Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato

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Compared with vaccine delivery by injection, oral vaccines offer the hope of more convenient immunization strategies and a more practical means of implementing universal vaccination programs throughout the world. Oral vaccines act by stimulating the immune system at effector sites (lymphoid tissue) located in the gut. Genetic engineering has been used with variable success to design living and non-living systems as a means to deliver antigens to these sites and to stimulate a desired immune response[1–4]. More recently, plant biotechnology techniques have been used to create plants which contain a gene derived from a human pathogen; the resultant plant tissues will accumulate an antigenic protein encoded by the foreign DNA[5–10]. In pre-clinical trials, we found that antigenic proteins produced in transgenic plants retained immunogenic properties when purified; if injected into mice the antigen caused production of protein-specific antibodies[6]. Moreover, in some experiments, if the plant tissues were simply fed to mice, a mucosal immune response occurred[7–10]. The present study was conducted as a proof of principle to determine if humans would also develop a serum and/or mucosal immune response to an antigen delivered in an uncooked foodstuff.

Enterotoxigenic *Escherichia coli* is a leading cause of diarrhea in infants in the developing world and in travelers to these areas. After ingestion of contaminated food or water, the bacterium colonizes the gut and secretes toxins which include the heat labile enterotoxin (LT). LT is comprised of six subunits: an enzymatically active protein (LT-A) which enters the epithelial cells of the gut and initiates cellular metabolic changes that lead to loss of water from the cells; and five identical enzymatically inactive proteins (LT-B) which form a pentamer that binds to GM_1 gangliosides in the membranes of epithelial cells[11]. Since binding of LT-B initiates transport of the active subunit inside cells (causing the onset of diarrhea), interference with binding will block the action of the toxin. The LT-B subunits can be isolated free of LT-A. When given orally, LT-B elicits a strong oral immune response without any symptoms of disease[12]. Oral immunization with these subunits results in the appearance of anti-LT-B immunoglobulins in serum (IgG and IgA) and in mucosal secretions (secretory IgA or sIgA). Secretory antibodies in mucosal fluids prevent LT-B binding to the epithelial cells and thereby interfere with the toxic effect of LT.

Oligomeric LT-B, which binds to GM_1 gangliosides, has previously been shown to accumulate in plants transformed with the bacterial gene encoding LT-B or a synthetic gene encoding an identical polypeptide[7,8]. One transgenic line of potatoes, designated #TH110-51, was selected for production of potatoes for pre-clinical trials in mice. After four feedings of the potatoes, the animals produced anti-LT-B IgG in serum and gut mucosal IgA. When challenged with LT derived from bacteria, the animals were partially protected from water loss into the gut[8]. Here we evaluate immune responses to the #TH110-51 potatoes when ingested by humans. LT-B is a good prototype mucosal immunogen because of its vigorous immunogenicity in humans and the safety record of the closely related B subunit of cholera toxin[12].

Fourteen healthy adult volunteers ingested either 100 g of transgenic potato, 50 g of transgenic potato, or 50 g of wild-type potato. Each potato contained 3.7–15.7 μg/g of LT-B. This variability may have been due to the tissue specificity of the promoter such that the cloned gene was expressed unevenly in the different tissues of the potato. The actual amount of LT-B ingested per 50 or 100 g dose ranged from approximately 0.4 to 1.1 mg/dose (mean 0.75 mg/dose).

The potatoes were generally well tolerated. Two volunteers who ate 100 g of raw transgenic potato complained of nausea, on the day of ingestion of the third dose in one of them and for 72 hours after ingesting the second dose in the other. Four of eleven volunteers who ate transgenic potatoes and one of three volunteers who ate wild-type potatoes noted at least one loose stool in the three days after ingestion (*P* not significant, Fisher's exact test).

Gut-derived antibody secreting cells (ASC) are cells which secrete specific antibodies and appear in the circulation about seven days after mucosal immunization. ASC are no longer detectable in the peripheral blood after about 14 days because they have migrated

Table 1 Antibody secreting cell (ASC) responses among volunteers who ingested transgenic or wild-type potatoes on days 0, 7 and 21

	Geometric mean IgA anti-LT ASC per 10^6 PBMC*					Geometric mean IgG anti-LT ASC per 10^6 PBMC				
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 0	Day 7	Day 14	Day 21	Day 28
Transgenic potato (*n*=11)	0.1	18.4	6.6	0.8	19.1	0	13.5	5.7	0.7	7.2
Wild-type potato (*n*=3)	0	0	0.7	0.3	2.4	0	0	1.8	0.4	0.6

* Peripheral blood mononuclear cells

Table 2 Serum and stool antibody responses to LT after ingestion of transgenic or wild-type potato on days 0, 7, and 21

		Serum IgG anti-LT Reciprocal Titers							Serum IgA anti-LT Reciprocal Titers							Serum Neutralizing Reciprocal Titers						Stool IgA Anti-LT Reciprocal Titers						
Volunteer	mg LTB per dose	Day 0	Day 7	Day 14	Day 21	Day 28	Day 59	Response	Day 0	Day 7	Day 14	Day 21	Day 28	Day 59	Response	Day 0	Day 7	Day 14	Day 21	Day 28	Day 59	Day 0	Day 7	Day 14	Day 21	Day 28	Day 59	Response
Transgenic Potato Recipients																												
1001-1	1.1	50	50	100	200	1600	800	+	<100	<100	<100	<100	400	100	+	0	0	10	10	1000	10	<1	22	<1	2	qns	1	+
1001-2	0.4	50	800	6400	3200	3200	3200	+	<100	400	400	400	400	100	+	0	1000	10,000	10,000	1000	1000	4	82	qns**	qns	1	ns	+
1001-3	0.6	50	800	1600	3200	3200	3200	+	100	100	400	400	200	100	+	0	1000	10,000	0	1000	1000	2	2	128	16	32	22	+
1001-5	0.6	100	100	3200	6400	1600	3200	+	<100	<100	200	400	100	100	+	0	10	100	1000	1000	100	2	4	1	qns	qns	qns	-
1001-6	0.6	50	100	800	400	800	800	+	<100	<100	400	200	200	<100	+	0	10	100	100	1000	100	1	1	1	ns***	ns	9	+
1001-7	0.9	50	800	800	1600	3200	800	+	100	100	100	200	200	<100	-	0	100	1000	1000	1000	1000	1	<1	<1	1	<1	<1	-
1001-9	0.9	200	200	100	200	200	100	-	100	100	100	100	100	<100	-	0	0	0	0	0	0	qns	<1	qns	5	1	2	possibly +
1001-10	0.8	100	100	100	50	800	400	+	<100	<100	<100	100	100	100	-	0	0	0	0	100	10	1	<1	<1	1	2	<1	-
1001-11	0.8	50	50	50	100	200	200	+	100	<100	<100	<100	100	<100	-	0	0	10	0	10	10	<1	4	3	qns	4	qns	+
1001-12	0.7	100	3200	6400	3200	3200	6400	+	<100	200	400	200	200	<100	+	0	1000	10,000	1000	1000	1000	1	1	<1	1	<1	2	-
1001-13	0.8	50	800	1600	800	1600	800	+	200	100	200	100	100	<100	-	0	1000	1000	1000	100	10	1	1	2	1	1	<1	-
Wild-type Potato Recipients																												
1001-4	0	100	100	200	100	100	100	-	100	<100	<100	100	<100	<100	-	0	10	100	10	0	10	2	1	<1	3	qns	3	-
1001-8	0	100	50	100	100	100	50	-	100	100	<100	100	100	<100	-	0	0	0	0	0	0	1	1	<1	2	2	<1	-
1001-14	0	<50	<50	<50	<50	<50	<50	-	<100	<100	<100	<100	<100	100	-	0	0	10	0	0	0	<1	1	qns	<1	<1	<1	-

**quantity not sufficient
***no sample

to a mucosal tissue site. The presence of these cells at seven to ten days after immunization reflects immunologic priming of the gut mucosal immune system. Before ingestion of potato, such cells could not be detected in the peripheral blood of volunteers (Table 1). At day seven, a geometric mean of 18.4 IgA anti-LT antibody secreting cells per 10^6 peripheral blood mononuclear cells (PBMC) (median 41, range 0–273) were detected among those who had ingested a single dose of transgenic potatoes (Table 1). Seven days after the second dose, a geometric mean of 6.6 IgA anti-LT ASC per 10^6 PBMC (median 6, range 0–60) were detected. On day 28, seven days after the third dose, a geometric mean of 19.1 IgA anti-LT ASC per 10^6 PBMC (median 20, range 0–126) were detected. A similar pattern of responses was observed for IgG anti-LT ASC (Table 1).

Serologic responses were also detected (Table 2). Ten (91%) of 11 volunteers who ingested transgenic potatoes and none of those who ingested wild-type potatoes developed 4-fold rises in IgG anti-LT at some point after immunization and six (55%) of these 11 volunteers developed 4-fold rises in IgA anti-LT. Five of the ten volunteers who developed IgG anti-LT had significant rises seven days after the first dose of potatoes. These IgG antibodies in all responders remained elevated when measured 59 days after ingestion of the first dose.

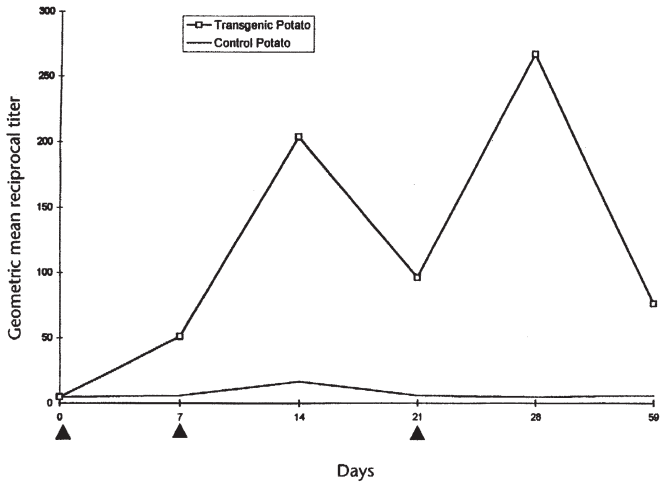

Fig. 1 Geometric mean LT neutralizing antibody titers among volunteers who ingested transgenic potatoes (n = 11) or wild-type potatoes (n = 3). Potatoes were ingested on days 0, 7 and 21 (arrows).

To test the function of the serum antibodies, LT neutralization assays were performed in Y-1 adrenal cells. Eight (73%) of 11 volunteers who ingested the transgenic potatoes developed neutralization titers > 1:100 at some point after immunization. The peak geometric mean titer (1:267) among transgenic potato eaters was measured on day 28, one week after the third dose (Table 2 and Fig. 1).

Stools were collected and assayed for sIgA anti-LT (Table 2). Five (50%) of ten volunteers who ingested transgenic potatoes developed 4-fold rises in sIgA, with a geometric mean peak reciprocal titer among responders of 24.

The LT-B content of potato ingested, which ranged from approximately 3.7 to 15.7 μg/g of potato, did not correlate with either symptoms or the extent or timing of the ASC or serum or stool antibody responses. Although the absolute amount of LT-B ingested per dose for an individual volunteer varied from about 0.4 to 1.1 mg, even the lowest dose was above the threshold dose needed to induce serum or mucosal immune responses in these volunteers. Increasing the dose of LT-B further over a 2–3-fold range did not increase the response in this small number of volunteers. Other factors, presumably related to the host, must explain the variation in responses among volunteers.

In this study, a vaccine antigen delivered by an edible transgenic plant was processed by the human immune system in a way that resulted in mucosal and systemic immune responses. The ASC responses after ingestion of transgenic potatoes were not unlike those measured when volunteers were challenged with 10^9 virulent enterotoxigenic *E. coli* organisms given with 2 g of sodium bicarbonate in a previous study[3] (unpublished data). In this study in which ten volunteers received 10^9 cfu of virulent enterotoxigenic *E. coli* with buffer, the geometric mean number of IgA anti-LT ASC was 28.6 per 10^6 peripheral blood mononuclear cells on day seven. In the current study of 11 volunteers who ingested potato vaccine, the mean number of IgA anti-LT ASC was 18.4 per 10^6 peripheral blood mononuclear cells on day seven (P = 0.67, t test comparing means of log transformed reciprocal titers). Although LT produced and secreted by *E. coli* cells stimulated somewhat more ASC at day seven in challenged volunteers, it is remarkable that LT-B delivered in a potato cell, without buffering, induced a similar degree of mucosal B-cell priming in volunteers who ingested the potato vaccine.

These results offer a new strategy for developing safe and inexpensive vaccines against diseases for which a protective antigen has been defined, such as tetanus, diphtheria and hepatitis B. The

potato is a convenient model system for developing this strategy. Future edible vaccines may be contructed in other plants, such as banana plants, which are grown in many parts of the world and are traditionally eaten raw.

It is possible that other proteins, less immunogenic than LT-B, may not stimulate a vigorous immune response when given in an edible transgenic plant or may even induce tolerance when delivered in this way. Further studies are needed to evaluate other vaccine antigens and the possible need for mucosal adjuvants to overcome or avoid tolerance.

Methods

Development of potato vaccine. We have designed a plant transformation vector (pTH-110) which minimizes limitations of transcription and translation of the bacterial gene in plant cells and thereby causes LT-B accumulation in plant cells[8]. A synthetic DNA sequence was constructed which encodes a protein of the same amino acid sequence as the authentic bacterial LT-B but which was designed to optimize plant codon preferences and also to remove spurious mRNA processing signals. The synthetic coding sequence for LT-B was linked to a nominally constitutive promoter (35S from cauliflower mosaic virus) and a plant-specific termination sequence derived from a soybean vegetative storage protein gene. The vector was used to transform potatoes (Frito Lay variety 1607) and individual transgenic lines were evaluated for LT-B expression by ganglioside-dependent ELISA assays. From these, plant line #TH110-51 was selected based upon its yield of edible potatoes under growth-room conditions and a comparatively high level of LT-B expression. Potato tubers were planted in soil and grown to maturity. Harvested potatoes were stored at 4 °C and used in clinical trials within three months of collection.

Clinical study. Fourteen adult volunteers 18–60 years of age, in excellent physical and mental health, were enrolled in the study after signing consent forms. Screening consisted of a medical history and a battery of blood tests. In addition, all volunteers had serum anti-LT titers ≤ 1:100 before enrollment; on repeat at day 0, one volunteer had a titer of 1:200. Volunteers were randomized in a double-blind manner to receive either 100 g of transgenic potato ($n = 6$); 50 g of transgenic potato ($n = 5$); or 50 g of wild-type untransformed potato ($n = 3$). These dosage sizes were chosen to assess side effects of eating potato, not to deliver different amounts of antigen. Potatoes of lot # TH110-51 were used—the amount of LT-B in potatoes of this lot grown in different pots varied from 3.7 to 15.7 µg/g of potato. Volunteers had nothing to eat or drink for 90 minutes before and after ingesting potato. The potatoes were peeled immediately before ingestion to remove the skin containing solanine, an alkaloid present in all green tissues of potatoes which can cause abdominal discomfort, nausea or a bitter taste. Each potato was then cut into uniform bite-size pieces. A second and third dose of transgenic or wild-type potato were given on days 7 and 21. Volunteers recorded symptoms each day for three days after ingesting each dose of potatoes.

Immunology. Whole blood was collected for antibody secreting cell (ASC) assays on days 0, 7, 14 and 28. ASC producing antibody against LT were measured by ELISPOT assays[13]. Peripheral blood mononuclear cells were separated from heparinized blood in a Ficoll gradient. Microdilution plates were coated with 1 µg/ml of LT and washed. Viable cells were suspended at 2.5×10^6 cells per milliliter in RPMI 1640 medium with 10% FCS, 2 mmol L-glutamine/l, and 15 µg/ml of gentamicin and dispensed in 100 µl portions into four replicate wells. After overnight incubation at 37 °C, wells were washed and 100 µl of a 1:1,000 dilution of goat anti-human IgA alkaline phosphatase conjugate or 1:5,000 dilution of an IgG conjugate was added to each well. Wells were washed again after a 1 hour incubation at 37 °C, and 100 µl of a molten agarose-substrate overlay was added. After incubation, the presence of LT-specific IgA or IgG secreted by individual lymphocytes was visualized as dark-blue spots which could be counted by a stereomicroscope and recorded as ASC per 10^6 peripheral blood mononuclear cells.

Blood was collected before and 7, 14, 21, 28 and 59 days after potato ingestion for measurements of antibodies to LT by ELISA[14] and for measurement of serum neutralizing antibody activity[15]. For the ELISA, plates were coated with 1.0 µg/ml of purified LT, washed, blocked with phosphate-buffered saline and 5% fcs, incubated for 1 hour at 37 °C, and washed again. Test serum samples were diluted from a titer of 1:50 to 1:12,800 in the wells, incubated for 1 hour at 37 °C, and washed. Goat anti-human IgG (1:5,000) or anti-human IgA (1:1,000) conjugated to alkaline phosphatase was added and the plates incubated for 1 hour at 37 °C, and washed. The reaction was developed with phosphatase substrate and read in an ELISA reader spectrophotometer. The endpoint titer was defined as an optical density of 0.2 for IgG and 0.1 for IgA. For the toxin neutralization assay, the Y-1 adrenal cell assay in miniculture was used[15]. Y-1 mouse adrenal cells change morphology from flat to round in response to LT via adenyl cyclase production. The titer was defined as the highest serum dilution which showed complete neutralization of biological activity of 100 pg of toxin.

Stool was collected before and 7, 14, 21, 28 and 59 days after potato ingestion. 10% stool supernatants were prepared and each sample was standardized to 2 mg% of total IgA before the ELISA assays were performed as above. A response was defined as a 4-fold or greater rise in titer of serum IgG or IgA and stool IgA antibody. A neutralizing titer > 100 was considered significant.

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