

Analysis of Immune Response in Young and Aged Mice Vaccinated With Corn-Derived Antigen Against *Escherichia coli* Heat-Labile Enterotoxin

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Abstract

Enterotoxigenic strains of *Escherichia coli* produce a heat-labile holotoxin (LT), which causes diarrhea. We engineered corn seeds to produce LT-B, the nontoxic subunit of LT, to serve as a plant-derived vaccine to traveler's diarrhea and as an adjuvant for co-administered proteins. We previously demonstrated that a strong mucosal and systemic antibody response is elicited in young mice with oral administration of corn-derived LT-B. The present study examined systemic and mucosal antibody responses to LT-B in young and aged mice, and recall responses to oral administration and injection of LT-B in aged mice. Specific IgA and IgG antibodies were detectable during an 11-mo period, although the concentration of antigen-specific antibodies declined gradually. Booster by feeding or injection dramatically increased the concentration of specific IgA from that seen in young mice. Specific IgG levels were boosted to concentrations similar to those in young mice. This effect may be age-dependent and related to prior immunization exposure. Analysis of the antibody response of naïve aged mice against corn-derived LT-B demonstrated an age-related suppression in specific IgG production, but not specific IgA. These results may provide important information for edible vaccine strategies for young and aged individuals.

Index Entries: Plant-derived vaccines; immune memory; LT-B.

1. Introduction

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

IgA, both at the inductive site where the pathogen is encountered first and in the effector sites throughout the CMIS (1).

The ability of the mucosal system to produce antigen-specific responses is the driving force for attempts to develop edible vaccines by which antigenic proteins are synthesized in edible parts of plants and administered orally to animals. Many studies show that recombinant proteins produced in plants are immunogenic when given orally and also provide protection in animal models of infectious disease (2). Phase I human clinical trials using three different recombinant antigens produced in plants show similar results as evidenced by the induction of a systemic and secretory antibody response (3–5).

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Although oral vaccines may stimulate a mucosal response, the extremely low pH and presence of gastric peptidases in the stomach present a challenge for the use of small proteins as oral vaccines when administered alone. Co-administration of adjuvants can increase immunogenicity of mucosally administered antigens (6–8). Two such mucosal adjuvants, heat-labile enterotoxin (LT) from enterotoxinogenic strains of *Escherichia coli* and its homolog cholera toxin (CT) from *Vibrio cholera*, are strongly immunogenic when administered through the intestinal tract, respiratory tract, or parenteral route (9–11). Both toxins cause diarrhea by a mechanism involving binding of the B subunit of the toxin to the G_{M1} receptors on intestinal epithelial cells. Binding of the B subunit enables entry of the enzymatically active A subunit to the cytosol. This results in secretion of water and chloride ions from epithelial cells to the intestinal lumen as a result of overactivation of adenyl cyclase (12). Because neither of the holotoxins is suitable for use as a mucosal adjuvant in humans, much effort has focused on the utilization of the nontoxic B subunit (LT-B and CT-B) and attempts to dissociate toxicity from adjuvanticity (13–15). Besides potential use as mucosal adjuvants, the two B subunits can also serve as vaccines against diarrhea caused by LT and CT as the B subunit induces high levels of anti-LT-B and anti-CT-B secretory IgA that provide protection against subsequent toxin challenge (12,15–18). In addition, our previous studies indicate that oral administration of LT-B corn can induce significant concentrations of anti-LT-B IgG in serum (17).

Immunogenicity and adjuvanticity of LT-B and CT-B have been studied extensively in young mice. However, immune responses in aged individuals can be different from cellular and molecular alterations associated with aging. Examples of age-related changes include dysfunctional alterations in mitogenic responses, phenotype, and cytokine expression of T cells. Changes in T-cell responses can result in an altered mode of help for B cells, ultimately leading to impaired antibody responses (6). For example, vaccines against

influenza and tuberculosis are less efficient in aged individuals when compared to young adults (19,20). Aged mice that were given CT and CT-B as an adjuvant for the co-administered antigen ovalbumin demonstrate a diminished capacity to produce antigen-specific antibodies mucosally (IgA) and systemically (IgG) when compared to young mice (6,21). However, immunogenicity and adjuvanticity of LT and its components in aged mice remains to be elucidated. Although LT-B and CT-B share 80% homology in terms of amino acid sequence, they have distinct biochemical and immunological features. For example, LT-B is more potent than CT-B in terms of induction of an antibody response. This could be to the result of the greater stability of LT-B at low pH and high temperature (22). Therefore, administration of LT-B to aged mice could result in a different outcome than that of CT-B and CT.

Earlier studies of recombinant LT-B and CT-B produced from transgenic plants report antibody responses with efficacy in protecting animals from toxin challenge. However, these studies measured antibody responses over a short time interval in young mice (17,23–27). In the present work, we aimed at investigating immune responses to corn-derived LT-B in young and aged mice. Our objectives were to carry out a kinetic assay to determine the time interval in which anti-LT-B antibody levels remain significantly elevated after feeding in young mice and to investigate the effect of booster administration of LT-B using both oral and intraperitoneal injection methods in aged mice. In addition, we measured the systemic and mucosal immune response against LT-B in elderly naïve mice when fed with corn-derived LT-B antigen.

2. Materials and Methods

2.1. Preparation of Feeding Pellets

Transgenic maize seeds (R_4 generation) expressing LT-B were used for making food pellets as described previously (17). Each pellet made of ground transgenic maize seeds and phosphate-buffered saline (PBS) weighed 1.87 g and contained 10 μ g of LT-B. Pellets were allowed to

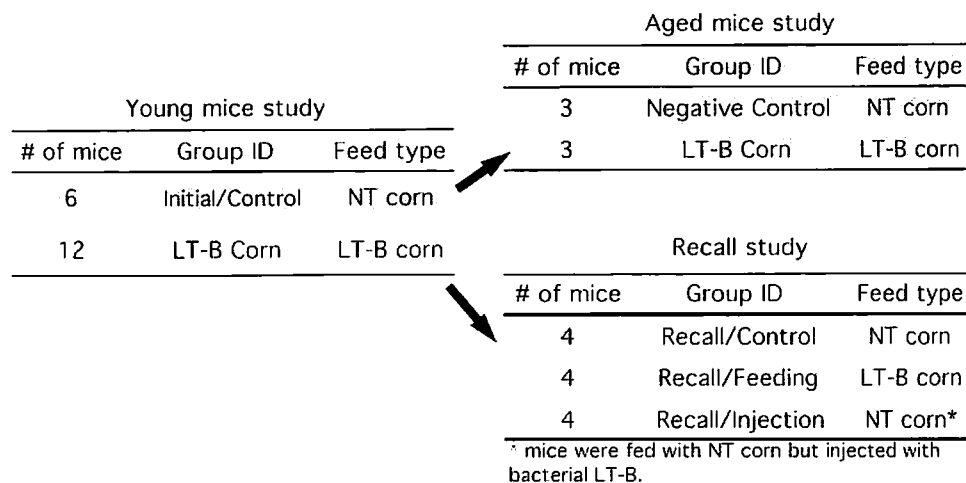


Fig. 1. Study design for mouse immunizations.

air-dry overnight. Nontransgenic (NT) maize seed powder was used to make pellets of similar weight to serve as a negative control. For each feeding, one extra pellet was prepared to serve as an LT-B dose control to be analyzed at the end of the experiment.

2.2. Immunization of Mice: Young Mice Study, Aged Mice Study, Recall Study

All animal procedures were approved through the Iowa State University (ISU) Committee on Animal Care. Ten-week-old female BALB/c mice were obtained from Harlan (Indianapolis, IN). Before the start of the experiment, mice were allowed a 2-wk adjustment period with a reverse light–dark cycle (lights off at 9 AM) in the ISU animal facility where they were housed throughout the experiment.

The design of the overall study is given in Fig. 1. Mice were fed a basic diet of mouse chow with water *ad libitum*. The mouse chow was removed overnight (during the light phase) before feeding corn pellets on d 0, 7, 21, and 35 of the study. Initial LT-B immunization was done when the mice were 3 mo old (young mice study). They were divided into two groups: the initial control group mice were fed with NT corn pellets (NT Corn) and the LT-B corn group mice were fed with LT-B corn pellets. Throughout the study,

two mice were housed in each cage. However, during feeding of control or vaccine pellet they were caged individually and one maize pellet was placed in each cage (without bedding) 30 min before lights off. Mice were allowed to eat the maize pellet in the dark cycle for 3 h until the pellet was completely consumed and then returned to their home cage with their normal mouse chow.

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

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

2.3. Preparation of Plasma and Fecal Samples and Determination of Anti-CT-B and Anti-LT-B Antibodies

For the initial LT-B immunization study, fecal and blood samples (approx 100 μ L) were obtained from mice during an 11-mo period (d -1, 6, 13, 20, 27, 34, 41, 51, 63, 94, 270, and 320). In the recall study, samples were collected before feeding (d 336) and on d 343 and 350 (6 and 13 d after feeding) of the study. For analysis of LT-B immunization in naïve 14-mo-old mice, fecal and blood samples were collected weekly from d -1 to d 41. Mice were bled through the sephalous vein. Plasma and fecal samples were stored at -20°C until analysis by enzyme-linked immunosorbent assay (ELISA) as described before (17). Briefly, plates were coated with 5 μ g of mixed gangliosides (G2375; Sigma, St. Louis, MO) per well diluted in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3 mM NaN₃ at pH 9.6) for 1 h at room temperature. After blocking with 5% powdered milk (diluted in PBS), plates were incubated with pure LT-B (John Clements) or CT-B (1 μ g/well; Sigma) at 37°C for 1 h. Next, diluted serum samples and fecal extracts were added and the plates were incubated at 37°C for 1 h. Plates were incubated with biotinylated antimouse IgG obtained from rabbit sera (B-8520; Sigma; diluted 1:100,000 in PBS containing 1% powdered milk) and anti-IgA from goat sera (B-2766; Sigma; diluted 1:20,000 in PBS containing 1% powdered milk) for 1 h at 37°C to determine IgG and IgA levels, respectively. Next, the enzyme conjugate, streptavidin-horse radish peroxidase (554066; BD Biosciences, San Diego, CA), was added to the wells (diluted 1:1000 in PBS containing 1% powdered milk) and the plates were incubated at room temperature for 30 min. Finally, wells were coated with ABTS substrate (3-ethylbenz-thiazoline-6-sulfonic acid; A-1888; Sigma) in substrate buffer (1.37 M ABTS, 0.1 M citric acid at pH 4.35) and incubated at room temperature for 30 min allowing color development. At the end of the incubation period end point readings were performed using absorbance at 405 nm. Antibody concentrations were determined as micrograms per millili-

Table 1
Specific Antibody Ratios^a

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

^aSera from four mice from LT-B Corn group analyzed for anti-LT-B and anti-CT-B antibodies on the indicated days.

ter by using standard curves derived from pure mouse IgG (MOPC 21, M-9269; Sigma) and pure mouse IgA (MOPC315, M-2046; Sigma).

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

2.4. Determination of Total IgG₁ and IgG_{2a} Levels

For determination of total IgG₁ and IgG_{2a}, sera from d -1, 6, 13, 20, and 27 from the initial immunization part of the study and sera from d 6, 13, 20, 27, and 34 from the aged mice study were analyzed. A total of 11 serum samples from mice fed with NT corn and 15 serum samples from mice fed with LT-B corn in the initial immunization part of the study were analyzed along with serum

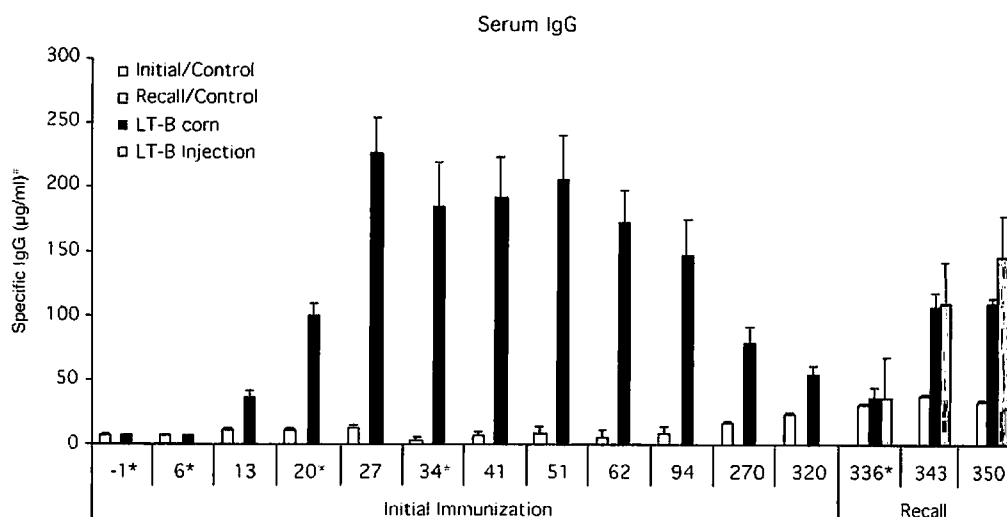


Fig. 2. Antigen-specific serum IgG concentrations in mice orally immunized with corn-derived LT-B and recall to reimmunization through oral and intraperitoneal administration of the toxin. The x-axis indicates days of initial immunization and recall study. Initial immunization regime involved feeding on d 0, 7, 21, and 35. A booster dose was administered once (d 337) and antibody response was monitored for 2 wk. *Indicates the blood collection day immediately before a feeding or injection. [#]Indicates specific IgG measured with bacterial CT-B. Antibody concentrations are presented as mean \pm SE.

samples from the aged mice study (three mice in each group; NT corn and LT-B corn). Purified IgG₁ (MI10-102; Bethyl Laboratories, Inc.) and IgG_{2a} (0103-01; Southern Biotechnology Associates, Inc.) were used as standards. The same standard range was used for both total IgG₁ and IgG_{2a} assays (2.5–0.019 μ g/mL). Standards and samples (diluted at 1:150) in sodium carbonate coating buffer (described in **Subheading 2.3.**) were directly coated on the 96-well high-binding ELISA plates and were blocked with 150 μ L of 5% milk. Total IgG₁ and IgG_{2a} samples were probed with goat antimouse IgG₁ (M-8770; Sigma; dilution 1/3000) and biotin-conjugated goat antimouse IgG_{2a} (1080-08; Southern Biotechnology Associates, Inc.; dilution 1/10,000), respectively. For the IgG₁ assay a secondary antibody conjugated to biotin (A-5420; Sigma; dilution 1/40,000) was used. Incubations with enzyme and substrate and end point readings were carried out as described previously (*see Subheading 2.3.*).

A ratio of IgG_{2a}/IgG₁ was used to estimate changes in Th1/Th2 responses. Although, the IgG_{2a}/IgG₁ ratio is only indicative of the T-helper balance, the isotypes have been correlated with the production of Th1 and Th2 cytokines, respectively (28). Earlier experiments attempting to stimulate T-cell cytokine production in splenocytes from LT-B immunized mice using purified recombinant bacterial LT-B were inconclusive because of the abundance of endotoxin associated with the LT-B. Thus, we are unable to measure LT-B-induced stimulation of antigen-specific T cells and their cytokines at this time, which would provide a direct measure of Th1 vs Th2 responses.

2.5. Statistical Analysis

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

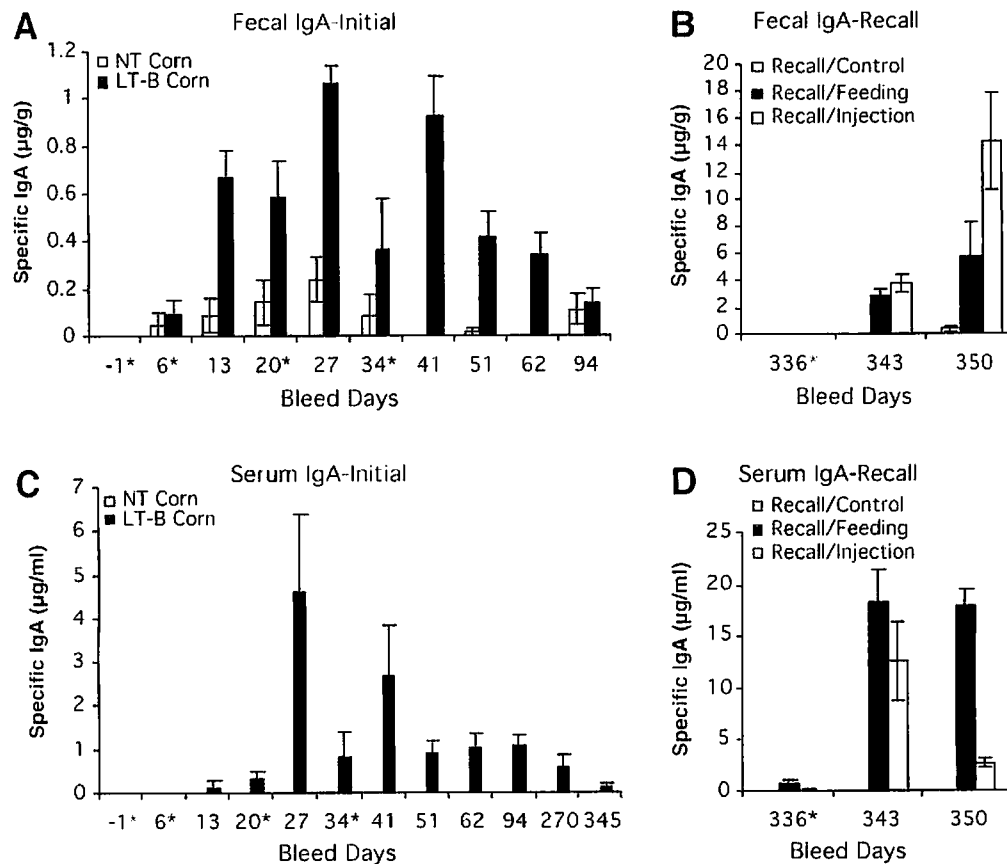


Fig. 3. LT-B-specific fecal IgA (A and B) and serum IgA (C and D) concentrations for initial immunization in young mice (A and C) and recall in aged mice to reimmunization (B and D). In initial immunization study, mice were fed with meal pellets from nontransgenic corn (NT Corn) and transgenic corn expressing LT-B (LT-B Corn) on d 0, 7, 21, and 35 when mice were 3 mo old. Mice were given a booster dose of the toxin through oral route and through injection when they were 14 mo old (d 337). *Indicates sample collection day immediately before a feeding and injection. Specific fecal IgA was measured with bacterial LT-B (A and B), specific serum IgA was measured with bacterial CT-B (C and D). Antibody concentrations are presented as mean \pm SE.

3. Results

3.1. Antibody Response in Mice With Initial Immunization and a Booster LT-B Administration

Twelve young mice (3 mo old) were initially fed four times with LT-B expressing transgenic corn pellets (LT-B Corn group) and six mice were initially fed four times with NT corn pellets (Initial/Control group). When they reached 14 mo of age, 12 mice from the LT-B Corn group were divided equally into three groups to test a booster response. The three groups included: (1) Recall/

Control group (fed once with NT corn pellets); (2) LT-B Corn group (fed with transgenic corn pellets); and (3) LT-B Injection group (fed with NT corn pellets but injected with purified bacterial LT-B IP). Figure 2 shows antigen-specific IgG concentrations during the 11-mo period after the initial immunization regime on d 0, 7, 21, and 35 and the secondary response after a single recall dose of LT-B administered when the mice were 14 mo old (d 337). On d 13, specific IgG antibody concentrations were detected at a level significantly higher than that of the control group ($p = 0.0034$) and remained significantly higher through-

out the remaining assay dates ($p < 0.005$). Specific IgG concentrations steadily increased and reached a peak at d 27 with a mean concentration of 226.21 ± 26.71 mg/mL. Specific IgG concentrations decreased gradually after the last immunization (d 35), but did not significantly differ from d 27 until d 270 ($p = 0.0037$).

In the recall experiment, 14-mo-old mice were given one single dose of LT-B orally (LT-B Corn group) or IP (LT-B Injection group) on d 336. We observed remarkably high levels of specific IgG in sera of both groups as early as 7 d (d 343) after the booster antigen administration, whereas the recall control group showed no change in levels of specific IgG. The recall responses on d 343, 1 wk after a single dose of LT-B by feeding and injection, were not significantly different from the specific IgG level on d 41. These two dates (d 41 and 343) both represent secondary responses 1 wk after immunization and were significantly higher than specific antibody concentrations observed on d 6 and 13 in young mice ($p < 0.0001$). Two weeks after the d 336 booster dose, specific IgG concentrations increased with a higher level of antibody in sera of the group that received LT-B by injection. The specific IgG levels in the LT-B Injection group on d 350 were significantly higher than that of the LT-B Corn fed group ($p < 0.0014$).

Figure 3 shows concentrations of anti-LT-B specific IgA measured in fecal material (**Fig. 3A,B**) and serum (**Fig. 3C,D**) of mice fed or injected with LT-B. As shown in **Fig. 3A**, concentration of LT-B-specific secretory IgA in feces increased gradually after the first immunization on d 0. The highest concentration was recorded on d 27 (1.07 ± 0.07 $\mu\text{g/g}$) and 41 (0.93 ± 0.16 $\mu\text{g/g}$) 1 wk after feedings. We observed a lower concentration of specific fecal IgA on d 34 and 41, approx 2 wk after feeding. However, antigen-specific fecal IgA remained significantly higher than control through d 62, 1 mo after feeding.

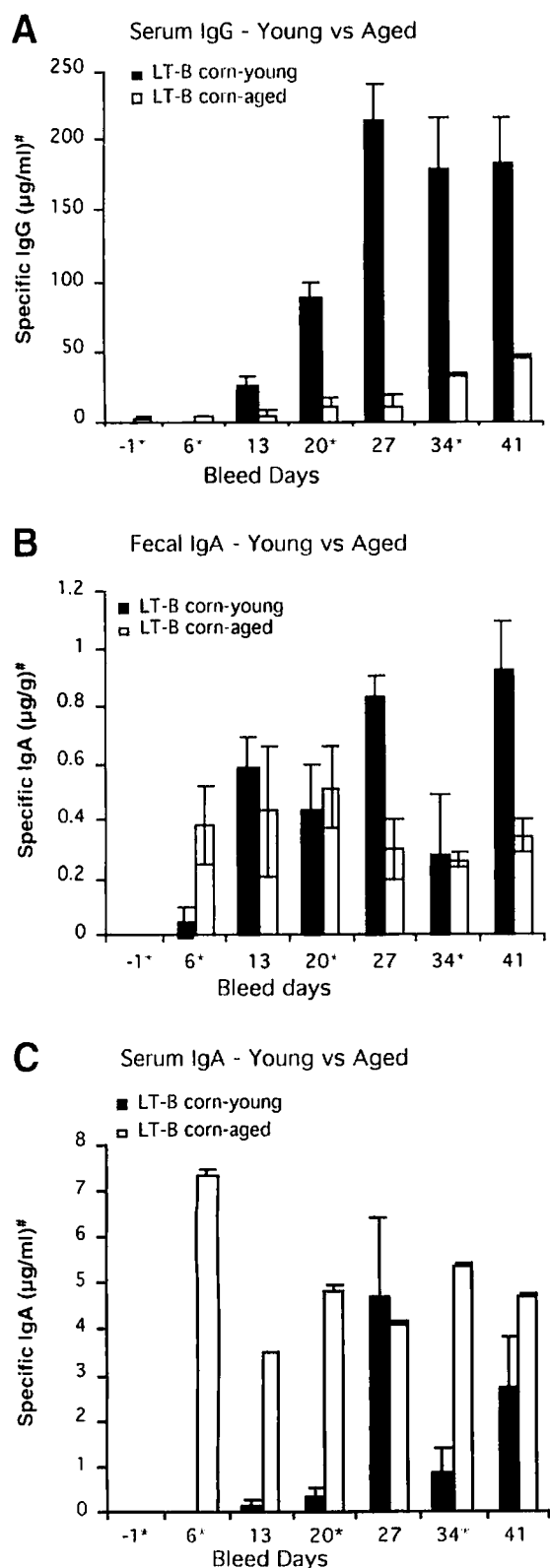
Figure 3B shows the fecal anti-LT-B IgA response after booster antigen administration when the mice were 14 mo old. Fecal anti-LT-B secretory IgA was not detectable before the administration of the booster dose. As in the case of antigen-specific serum IgG response to recall, a significant

increase is observed as early as 7 d after the administration of the antigen through either route (oral or injection). It is noteworthy that in 14-mo old mice the specific fecal IgA response to the booster on d 343, 6 d after recall immunization, was more robust than that of specific IgG. The levels of specific IgA were 2.66 and 3.53 times higher (2.84 ± 0.41 and 3.77 ± 0.68 $\mu\text{g/g}$ for recall feeding and recall injection groups, respectively) than the peak level (1.07 ± 0.07 $\mu\text{g/g}$) of specific IgA during the initial immunization and represent a significant difference in response ($p < 0.0001$). Specific IgG concentrations of the recall response did not exceed responses seen in animals during the initial immunization period. In the recall experiment, the specific fecal IgA concentrations continued to increase for both groups through d 350 (2 wk after recall exposure), but the LT-B injection group had significantly higher specific fecal IgA compared to the LT-B corn-fed group ($p < 0.0001$).

During the initial immunization phase, antigen-specific serum IgA was first detected on d 13 and reached a peak on d 27 (**Fig. 3C**). As with fecal IgA, we observed a significant decrease on d 34 compared to d 27 ($p < 0.0001$) 2 wk after a feeding and a large increase on d 41 compared to d 34 ($p < 0.0001$) 1 wk after a feeding. Specific serum IgA antibodies declined gradually, but were detectable during an 11-mo period. In the recall experiment, a robust-specific serum IgA response was observed with booster dose administration in 14-mo-old mice. The aged mice had a more rapid and stronger specific serum IgA response as compared to young mice (**Fig. 3D**). In addition, the concentration of specific serum IgA in the LT-B Corn-fed group remained constant from d 343 to d 350, whereas the level of the LT-B Injection group decreased significantly ($p < 0.0001$) compared to d 343. This differs from the result seen with the antigen-specific fecal IgA response in the recall mice (cf. **Fig. 3B and 3D**).

3.2. Antibody Response in Naïve Aged Mice With Oral LT-B Administration

Two groups ($n = 3/\text{group}$) of aged mice (14 mo old), which were never exposed to LT-B when younger, were used in a separate feeding study to



elucidate the immune response to corn-derived LT-B in aged mice. One of the groups was fed with LT-B corn pellets, each containing approx 10 µg of LT-B on d 0, 7, 21, and 35, whereas the other group was given NT corn pellets on the same days. **Figure 4** displays the results of feeding young and aged mice with LT-B-containing corn. We observed a significant effect of age on the antigen-specific IgG concentrations ($p < 0.0001$). As indicated in **Fig. 4A**, specific serum IgG antibodies were first observed on d 13; however, the concentration of specific IgG antibodies in aged mice were significantly lower than in sera of young mice on d 13 ($p < 0.001$) and remained significantly lower throughout d 20–41 ($p < 0.05$).

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

Figure 4C shows the antigen-specific serum IgA concentrations in young vs aged mice during an initial immunization regimen. Serum IgA was measurable in aged mice as early as d 6 after the first feeding and remained relatively constant during 42 d. Peak antigen-specific IgA levels in sera

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

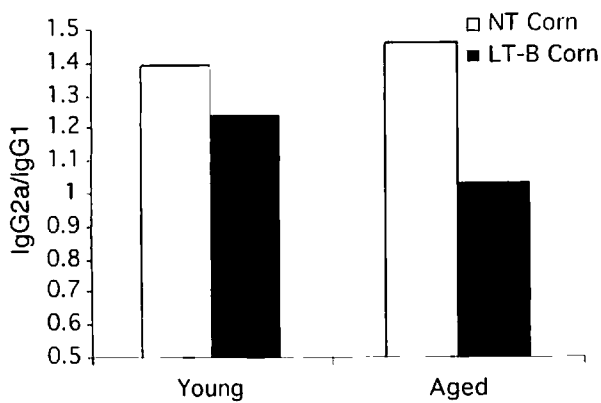


Fig. 5. Comparison of IgG_{2a}/IgG₁ ratios in young and aged mice.

of aged mice ($4.1 \pm 0.02 \mu\text{g/mL}$) were comparable to the peak anti-LT-B concentration recorded on d 27 in young mice ($4.63 \pm 1.76 \mu\text{g/mL}$). However, we observed a significant overall age effect for serum IgA levels in young vs aged mice ($p < 0.001$) as anti-LT-B IgA antibody concentrations in sera of aged mice were significantly higher than that measured in sera of young mice ($p < 0.05$) on d 6, 13, 20, and 34.

Both in fecal extracts and in sera collected from the mice in this part of the study, anti-LT-B IgA antibodies were observed as early as 1 wk after the initial LT-B corn administration. This rapid specific IgA response led us to preliminarily investigate changes in Th1 and Th2 responses in these mice as a possible explanation for these results. Using the serum samples collected in this experiment we measured total IgG_{2a} (a Th1 antibody) and IgG₁ (a Th2 antibody) concentrations. We were not able to measure antigen-specific forms of the antibody, because of the lack of sufficient serum. Figure 5 demonstrates the ratio of total IgG_{2a} and IgG₁ in young and old mice. The IgG_{2a}/IgG₁ ratio was significantly lower in sera of LT-B fed mice ($p < 0.01$). The main effect of LT-B feeding was because of the large decrease in IgG_{2a}/IgG₁ ratio seen in the aged mice ($p < 0.05$) after immunization. A comparison of young mice fed NT corn and LT-B corn did not demonstrate a significant shift in total IgG_{2a}/IgG₁ ratio.

4. Discussion

In this study, we monitored antigen-specific IgG and IgA concentrations in sera and fecal samples of mice immunized with corn-derived LT-B during 11 mo. The peak antibody production was reached after a minimum of three feedings during 4 wk in young mice. This is similar to data published previously by us and other investigators using either recombinant bacterial LT-B (29) or plant-derived LT-B (2). We previously demonstrated that the specific antibodies can provide protection from challenge with whole *E. coli* heat labile toxin (17). The present study expanded our previous research to show specific antibody remained elevated over background for an 11-mo period after the initial immunization paradigm.

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

Other researchers have reported a shift in the type of immune response in aged animals (30–32). They have noted that aged animals respond with more of a Th2-type immune response and have a decreased Th1 response in comparison to young mice. This shift may help explain our robust IgA (Th2 type) response in aged mice. The Th1 cells support production of serum IgG_{2a}, whereas Th2 cells induce production of IgG₁ serum antibodies (33). We measured total IgG_{2a} vs IgG₁ in the serum samples from immunized young and aged mice as an indicator of the Th1 to Th2 bal-

ance. Immunization at the mucosal surface favors a Th2-type of response (34). Thus, it is not surprising that our analysis indicated that the IgG_{2a}/IgG₁ ratio of the LT-B corn treatment group is significantly lower than that of the NT control group. The suppression of the IgG_{2a}/IgG₁ ratio was significant in the immunized aged mice, but not in the immunized young mice. This result is indicative of a shift toward a Th2-type response in the aged mice that could account for the rapid production and high levels of antigen-specific IgA antibodies.

In addition to a shift from Th1 to Th2 responsiveness, there have been reports of a general decline in immune responses to antigen in the elderly (35,36). In this study we also compared the initial immune response against corn-derived LT-B in young and aged naïve mice. Aged mice immunized with LT-B for the first time demonstrated a delayed and lower peak anti-LT-B IgG production through four feedings and 7 wk of monitoring as compared to young mice. In contrast to the delayed IgG response, the aged mice demonstrated maximal specific IgA production by d 6 after the first feeding. This is much sooner than the IgA response detected in young mice fed LT-B. One other difference between the initial antigen-specific IgA response of young and aged mice was that the response of young mice appears to increase 1 wk after each feeding and decrease at 2 wk after each feeding. This corresponds well with the half-life of IgA (6 d in serum). In aged mice there was no fluctuation of antigen-specific IgA concentrations seen in serum or in fecal samples in relation to feeding. These differences may be due in part to age-related changes in immune regulation.

Although the concentrations of specific IgA in serum of young and elderly mice were comparable, the fecal IgA levels in aged mice did not reach the same concentration observed in young mice on d 41. This observation is not surprising as other researchers have noted increased serum IgA in elderly subjects (37,38). Schmuker and Owen (35) suggest that the detection of higher serum immunoglobulin levels in the elderly could

be caused by altered epithelial cell transport to mucosal surfaces.

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

Acknowledgments

We are thankful to John Clements of Tulane University for providing bacterial LT-B, to Jennifer McMurray for her technical assistance, and to Nancy Boury for the critical reading of the manuscript. This work was partially supported by the Plant Science Institute of Iowa State University and Iowa State University Research Foundation to K. W. and the Scientific and Technical Research Council of Turkey to S. K.

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