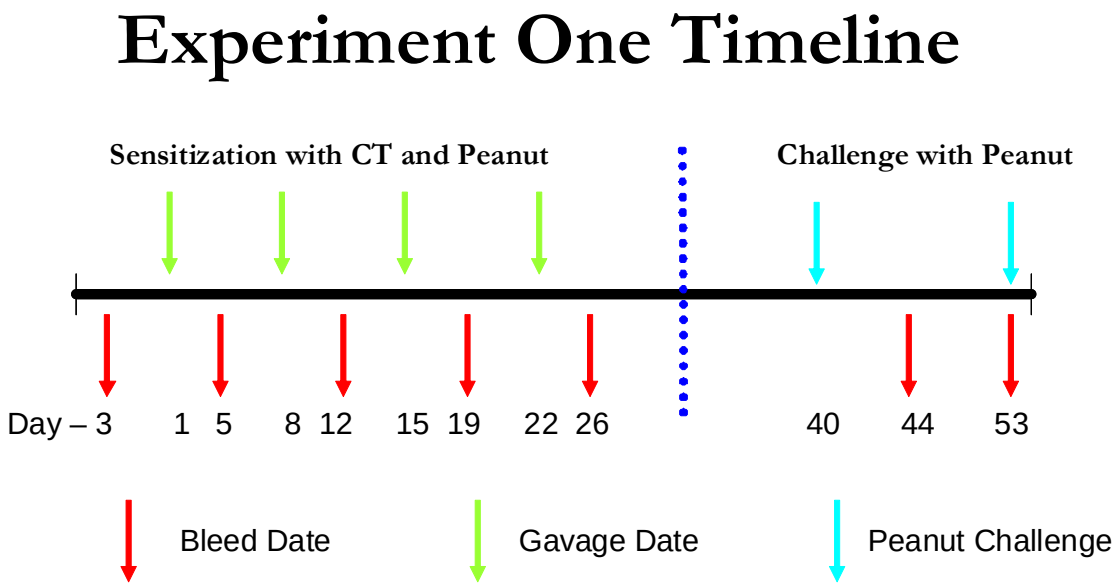

Figure 1: Timeline for Experiment one. Experiment one was run in two phases; the sensitization phase and the challenge phase, which are separated by a dotted line. Bleed days (-3, 5, 12, 19, 26, 44) gavage days (1, 8, 15, 22), and challenge days (40, 53) are all indicated by arrows.

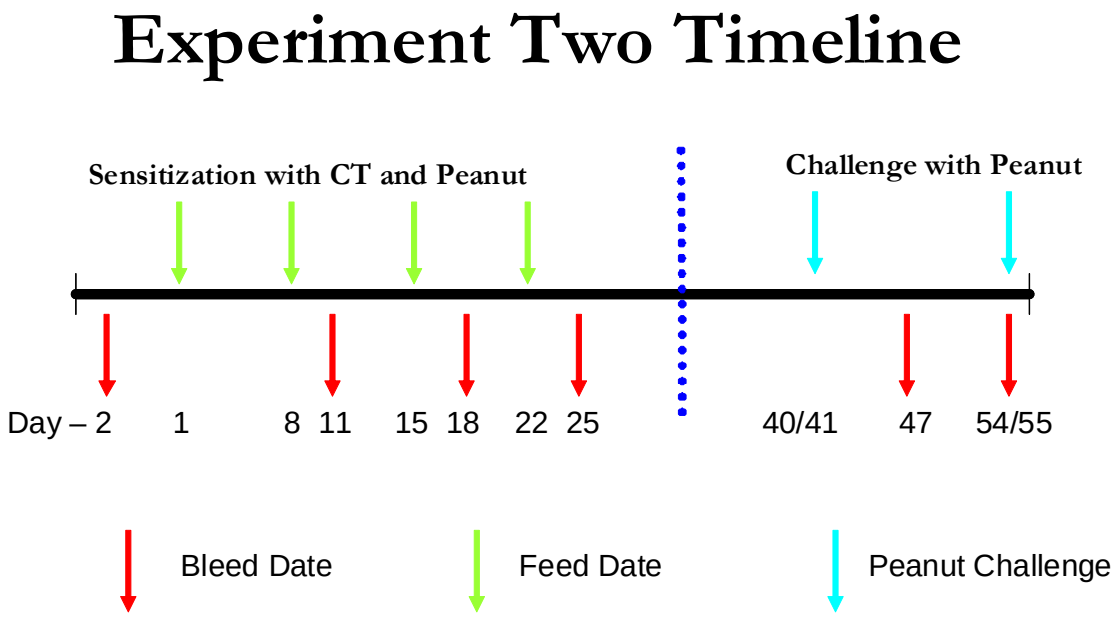

Figure 2: Timeline for Experiment two. Experiment two was run in two phases; the sensitization phase and the challenge phase, which are separated by a dotted line. Bleed days (-2, 11, 18, 25, 47) feed days (1, 8, 15, 22), and challenge days (40 & 41; 54 & 55) are all indicated by arrows.

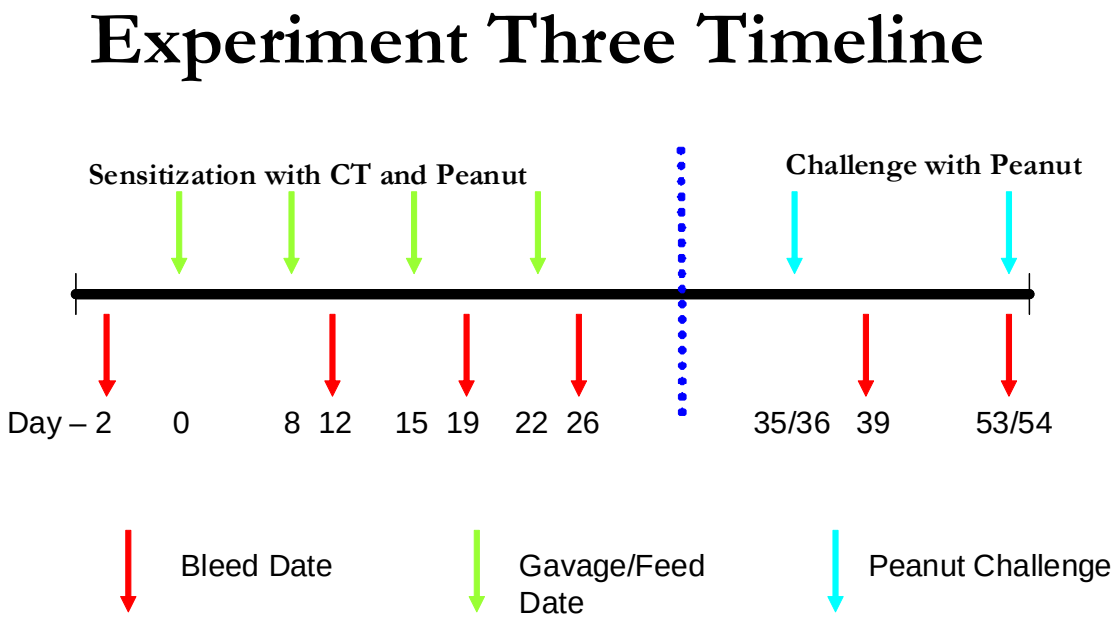

Figure 3: Timeline for Experiment Three. Experiment Three was run in two phases; the sensitization phase and the challenge phase, which are separated by a dotted line. Bleed days (-2, 12, 19, 26, 39) gavage days (0, 8, 15, 22), and challenge days (35 & 36, 53 & 54) are all indicated by arrows.

Experiment One

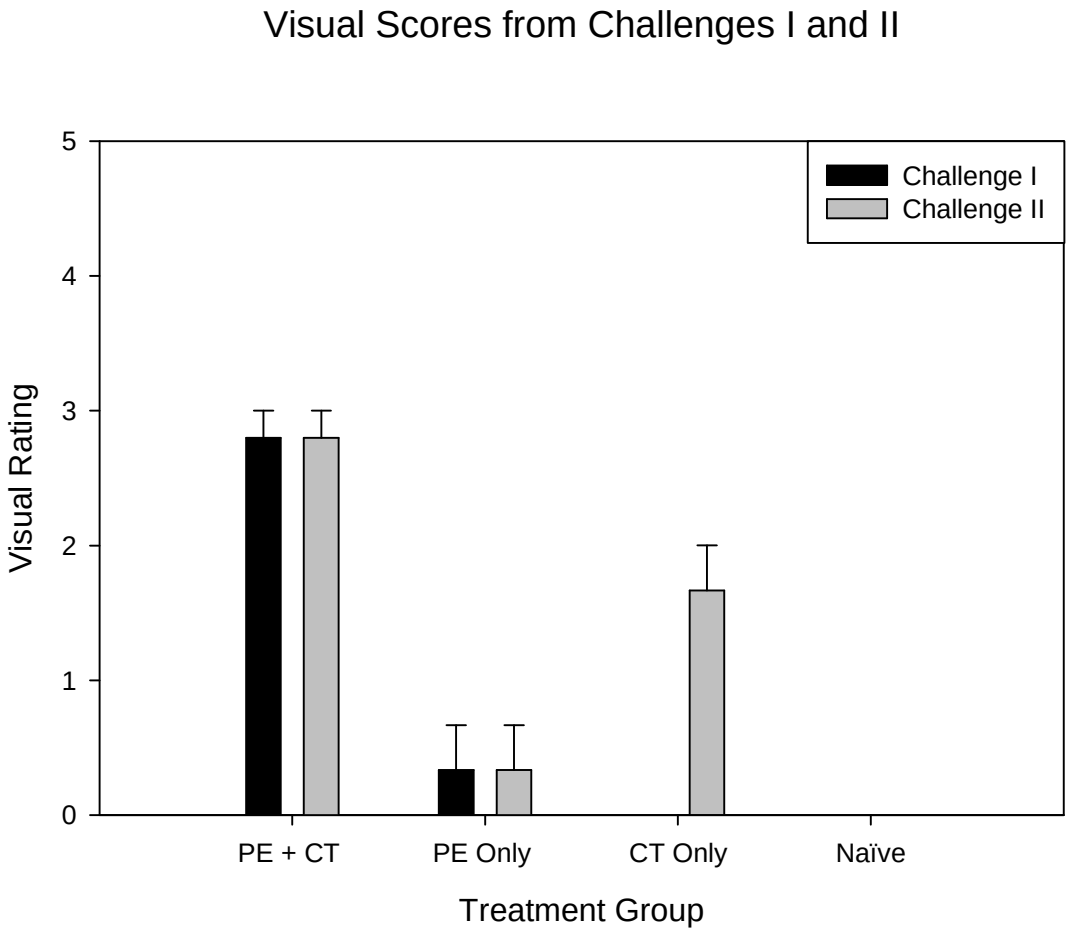

Figure 4: Visual scores from both peanut extract challenges on Day 40 and Day 54. N = 5 for the CT and PE group, n = 3 for all other groups. Each bar represents the group mean ± standard error.

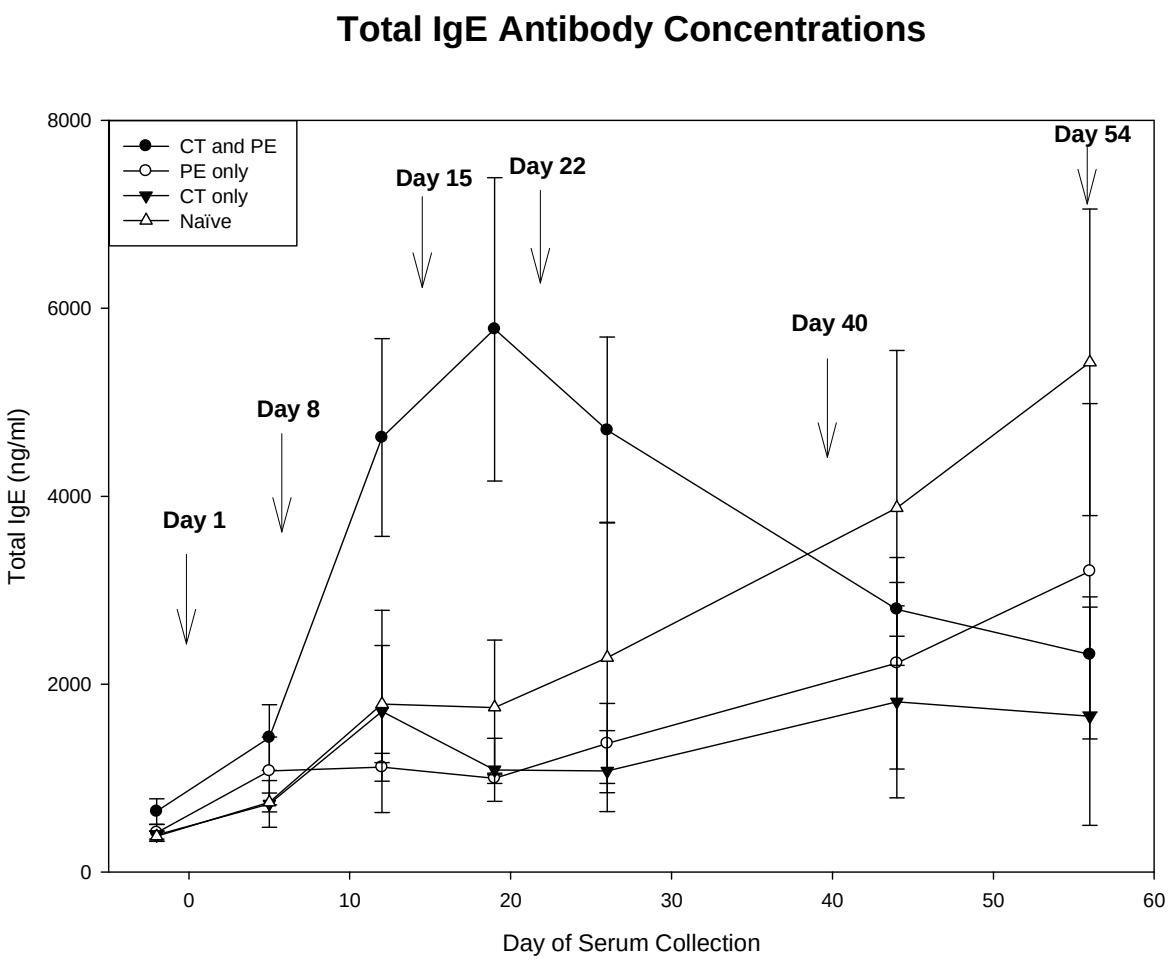

Figure 5: Total serum IgE concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (1, 8, 15, & 22) in which the mice received their individual treatments or challenge days (40 & 54) in which mice received 5 mg peanut extract gavages. N = 5 for the CT and PE group, n = 3 for all other groups. Each point represents the group mean ± standard error.

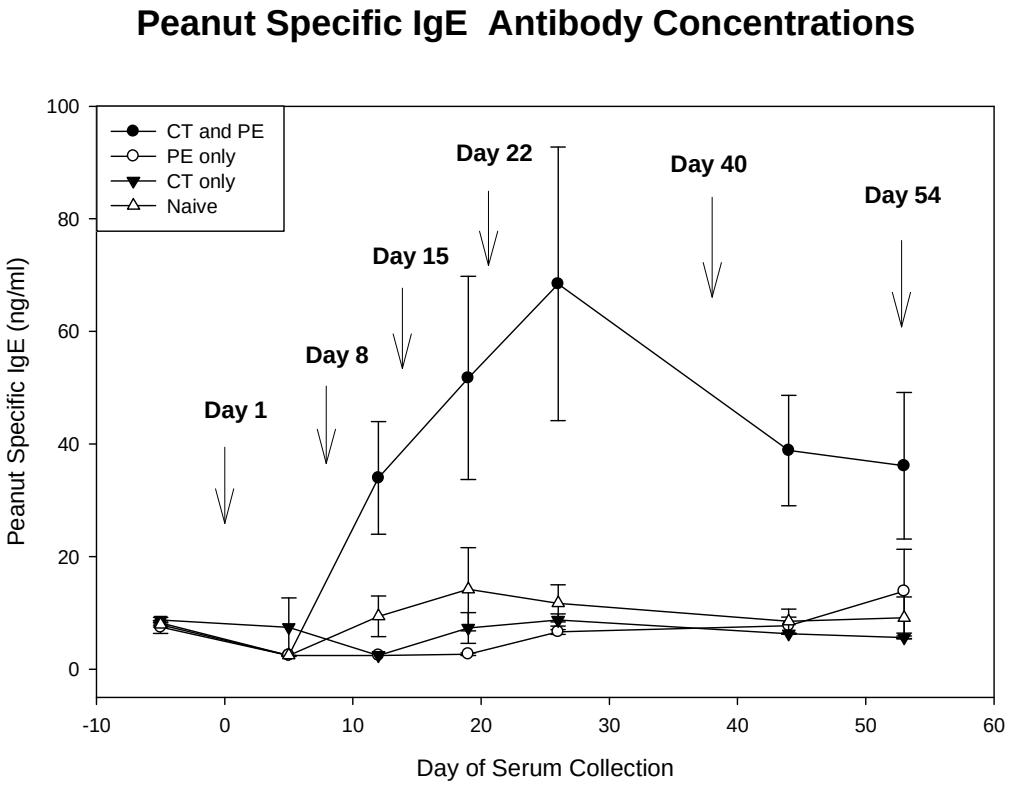

Figure 6: Peanut specific serum IgE concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days 1, 8, 15, & 22 in which the mice received their individual treatments or challenge days 40 & 54 in which mice received 5 mg peanut extract gavages. N = 5 for the CT and PE group, n = 3 for all other groups. Each point represents the group mean ± standard error.

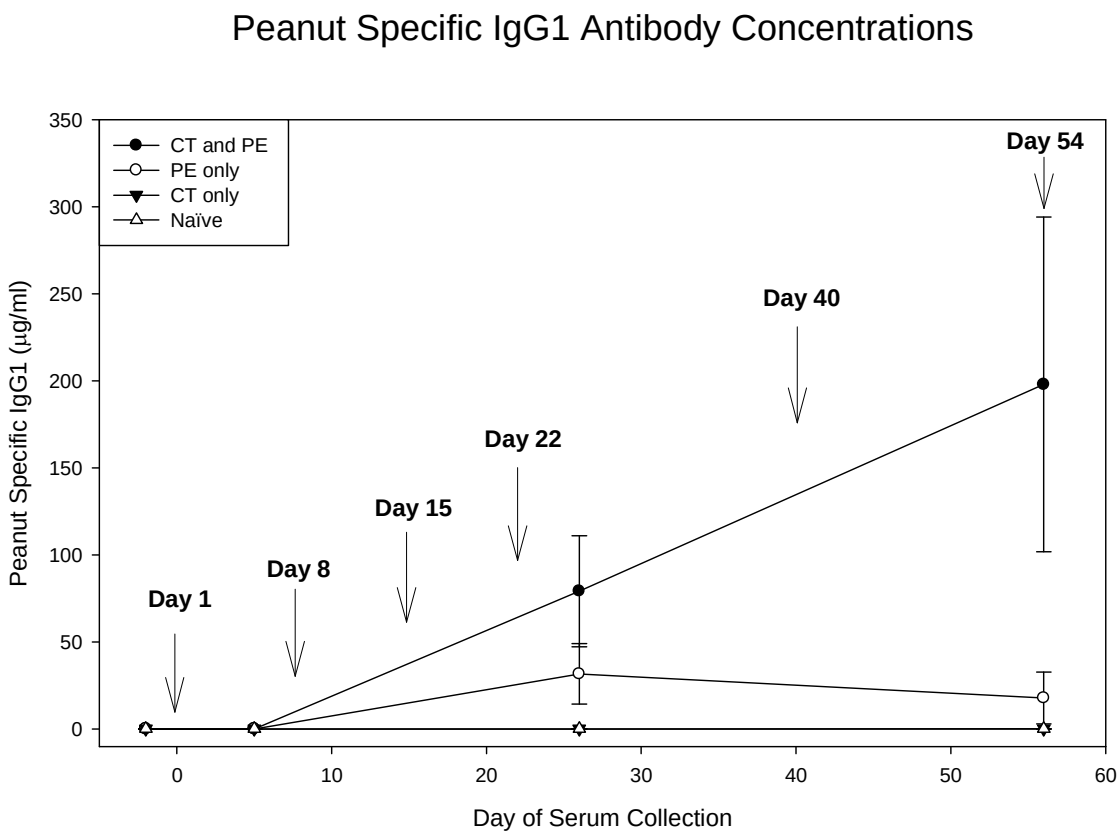

Figure 7: Peanut specific serum IgG1 concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (1, 8, 15, & 22) in which the mice received their individual treatments by gavage or challenge days (40 & 54) in which mice received 5 mg peanut extract gavages. N = 5 for the CT and PE group, n = 3 for all other groups. Each point represents the group mean ± standard error.

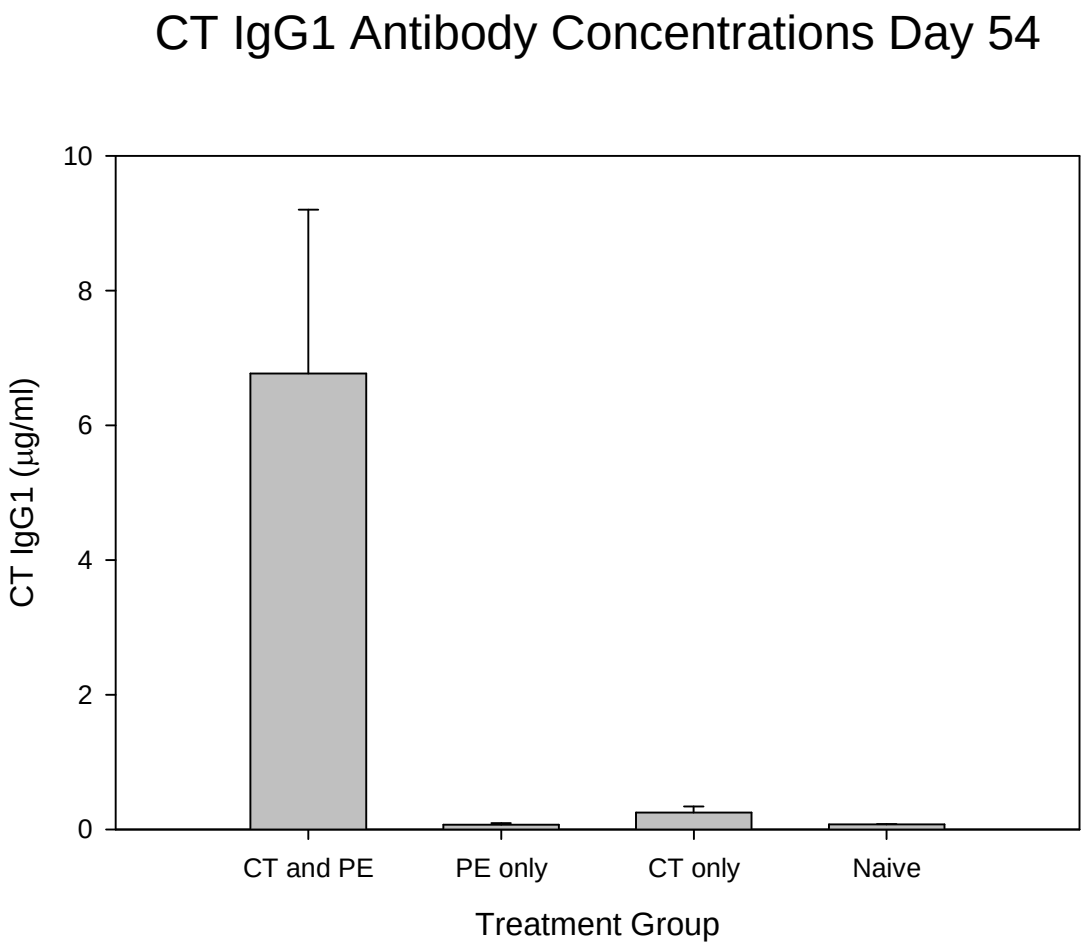

Figure 8: Cholera toxin specific serum IgG1 concentrations collected on the last day of the experiment, Day 54, when mice received 5 mg peanut extract by gavage. N = 5 for the CT and PE group, n = 3 for all other groups. Each point represents the group mean ± standard error.

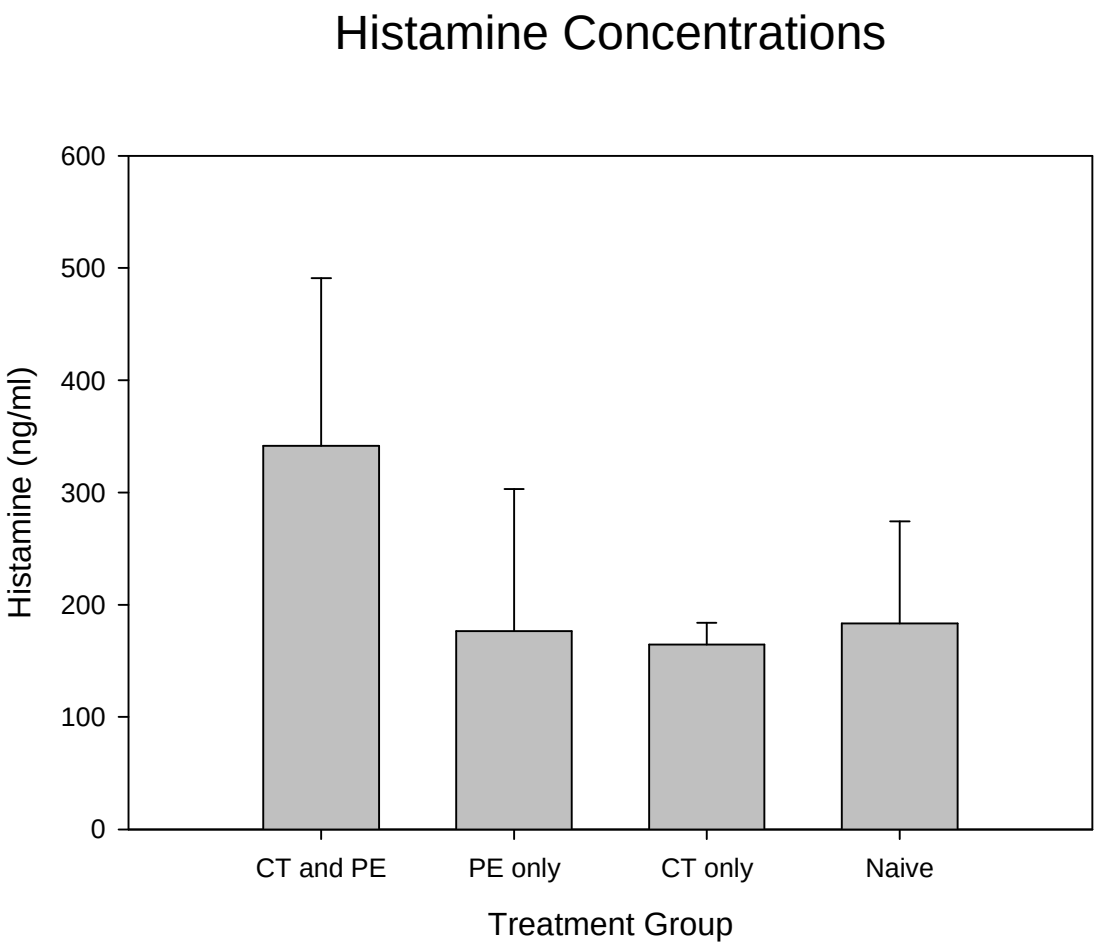

Figure 9: Serum Histamine concentrations collected on the last day of the experiment, Day 54, when mice received 5 mg peanut extract by gavage. N = 5 for the CT and PE group, n = 3 for all other groups. Each point represents the group mean ± standard error.

Experiment Two

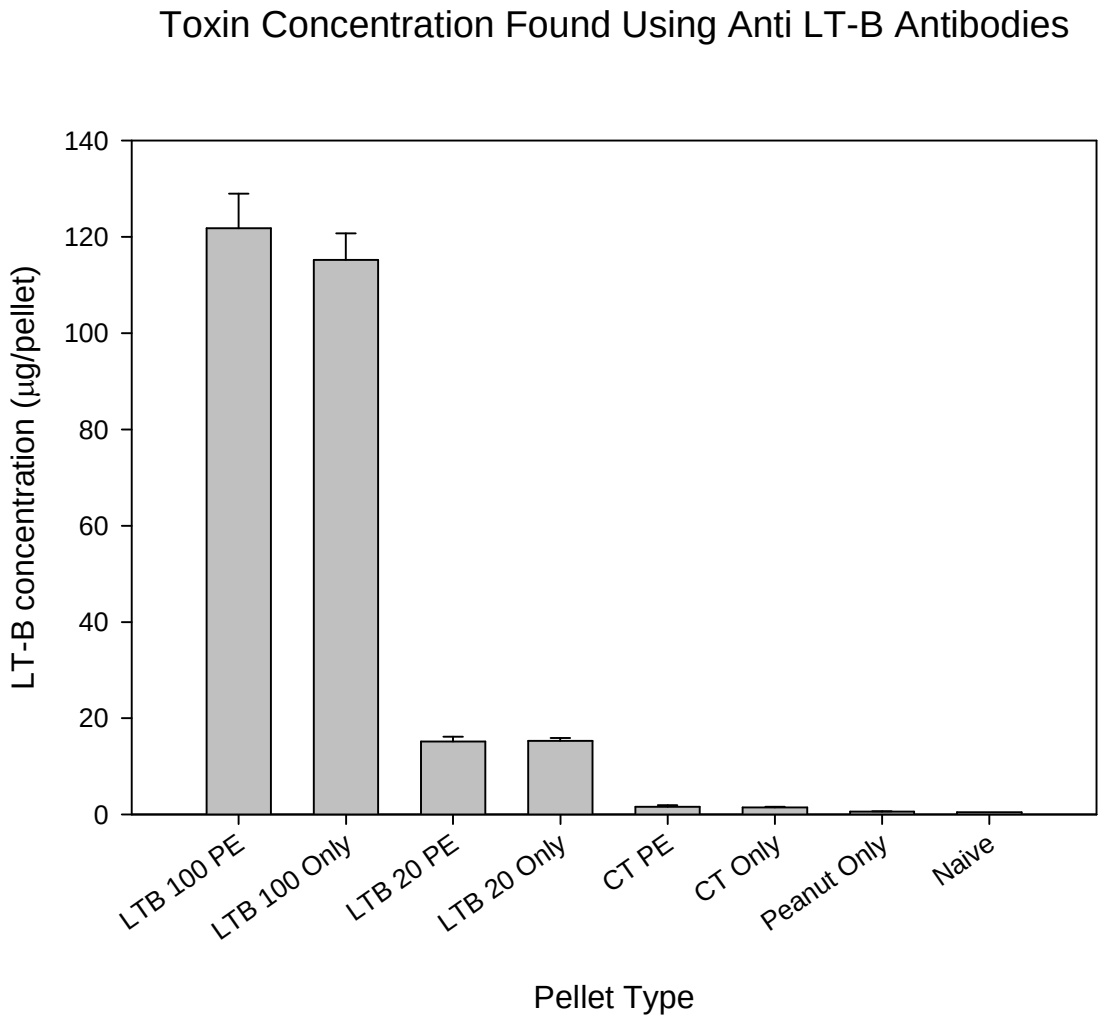

Figure 10: The toxins were extracted from mouse treatment pellets from the administrations performed on Day 8, Day 15, and Day 22. The pellets were averaged and graphed. Assays using anti LT-B antibodies were run. Due to the high degree of structural similarity between LT-B and CT, anti LT-B antibodies will react with CT, showing a reaction. Each bar represents the group mean ± standard error.

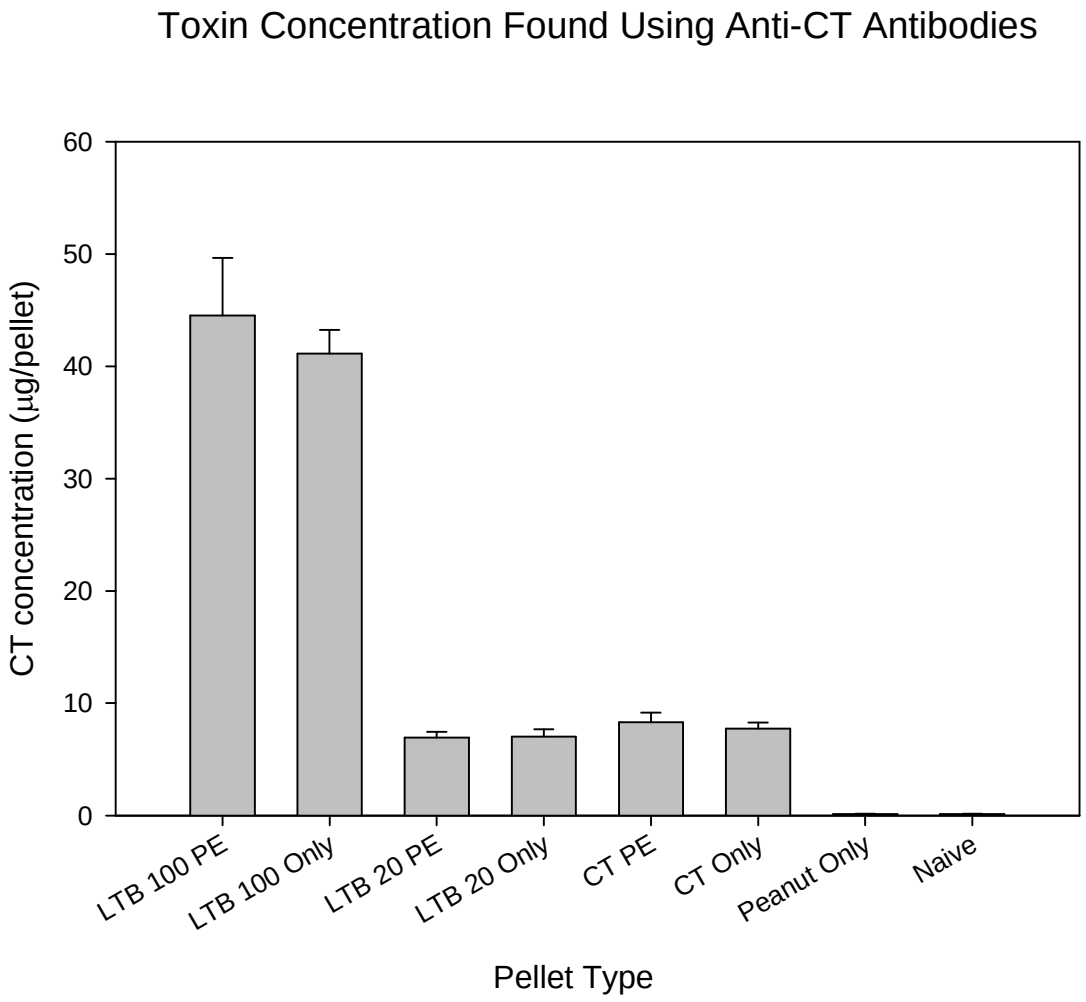

Figure 11: The toxins were extracted from mouse treatment pellets from the administrations performed on Day 8, Day 15, and Day 22. The pellets were averaged and graphed. Assays using anti CT antibodies were run. Due to the high degree of structural similarity between LT-B and CT, anti CT antibodies will react with LT-B, showing a reaction. Each bar represents the group mean ± standard error.

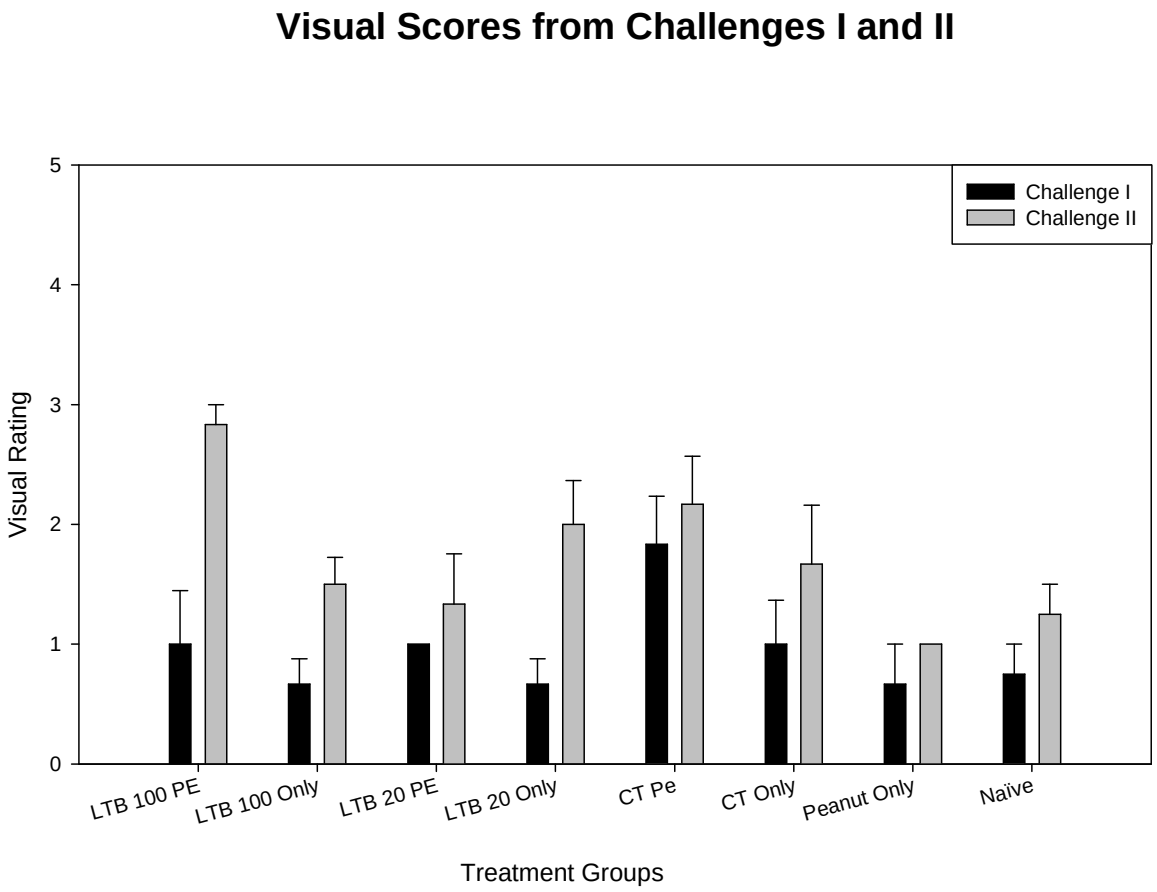

Figure 12: Visual scores from both peanut extract challenges on Days 40 & 41 and Days 54 & 55. N = 3 for PE only group, n = 4 for naïve group, n = 6 for all other groups. Each bar represents the group mean ± standard error.

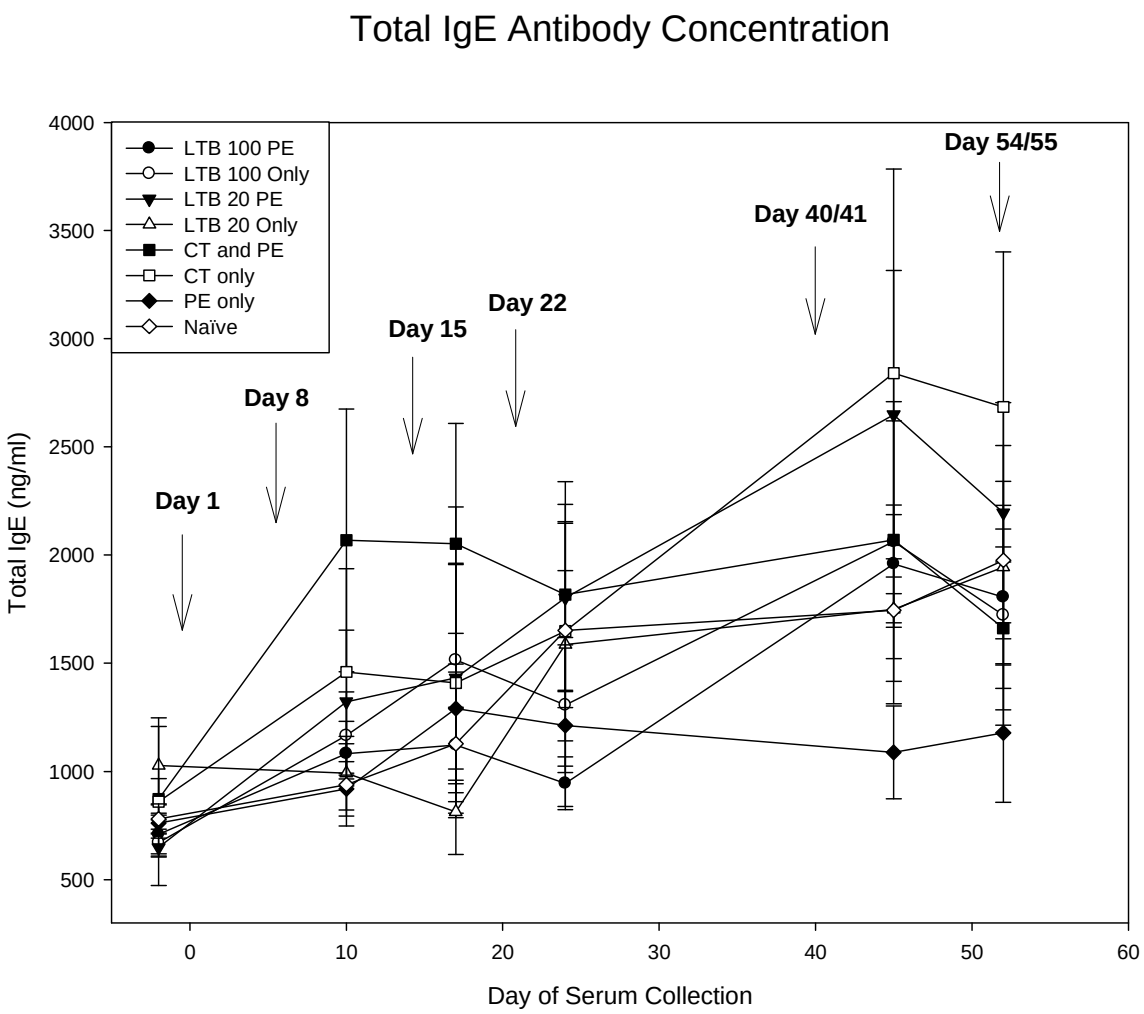

Figure 13: Total serum IgE concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (1, 8, 15, & 22) in which the mice received their individual treatment pellets or challenge days (40 & 41 or 54 & 55) in which mice received 5 mg peanut extract gavages. N = 3 for PE only group, n = 4 for naïve group, n = 6 for all other groups. Each point represents the group mean ± standard error.

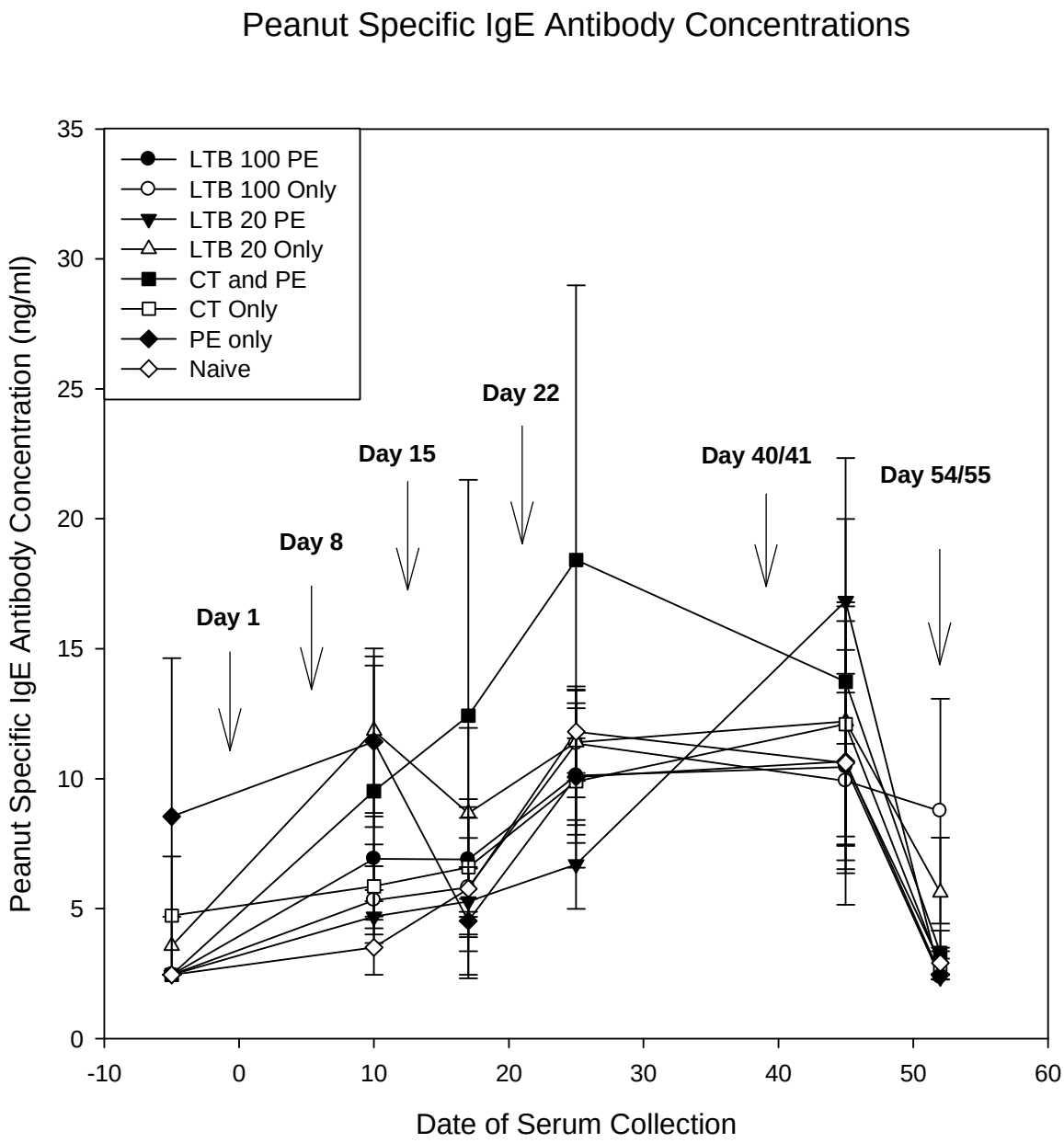

Figure 14: Peanut specific serum IgE concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (1, 8, 15, & 22) in which the mice received their individual treatment pellets or challenge days (40 & 41 or 54 & 55) in which mice received 5 mg peanut extract gavages. N = 3 for PE only group, n = 4 for naïve group, n = 6 for all other groups. Each point represents the group mean ± standard error.

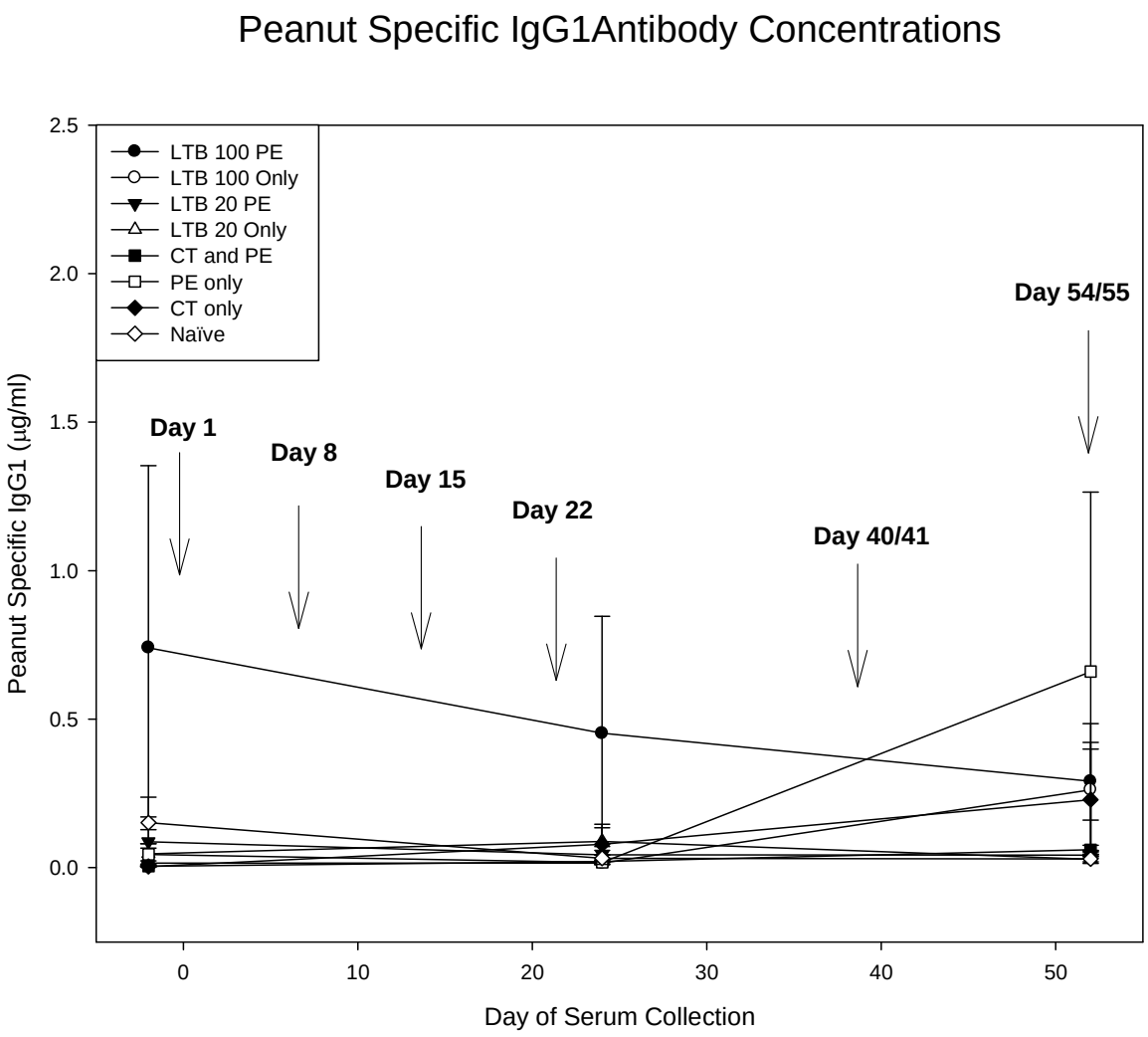

Figure 15: Peanut specific serum IgG1 concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (1, 8, 15, & 22) in which the mice received their individual treatment pellets or challenge days (40 & 41 or 54 & 55) in which mice received 5 mg peanut extract gavages. N = 3 for PE only group, n = 4 for naïve group, n = 6 for all other groups. Each point represents the group mean ± standard error.

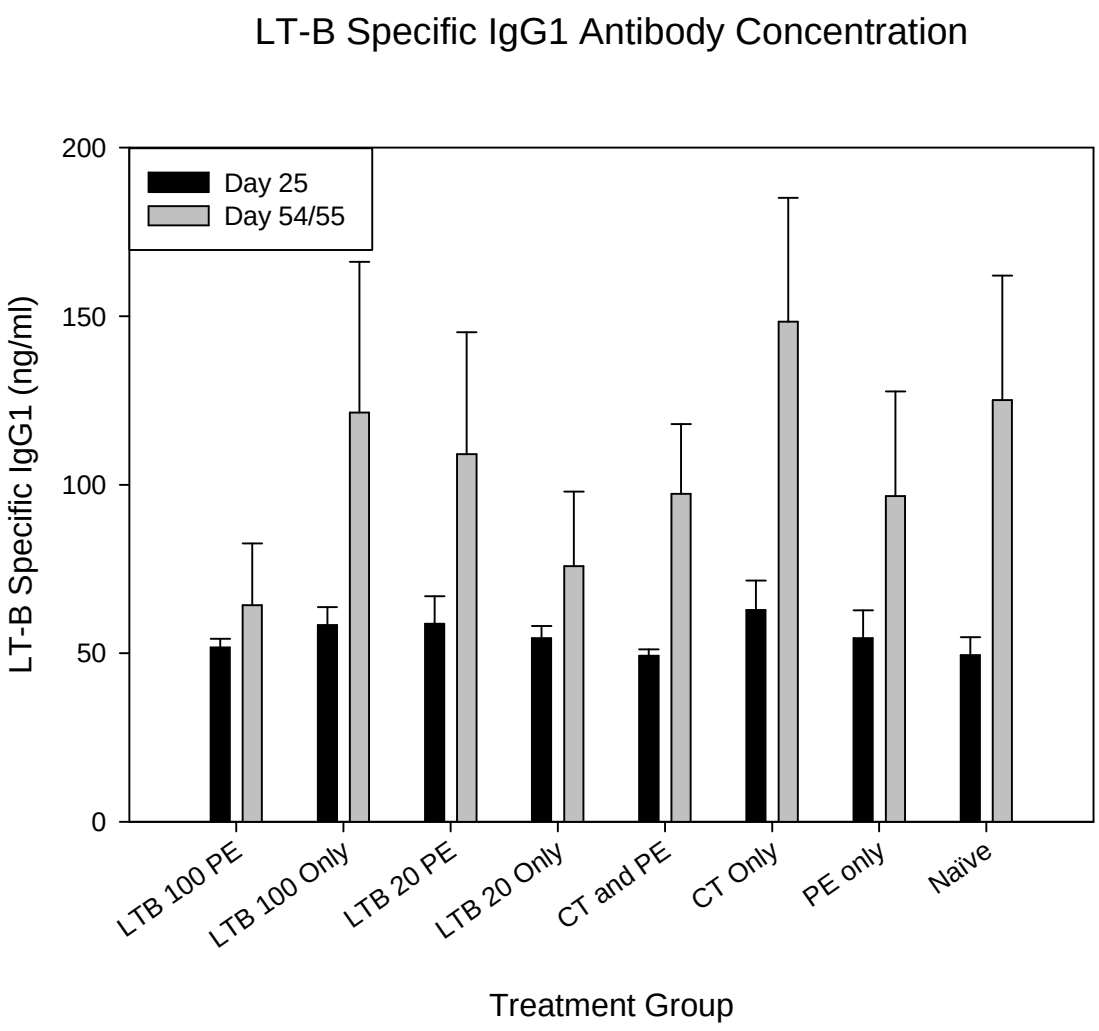

Figure 16: LT-B specific serum IgG1 concentrations collected on Day 25 and Days 54 & 55. N = 3 for PE only group, n = 4 for naïve group, n = 6 for all other groups. Each bar represents the group mean ± standard error.

Experiment Three

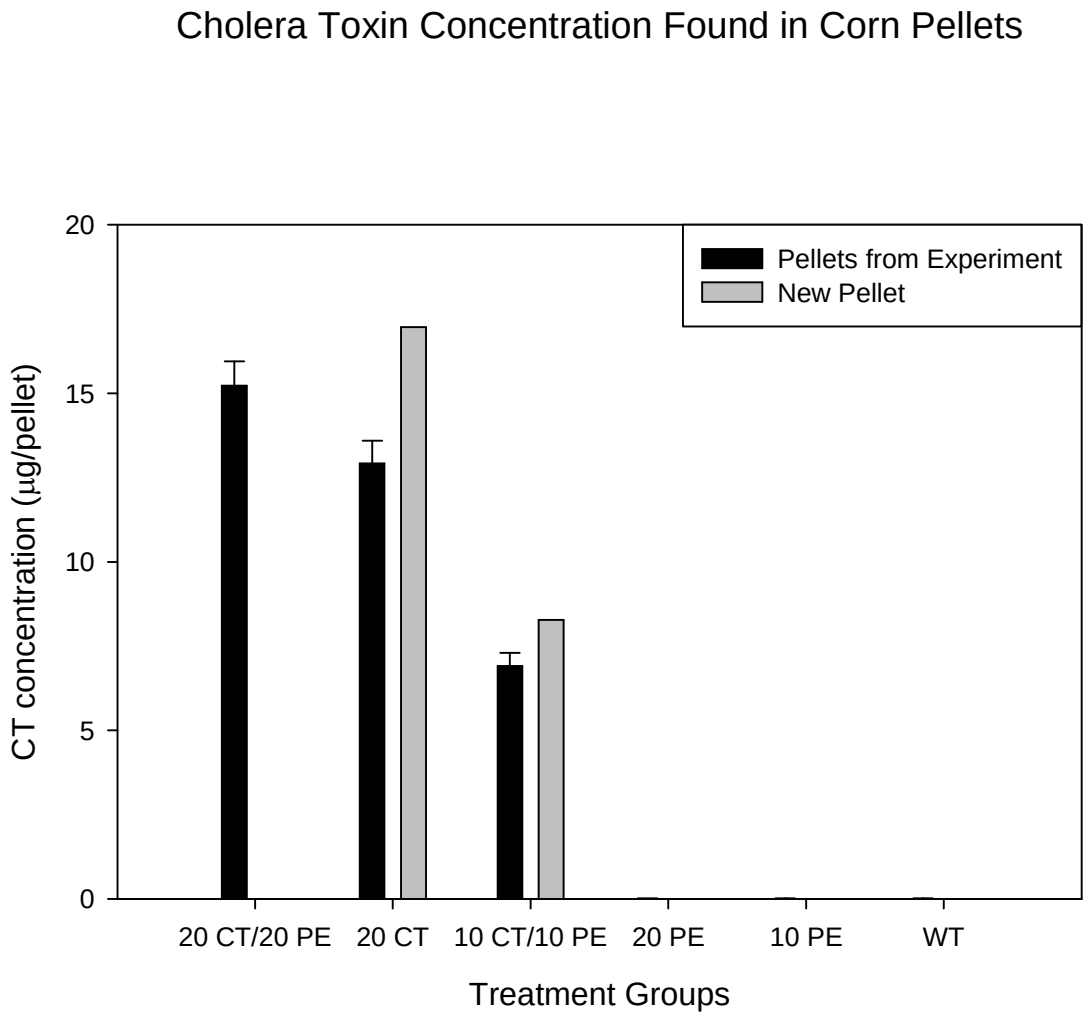

Figure 17: The toxins were extracted from mouse treatment pellets from the administrations performed on Day 0, Day 8, Day 15, and Day 22. The pellets were averaged and graphed. Due to duration of pellet storage, new pellets were made two days before assay was run for comparison. Each bar represents the group mean ± standard error.

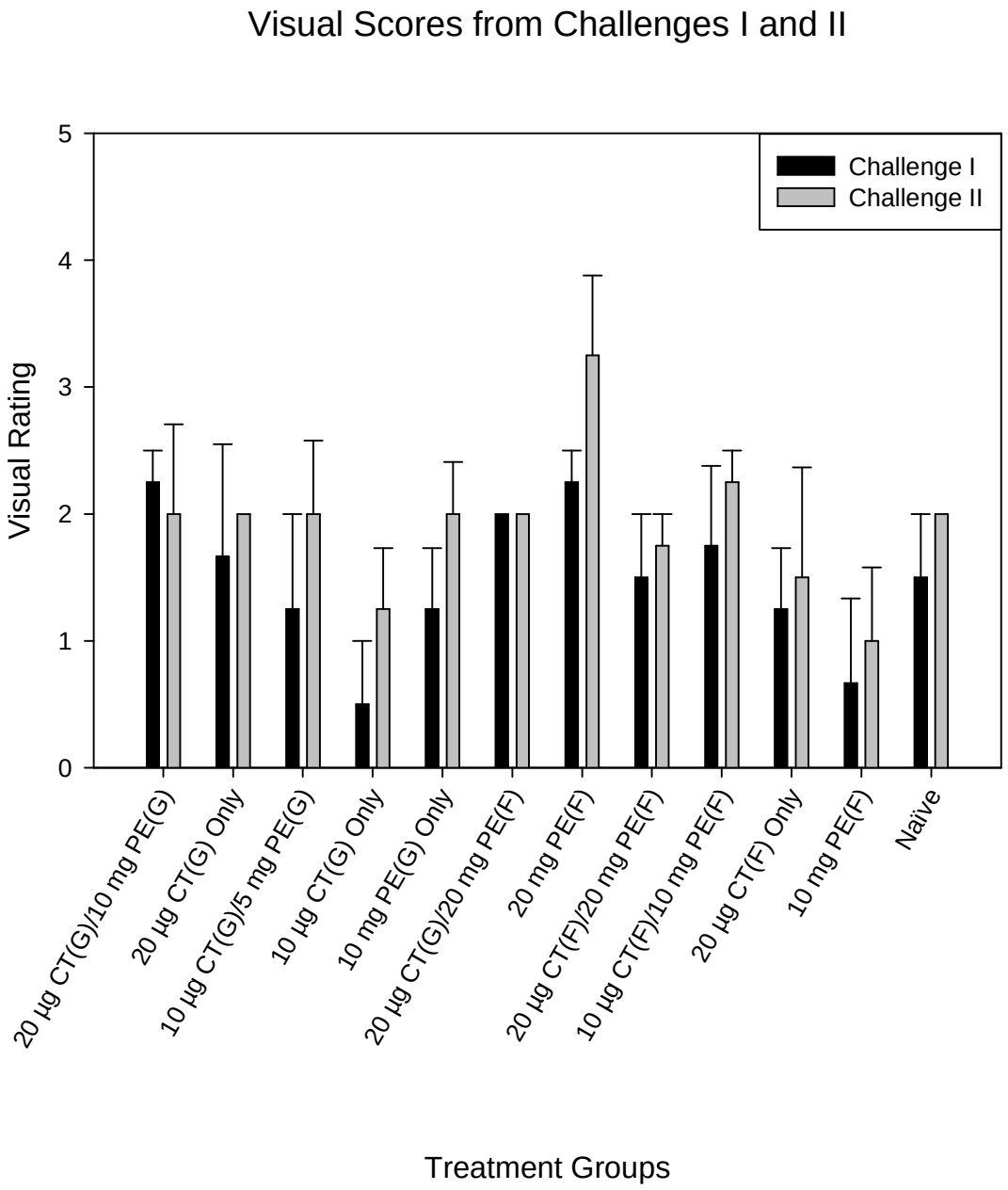

Figure 18: Visual scores from both peanut extract challenges on Days 32 & 33 and Days 53 & 54. N = 2 for the 20 µg CT (G)/20 mg PE (F) group; n = 3 for the 20 µg CT (G) only & 10 mg PE (F) only groups; n = 4 in all other groups. Each point represents the group mean ± standard error.

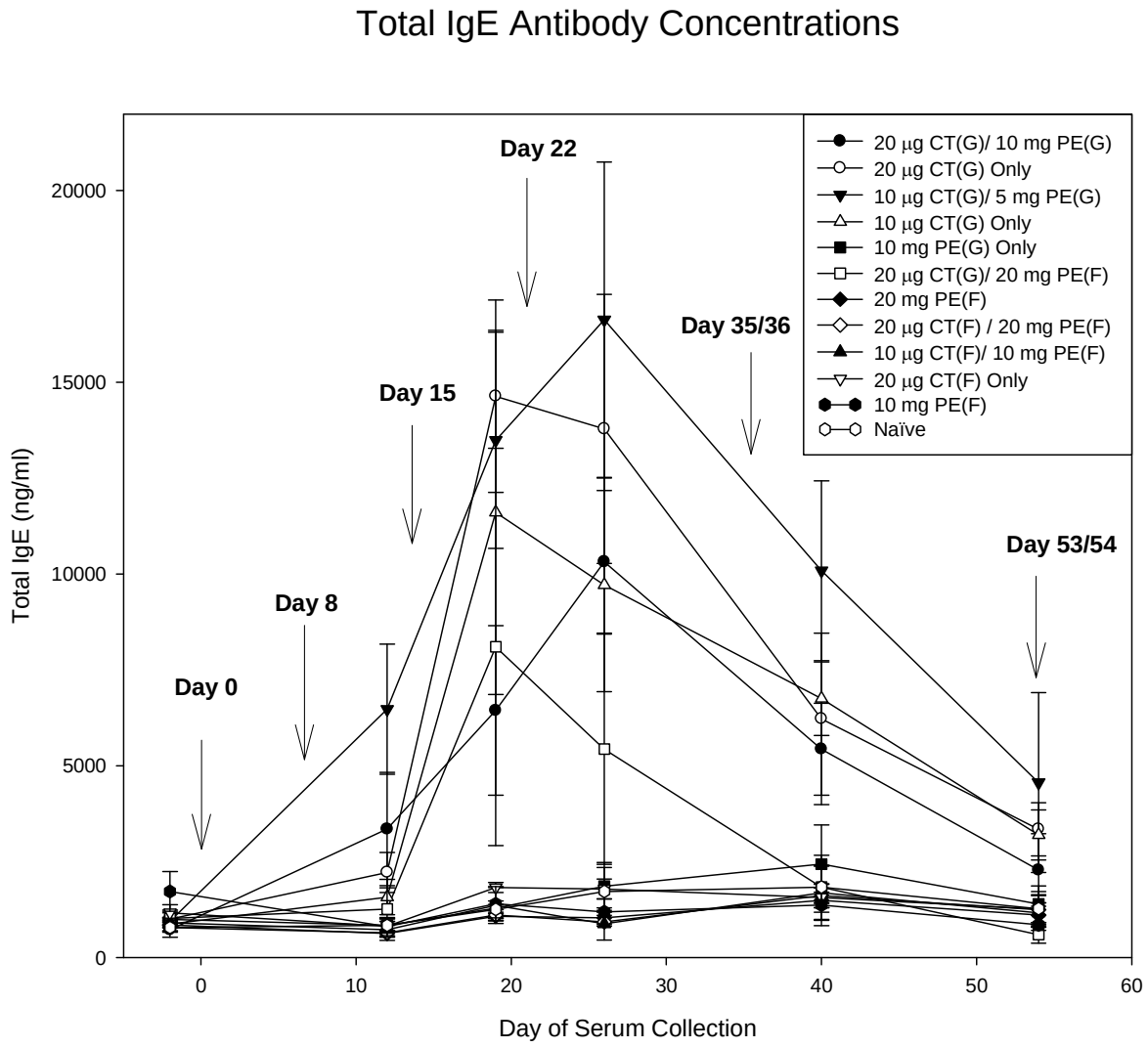

Figure 19: Total serum IgE concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (0, 8, 15, & 22) in which the mice received their individual treatment gavages/pellets or challenge days (35 & 36 or 53 & 54) in which mice received 10 mg peanut extract gavages. N = 2 for the 20 μg CT (G)/20 mg PE (F) group; n = 3 for the 20 μg CT (G) only & 10 mg PE (F) only groups; n = 4 in all other groups. Each point represents the group mean ± standard error.

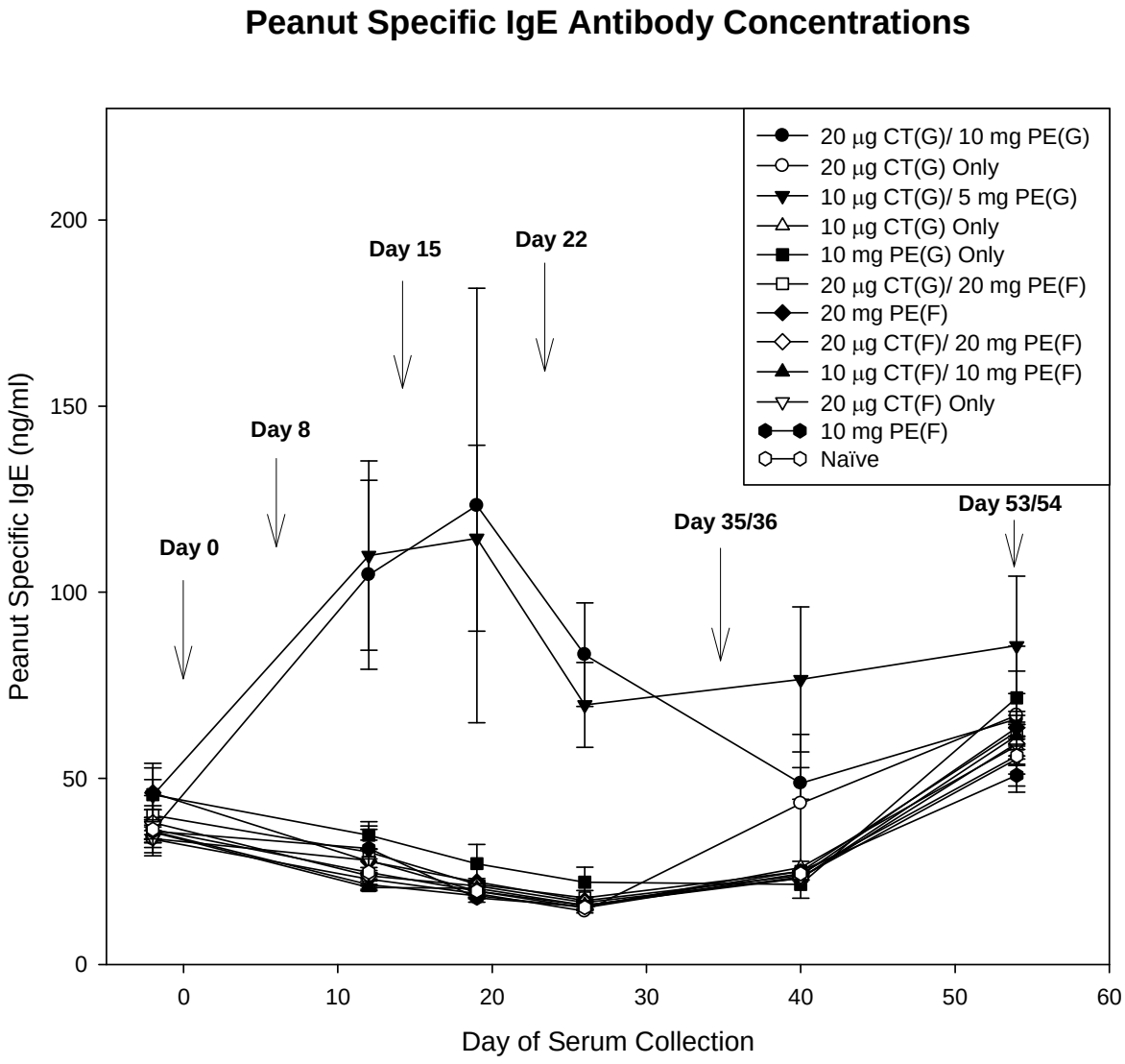

Figure 20: Peanut specific serum IgE concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (0, 8, 15, & 22) in which the mice received their individual treatment gavages/pellets or challenge days (35 & 36 or 53 & 54) in which mice received 10 mg peanut extract gavages. N = 2 for the 20 μg CT (G)/20 mg PE (F) group; n = 3 for the 20 μg CT (G) only & 10 mg PE (F) only groups; n = 4 in all other groups. Each point represents the group mean ± standard error.

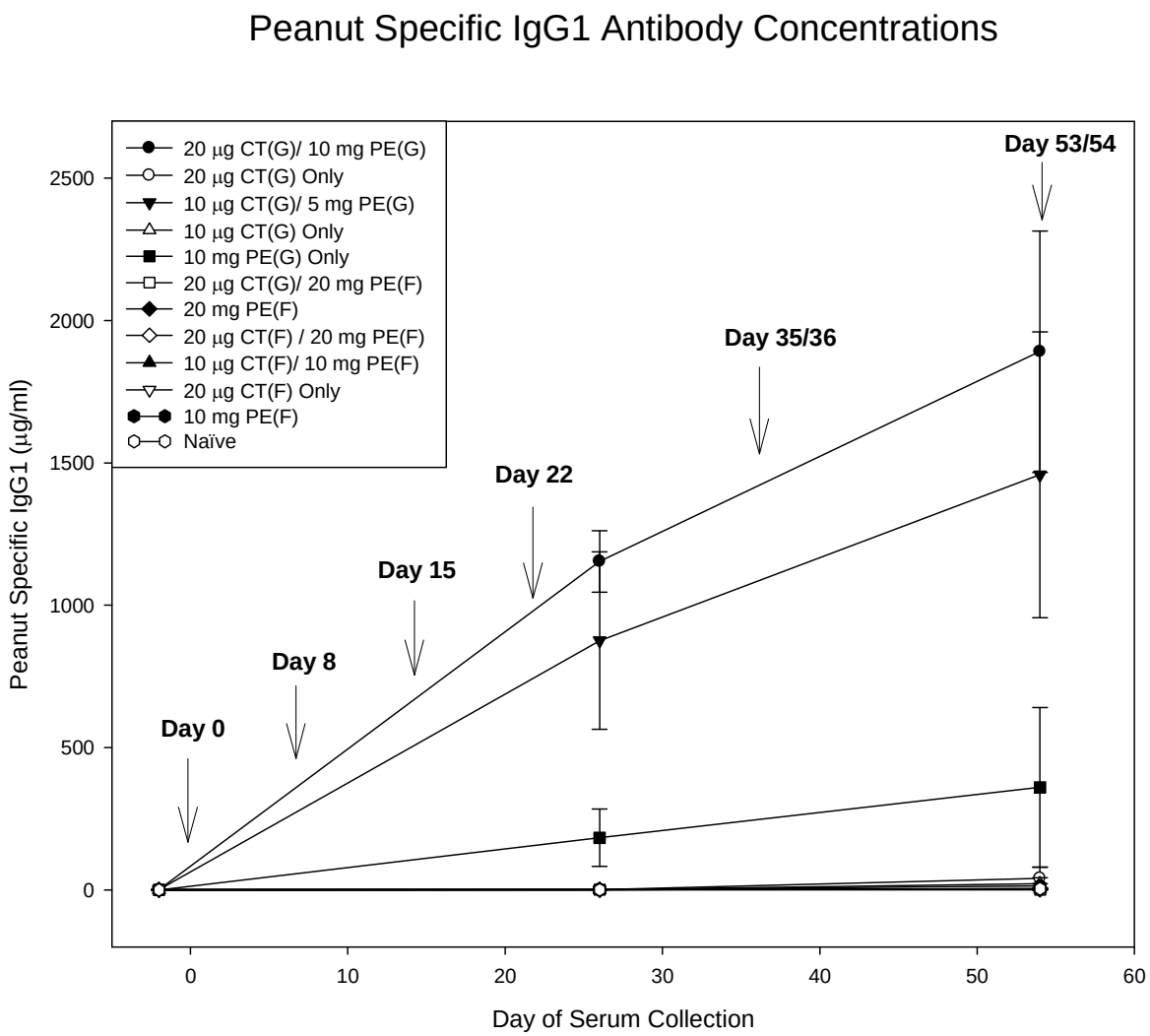

Figure 21: Peanut specific serum IgG1 concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (0, 8, 15, & 22) in which the mice received their individual treatment gavages/pellets or challenge days (35 & 36 or 53 & 54) in which mice received 10 mg peanut extract gavages. N = 2 for the 20 μg CT (G)/20 mg PE (F) group; n = 3 for the 20 μg CT (G) only & 10 mg PE (F) only groups; n = 4 in all other groups. Each point represents the group mean ± standard error.

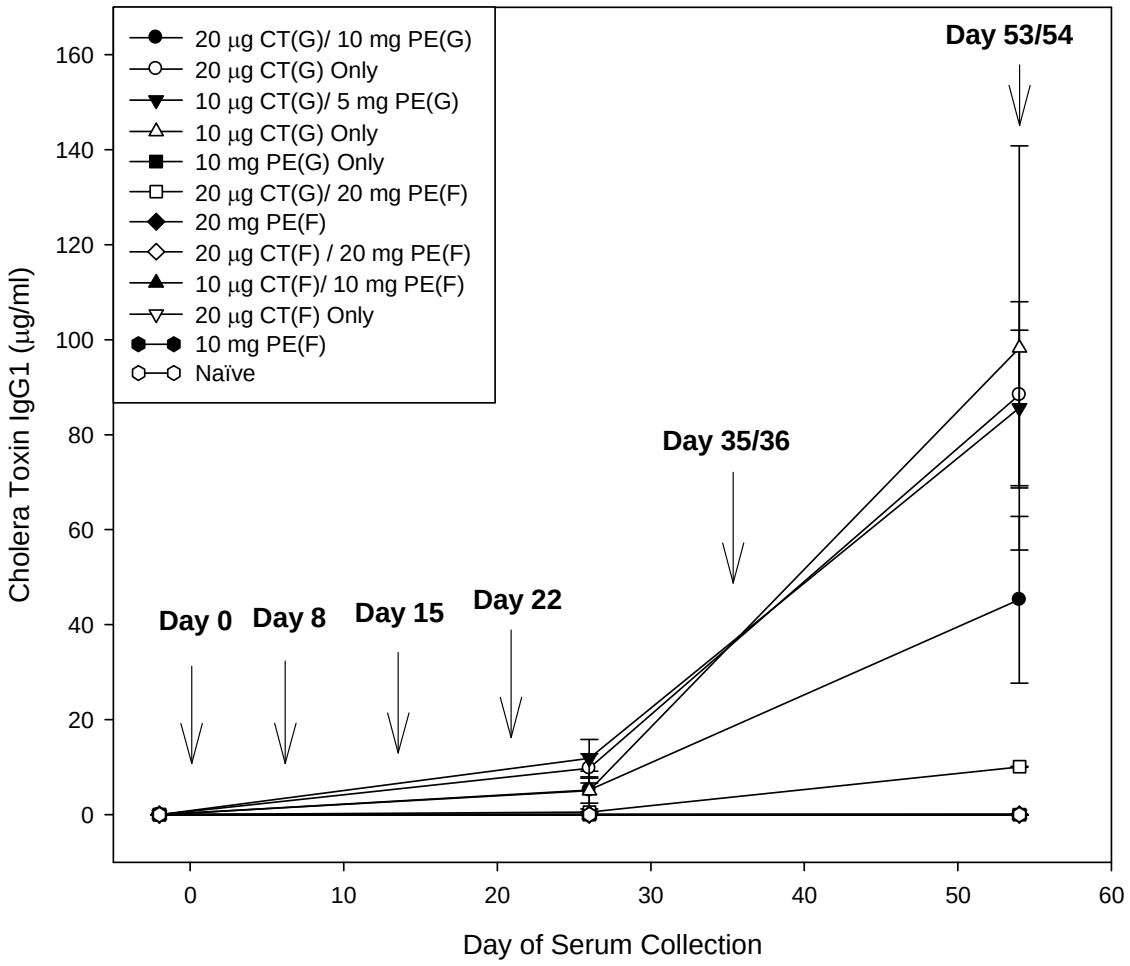

Figure 22: Cholera toxin specific serum IgG1 concentrations collected throughout both the sensitization and challenge periods. Arrows indicate sensitization days (0, 8, 15, & 22) in which the mice received their individual treatment gavages/pellets or challenge days (35 & 36 or 53 & 54) in which mice received 10 mg peanut extract gavages. N = 2 for the 20 μg CT (G)/20 mg PE (F) group; n = 3 for the 20 μg CT (G) only & 10 mg PE (F) only groups; n = 4 in all other groups. Each point represents the group mean ± standard error.

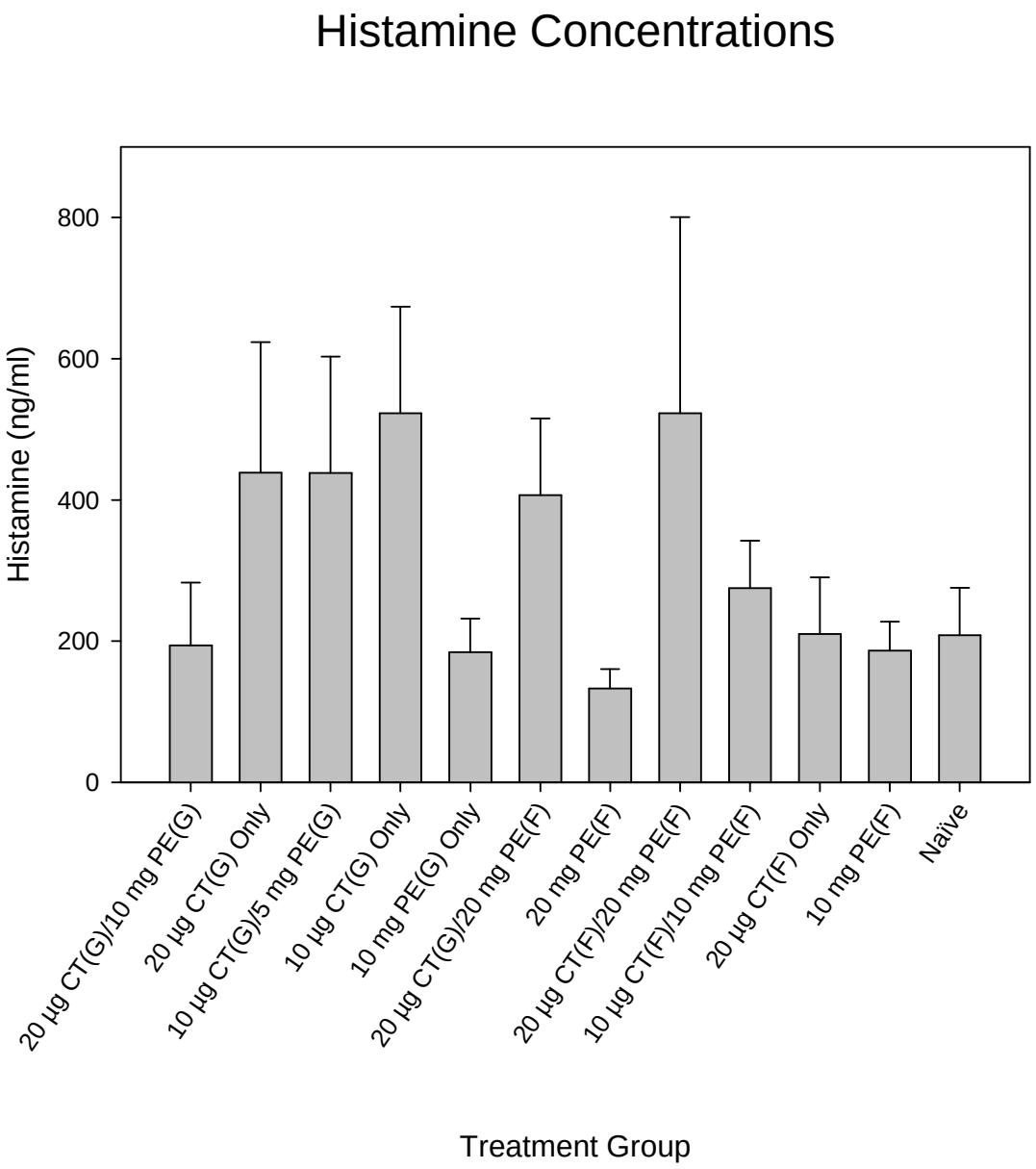

Figure 23: Serum histamine concentrations collected on the last day of the experiment, Day 53/54, when mice received 10 mg peanut extract by gavage. N = 2 for the 20 µg CT (G)/20 mg PE (F) group; n = 3 for the 20 µg CT (G) only & 10 mg PE (F) only groups; n = 4 in all other groups. Each bar represents the group mean ± standard error.

Acknowledgements

I would like to take a moment to acknowledge the help and support I have received during my time in graduate school. I would like to thank Dr. Joan Cunnick, my major professor, who provided the bulk of the information, guidance, and support during this time, as well as the other members of POS committee, Dr. Nancy Cornick and Dr. Kan Wang, for their guidance and recommendations.

I would also like to thank members of the Cunnick lab who provided support and assistance, including April Beyer, Raye Taylor-Vokes, and Zili Zhai. I would like to thank Kay Christiansen from the Iowa State University microbiology program and Lorena Moeller from the lab of Dr. Kan Wang, who provided a great deal of technical support during this time.

I would like to thank the laboratories of Dr. John Clements, which provided the generous gift of purified bacterial produced LT-B.

In addition, I would like thank my many friends and family members, who provided a great deal of love and support during a very stressful time.

This project was funded by BioPharmaceutical & BioIndustrial Initiative of Plant Science Institute at Iowa State University.