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GENETIC TRANSFORMATION AND HYBRIDIZATION

Ingestion of transgenic carrots expressing the *Escherichia coli* heat-labile enterotoxin B subunit protects mice against cholera toxin challenge

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Abstract Diarrheal diseases caused by *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) are worldwide health problems that might be prevented with vaccines based on edible plants expressing the B subunit from either the cholera toxin (CTB) or the *E. coli* heat labile toxin (LTB). In this work we analyzed the immunity induced in Balb/c mice by ingestion of three weekly doses of 10 μg of LTB derived from transgenic carrot material. Although the anti-LTB serum immunoglobulin G (IgG) and intestinal IgA antibody responses were higher with 10 μg-doses of pure bacterial recombinant LTB (rLTB), the transgenic carrot material also elicited significant serum and intestinal antibody responses. Serum anti-LTB IgG1 antibodies predominated over IgG2a antibodies, suggesting that mainly Th2 responses were induced. A decrease of intestinal fluid accumulation after cholera toxin challenge was observed in mice immunized with either rLTB or LTB-containing carrot material. These results demonstrate that ingestion of carrot-derived LTB induces antitoxin systemic and intestinal immunity in mice and suggest that transgenic carrots expressing LTB may be used as an effective edible vaccine against cholera and ETEC diarrhea in humans.

Keywords Cholera · ETEC · Edible vaccine · Protective immunity

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Introduction

Since the pioneering works of Curtis and Cardineau (1990) and Mason et al. (1998), edible vaccines based on genetically modified plants could be in a near future an alternative for vaccination on a global scale. This approach is based on the transformation of plants with relevant antigen-encoding genes inserted into the nuclear or chloroplast genome. Fruits or tissues from these plants can be used for oral immunization as inexpensive, safe and accessible vaccines (Daniell et al. 2001; Mason et al. 2002).

The diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) has been an attractive target disease for developing plant-based vaccine. ETEC is a common cause of infectious diarrhea in tropical climates where uncontaminated drinking water is not readily available. Most illness, in terms both of number of cases and severity of symptoms, occurs in infants. Another significant population at risk is travelers who lack recent exposure to ETEC (Black 1993). The *E. coli* heat-labile enterotoxin (LT) is the major ETEC pathogenic factor. LT subunit B (LTB) is of special interest because its immunogenicity, adjuvant activity and lack of toxicity make it a viable antigen to vaccinate against ETEC diarrhea (Belisle et al. 1984; Spangler 1992).

ETEC diarrhea has much in common with cholera. Both result from the ingestion of rather large inocula of gram negative bacteria that colonize the small intestine and produce toxins causing net secretion of fluid into the

intestinal lumen (Wolf 1997). LT structure and function are similar to those of cholera toxin (CT): both contain A and B subunits whose amino acid sequences show 80% identity and have the same mode of action (Sixma et al. 1991). Due to this homology, immunization with LTB raises an anti-toxin response that extends to the CTB subunit, thereby providing equally strong protection against oral LT or CT challenge (Klipstein et al. 1984).

In order to express recombinant LTB in plants several researchers have used the native bacterial (Haq et al. 1995; Mason et al. 1998) and optimized synthetic genes (Streatfield et al. 2001; Chikwamba et al. 2002a, b). Functional assays have shown that the recombinant LTB protein is able to form pentamers that bind its natural receptor and induce mucosal and systemic immune responses when administered by the oral route in mice. In addition, immunization with recombinant potato and maize has been shown to protect against oral LT and CT challenge (Mason et al. 2002).

Plants such as carrots, whose raw tissues are normally eaten by humans, are more attractive to develop plant-based vaccines than those that have to be cooked because the protein denaturation caused by cooking may affect the immunogenicity level. Our group has been able to express LTB encoded by an optimized synthetic gene driven by the CaMV 35S promoter in tap roots of adult carrot plants (Rosales-Mendoza et al. 2007). In this work we aimed to elicit an immune response via the mucosal system and it was shown that ingestion of transformed carrot material induces LTB-specific intestinal and systemic antibodies in mice and protects them against oral CT challenge.

Materials and methods

Bacterial strain, plasmid, plant material and selection of transgenic lines

The transgenic carrot was obtained as reported by Rosales-Mendoza et al. (2007). Briefly, *Agrobacterium tumefaciens* LBA4404 cultured on YM medium was used. Codon usage of the synthetic LTB gene was modified as per the codon bias for carrot genes maintaining the original amino acid sequence of the wild-type *Escherichia coli* gene. The LTB synthetic gene was subcloned in the pBI121 binary vector by replacing the *uid*A gene for transformation of carrot stem segments. Carrot seeds (*Daucus carota* L. var. Nantes) were obtained from Asgrow (USA) and germinated in MS medium (Murashige and Skoog 1962). Kanamycin resistant clones were regenerated by somatic embryogenesis removing plant growth regulators gradually over a period of 4 weeks. Then the plantlets were transferred to soil and grown in a greenhouse. PCR, Southern and RT-PCR analyses were performed in order to select the best lines for further in vivo trials. The transgenic line 1 exhibited the highest LTB protein content among the analysed samples and this was selected for mice immunization.

LTB content in carrots

Tap roots from adult plants of the transgenic carrot Line 1 expressing LTB were used. LTB quantitation in carrot material was performed with a GM1-ELISA. Briefly, 200 mg of plant material was ground in liquid nitrogen and resuspended in 1 ml of extraction buffer (100 mM NaCl, 25 mM sodium phosphate pH 6.6, 0.5% v/v Triton X-100, 10 μg/ml leupeptin); samples were then centrifuged at 12,000×*g* for 15 min at 4°C and the supernatants were assayed with the GM1-ELISA test of Chikwamba et al. (2002a). Mature transgenic and wild-type tap roots were freeze-dried and ground for use in the immunization trials.

Immunization

All animals were handled in accordance with Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico) and approved by the Institutional Animal Care and Use Committee. In all experiments 12–14-week-old Balb/c mice were used obtained from Harlan Sprague Dawley, Inc. USA. The average weight of the mice was 22 g. Each group contained 10 animals, to which three doses were administered on days 1, 7 and 14. Mice were fasted overnight before immunization. Freeze dried carrot material with 23 μg LTB/g, was hydrated in 1.0 ml of water to reach a final volume of 2.0 ml. The material was administered via gavage with a syringe without needle in a 2 h period with 0.2 ml in 10 doses. Treatments applied to each group were the following: (1) 10 μg pure recombinant LTB obtained from *E. coli* (rLTB, kindly donated by Dr. John Clements, Tulane University, New Orleans, USA); (2) 430 mg of carrot material from the transgenic L1 line (containing 10 μg of carrot-derived LTB); and (3) 430 mg of wild-type-carrot material as a negative control. Five mice from each group were sacrificed on day 21 to collect serum samples and intestinal content. The mice remaining in each group were challenged with cholera toxin (CT).

Cholera toxin challenge

The sealed intestinal loop model developed to measure protection against enterotoxin activity in mice was used

(Richardson et al. 1984). At day 21, 10 μg of CT (Sigma Aldrich Chemical Co., St Louis, MO) dissolved in 500 μl of 10% $NaHCO_3$ was intragastrically administered to two group of mice ($n = 5$ each), that had received either the transgenic carrot or the pure recombinant LTB. Two additional groups of five mice each that had received wild-type-carrot material were challenged with the same intragastric dose of CT or vehicle alone (500 μl of 10% $NaHCO_3$). Six hours after the challenge, the animals were weighed, sacrificed and their small intestine was ligated, dissected and weighed. Fluid accumulation (mg of intestine)/(g of body weight) was calculated with the formula $FA = G/(B - G) \times 1{,}000$, where G is the weight of the gut plus fluid in grams and B is the body weight in grams.

Sample collection

Serum samples were obtained from blood extracted by cardiac puncture from ether-anesthetized mice. The small and the large intestines were resected and 3 ml of cold RPMI medium was flushed through each intestinal segment. The flushed material was mixed, and 250 μl of 10 mM *p*-hydroxymercuribenzoate (dissolved in 150 mM Tris–base) were added to inhibit cysteine–proteinase activity. Samples were centrifuged at 12,000×*g* at 4°C for 10 min, and the supernatants stored at –70°C until their antibody content was analyzed.

Anti-LTB antibody content and subclass

Anti-LTB antibody levels in the serum and intestinal fluid samples were determined by an indirect enzyme-linked immunosorbent assay (ELISA). Plates were coated overnight at 4°C with rLTB (5 μg/ml) dissolved in carbonate/bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and blocked with 5% fat-free milk dissolved in PBS (100 mM NaCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.2). Serum samples were diluted 1:50 in PBST (0.05% v/v Tween-20 in PBS), and intestinal fluid samples were diluted 1:2. Duplicates from each sample were incubated overnight at 4°C; goat antimouse anti-immunoglobulin G (IgG) (Pierce, Rockford, IL), anti-IgA (Zymed Laboratories, San Francisco, CA), or biotinylated goat anti-mouse IgG1 or IgG2a (Zymed) were then added per well, and the plates were incubated for 2 h at room temperature. Plates incubated with biotinylated antibodies for IgG subclass analysis were washed with PBST; conjugated horseradish peroxidase–streptavidin was added to each well and the plates were incubated 2 h at room temperature. After 15 min incubation with substrate solution (0.5 mg/ml *o*-phenylenediamine, 0.01% H_2O_2, 50 mM citrate buffer, pH 5.2) the enzymatic reaction was stopped with 25 μl of 2.5 M H_2SO_4. Specific antibody levels in serum and mucosal samples were expressed as the corresponding optical density values measured at 492 nm (A_{492}) using a Multiskan Ascent (Thermo Electron Corporation, Waltham, MA) microplate reader. The antibody conjugates used to test IgG1 and IgG2a specific responses were previously tittered to be employed at the appropriate dilution and ensure that the optical density values read using each conjugate give comparable approximations of antibody levels.

Statistical analysis

The ELISA data shown are the geometric means of values obtained per group and the error bars represent standard deviations. Significant differences in antibody levels and fluid accumulation values between pairs of groups were determined using two-way analysis of variance followed by a Tukey test. P values <0.05 were considered statistically significant.

Results

LTB content of plant material

LTB content in fresh carrot tap roots was estimated by GM1-ELISA. The analysis revealed 3 μg of LTB per gram of tap root (Rosales-Mendoza et al. 2007). Plant material was ground in liquid nitrogen and freeze-dried. The doses used for oral immunization were 430 mg of carrot powder containing 10 μg of LTB.

Immunogenicity of carrot-derived LTB

Serum antibody response

LTB immunogenicity was determined after administering three intragastric doses of rLTB, carrot-derived LTB or wild-type carrot. Anti-LTB serum antibody levels are shown in Fig. 1a. The antibody response in control mice fed with wild-type carrot material was not statistically different from that of untreated animals, whereas mice fed with transgenic carrot expressing LTB elicited significant serum anti-LTB antibody responses but lower than those elicited by rLTB administration ($P < 0.05$). These findings indicate that carrot-derived LTB is immunogenic by the oral route, although its potency is lower than that of pure rLTB.

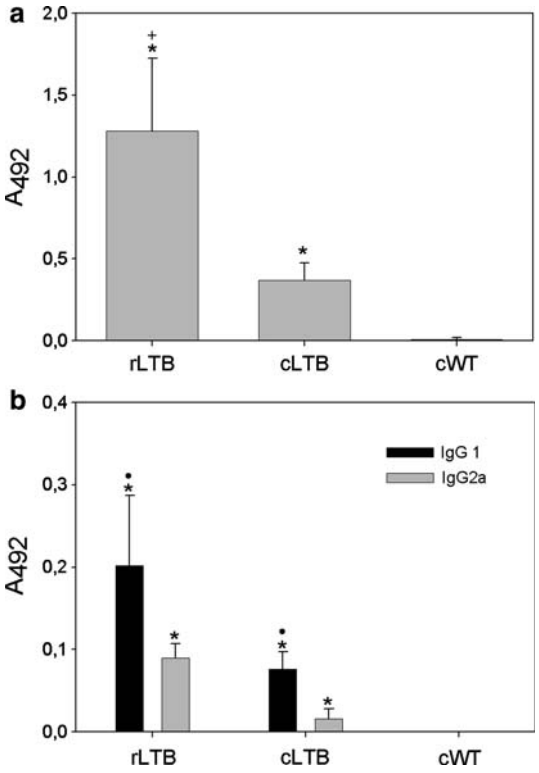

Fig. 1 Anti-LTB serum antibody responses. Three weekly 10 µg-doses of pure recombinant LTB (rLTB), carrot-derived LTB (cLTB), or untransformed wild-type carrot (cWT) were administered to Balb/c mice by the intragastric route. Anti-LTB IgG antibody levels (**a**), as well as IgG1 and IgG2a antibody levels (**b**) were determined by ELISA at day 21 in serum samples diluted 1:50. Mean A_{492} values ± SD from each experimental group ($n = 5$) are shown. Statistically significant differences ($P < 0.05$) in the antibody responses between groups are indicated as follows: *asterisks* versus control group (cWT); *plus* versus group immunized with cLTB; *filled circle* versus IgG2a subclass

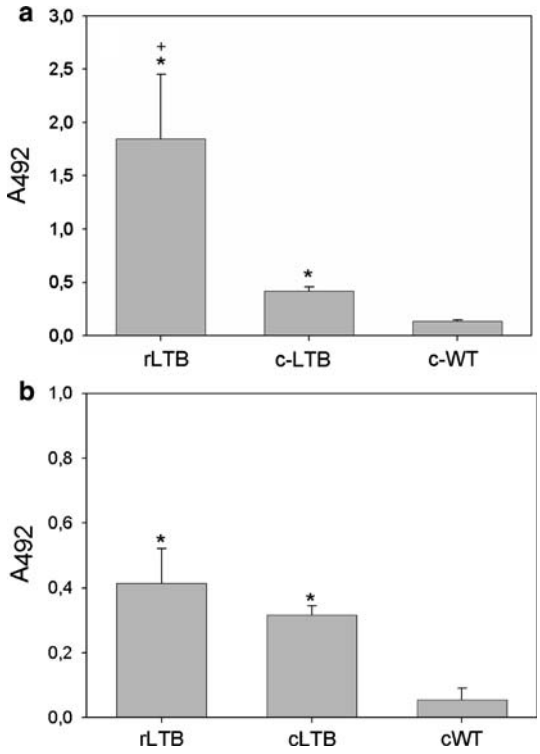

Fig. 2 Anti-LTB intestinal antibody responses. Three weekly 10 µg-doses of pure recombinant LTB (rLTB) or carrot-derived LTB (cLTB), as well as untransformed wild-type carrot (cWT) were administered to Balb/c mice by the intragastric route. Anti-LTB IgA antibody levels (**a**), and IgG antibody levels (**b**) were determined by ELISA at day 21 in fluid samples from the large intestine diluted 1:2. Mean A_{492} values ± SD from each experimental group ($n = 5$) are shown. Statistically significant differences ($P < 0.05$) in the antibody responses between groups are indicated as follows: *asterisk* versus control group (cWT); *plus* versus group immunized with cLTB

Specific anti-LTB serum IgG1 and IgG2a antibodies were elicited in mice immunized with carrot-derived LTB or rLTB (Fig. 1b).

Intestinal antibody response

After oral immunization with carrot-derived LTB, significant specific IgA antibody levels were detected in the large intestine, and the response to rLTB was significantly higher (Fig. 2a). In addition, carrot-derived LTB elicited a specific intestinal IgG response whose magnitude was similar to that elicited by rLTB (Fig. 2b). No significant antibody responses were detected in the small intestine (data not shown). These results demonstrate that carrot-derived LTB is an effective mucosal immunogen when administered by the oral route.

Protection against CT challenge

The sealed intestinal adult mouse loop assay was used to assess the neutralizing activity against an oral CT challenge (Fig. 3). In mice fed with untransformed carrot material the mean fluid accumulation (FA) value after CT challenge was 82.0 mg/g, significantly higher than that of 52.5 mg/g observed in the negative control group (naïve mice challenged with vehicle alone). In contrast, the mean FA value of mice immunized with rLTB was not significantly different from the negative control group and was indistinguishable from the group treated with carrot-derived LTB. However, the mean FA value of mice immunized with carrot-derived LTB was significantly higher than that of the naïve group challenged only with the vehicle.

These findings indicate that oral immunization with carrot-derived LTB induces protection against oral CT. Also although the antibody levels elicited in the rLTB group was quite a bit higher than in the cLTB group,

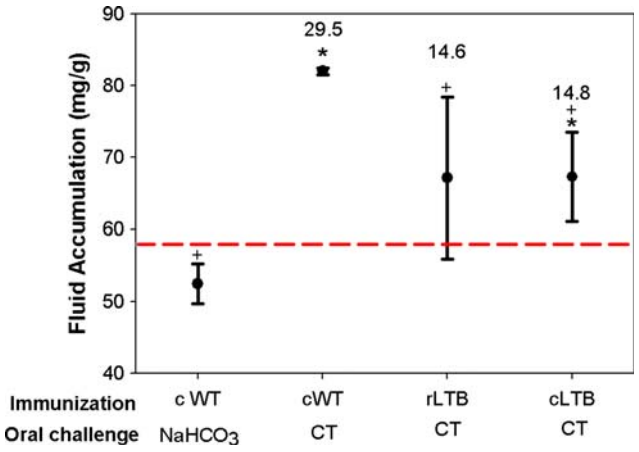

Fig. 3 Immune protection against oral cholera toxin challenge. Three weekly doses of wild-type carrot (cWT), pure recombinant LTB (rLTB), or carrot-derived LTB (cLTB) were administered to Balb/c mice by the intragastric route. At day 21 the cWT group received 500 µl of bicarbonate buffer (cWT $NaHCO_3$), whereas the other groups (cWT, rLTB, cLTB) were challenged with 10 µg of cholera toxin (CT) dissolved in the same buffer volume. After 6 h mice were sacrificed and their small intestine was ligated and dissected to assess fluid accumulation (FA). Mean FA values ± SD from each group ($n = 5$) are shown. The cutoff value (mean FA + 2 SD of the negative control group, cWT/$NaHCO_3$) is indicated by the *discontinuous horizontal line*, and the mean FA increase above it is indicated for each challenged group. Statistically significant differences ($P < 0.05$) in FA values are indicated as follows: *asterisk* versus the cWT/$NaHCO_3$ group (negative control), *plus* versus the cWT/CT group (positive control)

similar protection levels against CT challenge were achieved by both immunization treatments.

Discussion

Diarrheal diseases caused by bacteria are a global health problem. Among them cholera is the most severe but ETEC infections cause the largest number of cases (Nataro and Kaper 1998). An effective vaccine against cholera or ETEC diarrhea should induce an immune response at the intestinal mucosa capable of conferring protection by inhibiting the toxin activity. CTB and LTB are currently used for the formulation of oral vaccines because they are atoxic and each one induces effective immune responses against both toxins (Boedeker 2004).

Major limitations for the massive application of parenteral vaccines are their high costs of production, distribution and delivery. Development of stable, cold-chain-free material may have great relevance for immunization programmes against childhood and infant diarrheas, especially in developing countries, and for this reason needle-free vaccine delivery has become a priority of the World Health Organization (WHO 2005).

Several groups have demonstrated the oral immunogenicity of LTB expressed in diverse transgenic plants. Mason et al. (1998) first demonstrated the oral immunogenicity of potatoes containing 3.7–15.7 µg of LTB per gram of fresh tuber. They immunized mice with three doses of 20 or 50 µg of potato-derived LTB present in 5 g of fresh tuber. Although the animals were not completely protected against oral LT challenge, vaccination by potato ingestion compared favourably with pure bacterial LTB oral vaccination. Clinical studies also have demonstrated the feasibility of edible transgenic plants to deliver LTB, since transgenic potatoes induce vigorous anti-LTB intestinal immune responses in humans (Tacket et al. 1998).

LTB has been expressed in maize plants containing up to 350 µg/g of dry ground kernel (Chikwamba et al. 2002a). An immunization schedule of 10 µg LTB doses contained in 1 g of germ meal made from transgenic maize has been used in mice. Reduced intestinal fluid accumulation after oral LT and CT challenges has been demonstrated in transgenic maize-fed mice (Chikwamba et al. 2002b). LTB-producing maize has been shown to be immunogenic also in humans (Tacket et al. 2004).

Mature tap roots of the transgenic Line 1 use in the current work contain 3 µg of LTB per gram of fresh tap roots (23 µg/g of dry carrot material). In this study we found that three intragastric doses (each one containing 10 µg of carrot-derived LTB) elicited specific systemic and mucosal antibody responses in mice, although the IgG and IgA specific antibody levels were lower than those attained with three 10 µg-doses of pure rLTB. This difference in potency may be due to the carrot-derived LTB having a lower rate of mucosal delivery than the soluble rLTB, perhaps to the to plant cell encapsulation. However, the immunity attained with the transgenic carrot material was high enough to protect mice against the cholera toxin challenge. The protein content in carrots is low and perhaps the best way to administer the edible vaccine can be the freeze dried and ground tap root mixed blended with water or milk and increasing the dose could be a solution to obtain better protection results, we suggest 1 g of freeze dried material and several doses for infant immunization but future studies using different immunization schemes (e.g., increasing the amount of carrot-derived LTB and the number of doses) should be done in order to achieve a higher mucosal immune responses leading to a complete protection against the oral toxin challenge.

We conclude that the genetically modified carrot Line 1 tested in this work may be of use as a practical edible vaccine against cholera and ETEC diarrhea, since carrots are components of the diet that can be consumed raw and have a long shelf life (Simon 1984).

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