Chapter 10

AN ORAL VACCINE IN MAIZE PROTECTS AGAINST TRANSMISSIBLE GASTROENTERITIS VIRUS IN SWINE

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Keywords: antigen, mucosal immunity, transgenic plants, TGEV, enteric pathogen

Abstract Transmissible gastroenteritis (TGE) is a highly contagious enteric disease that is characterized by vomiting, severe diarrhea and high mortality in piglets less than two weeks of age. The development of edible vaccines offers the potential to aid in the control of enteric diseases such as TGE. Edible vaccines from plant material could be directly delivered in the feed and could be produced cheaply in large volumes thus avoiding many costs associated with the administration of conventional vaccines. Vaccines from plants are particularly suitable for stimulation of mucosal immunity, since edible plant products can be delivered orally to reach the gut mucosal tissue and elicit an immune response at mucosal surfaces. Recent advances in technology make it now possible to express vaccine antigens at high levels in plants. Corn expressing the S protein of TGEV was fed to 13-day-old piglets for ten days and subsequently challenged with a virulent Purdue strain of TGEV. This group of piglets was significantly protected from the disease as contrasted to the control group that was fed only corn. Results from a second trial duplicated these results demonstrating that the delivery of antigens delivered in an edible oral form are efficacious

1. INTRODUCTION

Swine transmissible gastroenteritis (TGE) (Saif et al., 1992) is recognized as one of the major causes of sickness and death in piglets particularly in areas with high concentrations of pigs. TGE is a highly contagious enteric disease that is characterized by vomiting, severe diarrhea and high mortality in piglets less

L. Erickson et al. (eds.),
Molecular Farming of Plants and Animals for Human and Veterinary Medicine, 223–236.
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than two weeks of age. The causal agent of TGE is a pleomorphic, enveloped single-stranded RNA virus belonging to the genus *Coronavirus* of the family Coronaviridae. Replication of virus in the villous epithelial cells of the small intestine results in the destruction or alteration of function of these cells. These changes lead to a reduction in the activity of the small intestine that disrupts digestion and cellular transport of nutrients and electrolytes. In small piglets this can lead to a severe and fatal deprivation of nutrients and dehydration. Following infection, pigs that have survived the infection are immune to subsequent infections presumably due to local immunity in the intestinal mucosa. Thus, since active immunity towards TGEV involves local immunity in the intestinal mucosa, presumably through the activation and secretion of intestinal SIgA, vaccines that target activation of the intestinal mucosa immune system are particularly attractive in the control of this disease. In particular, the development of edible vaccines offers the potential to aid in the control of enteric diseases such as TGE. Edible vaccines from plant material could be directly delivered in the feed and could be produced cheaply in large volumes thus avoiding many costs associated with the administration of conventional vaccines. Vaccines from plants are particularly suitable for stimulation of mucosal immunity, since edible plant products can be delivered orally to reach the gut mucosal tissue and elicit an immune response at mucosal surfaces. Recent advances in technology make it now possible to express vaccine antigens at high levels in plants.

A number of different plant systems have recently been under investigation for use as an edible oral delivery systems (Gomez et al., 1998; Tuboly et al., 2000) in the development of an oral vaccine for TGEV. Of these, a system based on the use of transgenic maize seed appears to be the most realistic for a number of different reasons. Among these reasons include the ability to introduce a grain-based product directly into a producer's feed system, the ability to utilize the already existing infrastructure for the production, harvesting, transportation, storage, and processing of the grain, the ability to deliver a product (both monovalent and multivalent) at a cost competitive with contemporary vaccines due to a low cost of goods, and a plant system amenable to transformation with highly developed and characterized genetics.

TGEV virions contain three major structural proteins: a nucleocapsid protein (N), a small membrane-bound glycoprotein (M), and large spike or peplomer glycoprotein (S). In this study, we generated transgenic maize plants that express the spike protein at high levels. Corn expressing the S protein of TGEV

was fed to 13-day-old piglets for ten days and subsequently challenged with a virulent Purdue strain of TGEV. This group of piglets was significantly protected from the disease as contrasted to the control group that was fed only corn. Results from a second trial duplicated these results demonstrating that the delivery of antigens delivered in an edible oral form are efficacious.

2. GENE CONSTRUCTS AND TRANSFORMATION OF MAIZE VIA AGROBACTERIUM

The amino acid sequences of the S, M, and N protein of an isolate of the Miller strain of TGEV were provided by Prem Paul, DVM, College of Veterinary Medicine, Iowa State University. The amino acid sequences of the various structural proteins of TGEV were back-translated using the Backtranslate program of the Wisconsin GCG Package against a codon table tabulated for highly expressed maize genes. The resulting DNA sequence was scanned for the presence of undesirable sequence, e.g. polyadenylation signals, 5' and 3' consensus splice sites, other mRNA destabilizing sequences, and undesirable endonuclease restriction enzyme sites. The DNA sequence was modified to eliminate these sites by choosing alternative codons. Alternative codons with a codon frequency of less than 10 percent for that amino acid were avoided. The resulting sequence was then constructed using a series of synthesized overlapping complementary oligonucleotides and the polymerase chain reaction (PCR) to amplify the resulting synthetic sequence. Convenient restriction sites were also engineered into the 5' and 3' ends of the optimized gene to facilitate cloning. The barley alpha-amylase signal sequence (Rogers J.C., 1985) was also synthesized using overlapping complementary nucleotides with maize-preferred codons. Inclusion of the translated leader sequence directs the protein of interest to the cell wall and allows high levels of accumulation.

Transgenic maize plants were generated using the method of Ishida et al. (1996). Essentially, maize corn ears were harvested at 9-12 days after pollination when embryos are approximately 1-2 mm in length. Whole ears were surface sterilized in 50% bleach (+teaspoon of Tween 20) for 30 min and given two rinses of sterile H_2O. Immature zygotic embryos (ZE) were sterilely isolated from the ears. Embryos were washed twice with co-cultivation medium and *Agrobacterium* was added directly by pouring bacterial solution into the ZE tube. Embryos with bacteria were vigorously vortexed for 30 seconds and

allowed to incubate at room temperature for 5 minutes. Embryos were placed scutellum side up onto co-cultivation medium and incubated at 19°C in the dark for 3-5 days. Keeping scutellum side up, embryos were transferred to antibiotic-containing medium without selection for three days in the dark at 27-28°C. every subsequent 2 weeks, embryos and herbicide resistant calli were transferred to fresh selection medium. When sufficient callus from a single event had developed on selection medium (approximately two plates), the callus was transferred onto regeneration medium. Mature somatic embryos were placed in the light and allowed to germinate. Ten plants from each event were transplanted to soil in the greenhouse and allowed to flower and produce seed. The resulting seed (T1 seed) was screened by ELISA to determine the levels of the recombinant protein of interest.

3. PROTOCOLS FOR FEEDING AND CLINICAL TRIALS

3.1 Transgenic Grain Production

Highly expressing seeds were backcrossed into maize lines of commercial interest. For this study, pollen from T1 seed was crossed to commercial maize hybrids in order to bulk up the seed as fast as possible. The resulting grain (approximately 30 lbs.) was ground to cornmeal (600-micron particle size). The levels of S protein in this fraction were estimated to be 0.004% (w/w). Piglets were fed about 50 grams per day of transgenic corn. That roughly amounted to 2 mg of S protein per dose per day.

3.2 Swine Feeding Trials

All swine feeding trials were conducted at Ames, Iowa in collaboration with David Carter, D.V.M., Veterinary Resources, Inc. and Mark Welter, M.S. at Oragen Technologies. 10-day-old SPF TGEV sero-negative pigs from a low disease incidence herd were utilized in these trials.

3.3 Vaccination of Feed Test Groups

For the appropriate consecutive days, all piglets were withheld from feed overnight (including the MLV vaccinates) and all feed-test groups were vaccinated first thing in the morning. In groups receiving the TGEV-S corn, 50 grams of TGEV transgenic corn was needed per day per pig. The dry corn was mixed with a wooden stick to ensure distribution of the transgenic corn. Medicated milk replacer to a total of not less than 300 ml and not more than 600 ml was used as a base to which the ground corn was added and mixed so as to produce a thick oatmeal-type meal. The corn was stirred in with a clean wooden stick until thick with just a little milk settling to the top. This amounted to approximately 1000 grams of feed representing 100 grams per piglet feeding, containing 50 grams of transgenic corn per pig feeding. A line of vaccine meal was placed on a clean dry floor and the piglets allowed to consume the vaccine. Attempts were made to ensure each piglet received an adequate vaccine portion. After the vaccine was consumed, regular water and medicated weaning rations were replaced in the pen. Pigs in the treatment group receiving the modified live vaccine (MLV) were orally vaccinated with MLV TGEV according to label directions at day 0 and 7 days later.

3.4 Virus Challenge

In the case of TGEV-1, on day 12 (2 days after last feed vaccination and 5 days after MLV TGEV) all animals were orally challenged with a 2 ml oral dose of virulent TGEV challenge (Purdue strain, titre of $10^{7.6}$ $FAID_{50}$'s per dose). In the case of TGEV-2, on day 18 (2 days after last feed vaccination for the 16-day groups and 11 days after MLV TGEV) all animals were orally challenged with a 2 ml oral dose of virulent TGEV challenge (Purdue strain, titre of $10^{7.6}$ $FAID_{50}$'s per dose). Previous work has determined that this challenge strain and levels will produce a clinically typical TGEV watery diarrhea in 21 to 28 day old piglets that persists for 7 to 10 days. No mortality values have been observed with this challenge model in this age animal.

3.5 Data and Sample Collection

Persons performing daily observations were blinded as to treatment.

1) Daily Observations: Piglets were observed twice daily and were scored for any signs of diarrhea as below:

0 (Normal)

2 (Creamy, piles up in pen)
4 (Watery)

Additional clinical signs which were observed such as dehydration or depression, anorexia, vomitus and death were scored as below and the number added to fecal observation for a total clinical score as shown below. Any animal that died or appeared moribund was sacrificed and necropsied. A sample from the jejunum of the small intestine was collected and observed for villous atrophy and providing that the sample was not too necrotic it was assayed for TGEV.

1 (Dehydration & Depression)
1 (Anorexia)
3 (Vomitus)
10 (Moribund or Death)

Attempts were made to isolate TGEV from the feces of watery scouring animals so as to confirm the challenge. A fecal sample was collected and TGEV isolation was conducted by inoculating confluent ST cells and staining by specific immunofluorescence.

2) Weights: All animals were weighed on day 0, day 12 and day 24.

3) Blood Samples: Blood was collected on day 0, day 12 and day 26. Blood was allowed to clot and serum collected and stored at 20° C until assay. Sera was assayed for TGEV neutralizing titers and titre values calculated using a Spermen Karber table.

4) Fecal Samples: Fecals were collected from randomly selected animals within a group that showed watery diarrhea and fecals were checked for TGEV activity.

3.6 Data Analysis

The total clinical scores for all animals within their group were divided by the number of observations to give a group clinical score. Statistical differences between groups were compared. The clinical symptom data are presented as: Percent Morbidity Incidence (number of animals with clinical signs >2 divided by total number of animals); Percent Morbidity Incidence and Duration (total number of clinical observations ≥ 2 divided by total number of pig days) and;

Clinical Severity Index (total clinical score value divided by total number of pig days).

3.7 Swine Feeding Trial #1 (TGEV-1)

The study consisted of three treatment groups. Group A was fed transgenic corn expressing the spike protein (S) of TGEV, Group B was fed non-transgenic corn, and Group C was vaccinated with a commercial modified live TGEV vaccine (MLV TGEV). All animals were challenged on day 12. Table 6 shows a summary of the design of the study.

Table 6. Summary of study design (TGEV-1)

Group	Number of pigs	Vaccine description	Amount/day	Route	Timing	Day of challenge
A	10	TGEV	50 g	Oral	0 to 10 days	Day 12
B	10	Control corn	50 g	Oral	0 to 10 days	Day 12
C	10	MLV TGEV	N.A.	Oral	0 & 7 days	Day 12

3.8 Swine Feeding Trial #2 (TGEV-2)

This study consisted of five treatment groups. Groups A , B and C were fed transgenic corn expressing the spike protein (S) of TGEV for 4, 8 or 16 days. Group D was fed non-transgenic corn for 16 days, and Group E was vaccinated with a commercial modified live TGEV vaccine (MLV TGEV) on days 0 and 7. All animals were challenged on day 18. Table 7 shows a summary of the design of the study.

4. RESULTS FROM CLINICAL TRIALS

4.1 Observations of Clinical Symptoms for TGEV-1

The results from this experiment are summarized in Table 8. The data for morbidity incidence represent a compilation of the significant (>2) clinical scores for each treatment group. These data show that 100% of the pigs that

were fed control corn developed TGEV clinical symptoms. Only 50% of those that received the TGEV corn exhibited symptoms and 78% of the pigs receiving the modified live vaccine developed symptoms.

Table 7. Summary of study design (TGEV-2)

Group	Number of pigs	Vaccine description	Amount/day	Route	Timing	Day of challenge
A	10	TGEV transgenic corn	50 g	Oral	0 to 4 days	Day 18
B	10	TGEV transgenic corn	50 g	Oral	0 to 8 days	Day 18
C	10	TGEV transgenic corn	50 g	Oral	0 to 16 days	Day 18
D	10	Control corn	50 g	Oral	0 to 16 days	Day 18
E	10	MLV TGEV	N.A.	Oral (0.5 ml)	0 & 7 days	Day 18

The data for the percent of morbidity incidence and duration are also a compilation of the significant (>2) clinical scores for each treatment group. As indicated by the data, 100% of the pigs that were fed control corn developed TGEV clinical symptoms. Only 50% of those that received the TGEV corn exhibited symptoms of similar duration, while 78% of the pigs receiving the modified live vaccine developed symptoms of similar duration.

Data for the clinical severity index for each treatment group are a compilation of the total clinical value divided by the total number of pig days for each treatment group. This data shows that the disease that developed in the pigs that were fed control corn was much higher than either the TGEV-S containing transgenic corn or the modified live vaccine treatment group.

4.2 Observations of Clinical Symptoms for TGEV-2

The results from this experiment are summarized in Table 9. As in TGEV-1, the data for morbidity incidence represent a compilation of the significant (>2) clinical scores for each treatment group. These data show that 36% of the pigs that were fed control non-transgenic corn developed TGEV clinical symptoms. Such symptoms were present in 50%, 0%, and 20% of the pigs that received 4

days, 8 days and 16 days of TGEV corn, respectively. 9% of the pigs receiving the modified live vaccine developed symptoms.

Table 8. Summary of clinical data for TGEV-1

Treatment group	Morbidity incidence	Morbidity incidence and duration	Clinical severity index
TGEV corn	50%	22%	0.96
Control corn	100%	38%	1.3
MLV TGEV	78%	18%	0.89

The data for percent morbidity incidence and duration in each of the treatment groups are also a compilation of the significant (>2) clinical scores for each treatment group. Pigs fed non-transgenic control corn scored 36% morbidity/duration, while pigs that received 4 days, 8 days and 16 days of TGEV corn scored 13%, 0% and 5% morbidity/duration. The corresponding score for pigs receiving the modified live vaccine was 2%.

Table 9. Summary of clinical data for TGEV-2

Treatment group	Dose duration	Percent morbidity incidence	Percent morbidity incidence and duration	Clinical severity index
TGEV corn	4 days	50	13	0.36
	8 days	0	0	0
	16 days	20	5	0.16
Control corn	16 days	36	5	0.15
MLV TGEV	NA	9	2	0.05

The data showing the clinical severity index are a compilation of the total clinical value divided by the total number of pig days for each treatment group. These data show that the disease that developed in the pigs that were fed control corn was much higher than either the TGEV-S containing transgenic corn or the modified live vaccine treatment group.

5. GENERAL DISCUSSION

Over the past decade, transgenic plants have been successfully used to express a variety of genes from bacterial and viral pathogens. Many of the resulting peptides induced an immunologic response in mice (Gomez et al., 1998; Mason et al., 1998; Wigdorovitz et al., 1999) and humans (Kapusta et al., 1999) comparable to that of the original pathogen. Characterization studies of these engineered immunogens have proven the ability of plants to express, fold and modify proteins in a manner that is consistent with the authentic source.

Numerous genes have been cloned into a variety of transgenic plants including many enzymes that have demonstrated the same enzymatic activity as their authentic counterparts (Hood et al., 1997; Moldoveanu et al., 1999; Trudel et al., 1992). Many additional genes have been expressed in plants solely for their immunogenic potential, including viral proteins (Gomez et al., 1998; Kapusta et al., 1999; Mason et al., 1996; McGarvey et al., 1995; Thanavala et al., Wigdorovitz et al., 1999) and subunits of bacterial toxins (Arakawa et al., 1997; Arakawa et al., 1999; Haq et al., 1995; Mason et al., 1998). Animal and human immunization studies have demonstrated the effectiveness of many plant-derived recombinant antigens in stimulating the immune system. The production of antigen-specific antibodies and protection against subsequent toxin or pathogen challenge demonstrates the feasibility of plant derived-antigens for immunologic use.

The utilization of transgenic plants for vaccine production has several potential benefits over traditional vaccines. First, transgenic plants are usually constructed to express only a small antigenic portion of the pathogen or toxin, eliminating the possibility of infection or innate toxicity and reducing the potential for adverse reactions. Second, since there are no known human or animal pathogens that are able to infect plants, concerns with viral or prion contamination are eliminated. Third, immunogen production in transgenic crops relies on the same established technologies to sow, harvest, store, transport, and process the plant as those commonly used for food crops, making transgenic plants a very economical means of large-scale vaccine production. Fourth, expression of immunogens in the natural protein-storage compartments of plants maximizes stability, minimizes the need for refrigeration and keeps

transportation and storage costs low (Kusnadi et al., 1998). Fifth, formulation of multicomponent vaccines is possible by blending the seed of multiple transgenic corn lines into a single vaccine. Sixth, direct oral administration is possible when immunogens are expressed in commonly consumed food plants, such as grain, leading to the production of edible vaccines.

Some of the first attempts to make edible vaccines included transgenic potatoes expressing the *E. coli* heat-labile enterotoxin (LT-B) (Haq et al., 1995), and a Norwalk virus surface protein (Mason et al., 1996). In both cases, mice fed the antigenic tubers produced serum and secretory antibodies specific to the authentic antigen. Subsequently, many plant-expressed antigens, including those referenced above, have been shown to elicit an immune response when administered through an oral route. Several of these antigens have shown sufficient promise to warrant human clinical trials (Mason et al., 1998; Saif et al., 1994).

One of the most promising aspects of edible vaccines is the ability of orally administered immunogens to stimulate a mucosal immune response (Ruedl et al., 1995). Mucosal surfaces, the linings of the respiratory, gastrointestinal, and urogenital tracts, play an important physical and chemical role in protecting the body from invading pathogens and harmful molecules. The mucosal immune system is distinct and independent of the systemic, or humoral, immune system, and is not effectively stimulated by parenteral administration of immunogens (Czerkinsky et al., 1993). Rather, the mucosal immune system requires antigen presentation directly upon the mucosal surfaces. Since most invading pathogens first encounter one or more of the mucosal surfaces, stimulation of the mucosal immune system is often the best first defense against many transmissible diseases entering the body through oral, respiratory and urogenital routes (Holmgren et al., 1994). Transgenic plants could produce large quantities of immunologically active recombinant antigen very economically for vaccine production. Multicomponent vaccines could easily be formulated from the seed of multiple transgenic plant lines to generate an increased chance for successful virus neutralization in a stand-alone vaccination strategy, as a booster, or in combination with other vaccines and vaccination routes.

Previously the full spike (S) protein has been expressed in *Arabidopsis* (Gomez et al., 1998). In this case, expression of the S protein was not detectable yet a plant extract injected intramuscularly into mice resulted in the production of detectable anti-S serum. More recently, the S protein has been expressed in tobacco (Tuboly et al., 2000). The S protein was expressed at levels that could

be detected by ELISA and of the expected size when analyzed by Western blotting. Leaf extracts from these plants were injected into 28-day old pigs. In contrast to pigs which were injected with nontransgenic plant extracts, the former produced measurable TGEV-specific antibodies, whereas the latter piglets did not.

We have extended these results by generating transgenic corn expressing the S protein which can be fed to pigs in a virulent TGEV challenge study. We report for the first time the protection of an economically important animal from a naturally occurring disease by an oral vaccination using an edible system. Moreover this system uses the conventional feed materials, e.g. corn, to deliver the antigen. One report (Modelska et al., 1998) has shown in the laboratory the amelioration of rabies symptoms in mice fed multiple doses of a chimeric plant virus expressing the rabies glycoprotein following challenge with an attenuated rabies strain. To our knowledge until our report no animals in conventional food animal husbandry have been vaccinated with edible vaccines and shown to be protected from the disease. The level of protection seen in this study includes general health and vigor, a decrease in clinical symptoms, lack of virus shedding and other observations known to be criteria for disease protection. The mode of protection is unknown but may be an active immune response by the animal, competitive inhibition of viral receptor sites leading to non-establishment of a viral infection, or interference with parts of the viral replicative process.

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