Plant Cell-Based Intimin Vaccine Given Orally to Mice Primed with Intimin Reduces Time of *Escherichia coli* O157:H7 Shedding in Feces

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Intimin is the primary adhesin of *Escherichia coli* O157:H7, the most common infectious cause of bloody diarrhea in the United States and the leading cause of acute kidney failure in children who develop hemolytic uremic syndrome. Cattle are the primary reservoir of *E. coli* O157:H7. Indeed, most cases of *E. coli* O157:H7 infection in the United States occur after ingestion of contaminated undercooked hamburger or produce that had contact with bovine manure. Because intimin is required for persistent colonization of neonatal calves and adult cattle, we hypothesized that an intimin-based vaccination strategy in calves would reduce colonization of cattle with *E. coli* O157:H7. To test this concept in a small-animal model, we developed transgenic tobacco plant cells that express the carboxy-terminal host cell-binding domain of *E. coli* O157:H7 intimin. Mice were either immunized intraperitoneally with intimin expressed from the plant cells, fed transgenic plant cells, or both. Here we show that these mice generated an intimin-specific mucosal immune response when primed parenterally and then boosted orally and also exhibited a reduced duration of *E. coli* O157:H7 fecal shedding after challenge.

Escherichia coli O157:H7 is the most common cause of bloody diarrhea, or hemorrhagic colitis, in the United States, with an estimated incidence of 73,480 cases per annum (7, 34). Moreover, hemolytic uremic syndrome, a sequela of *E. coli* O157:H7 infection, is the most frequent basis for acute kidney failure in U.S. children (7). These organisms are typically transmitted directly or indirectly from infected cattle to humans. Both beef and dairy cattle can be sporadically and asymptomatically colonized with *E. coli* O157:H7 and shed the bacteria, which can survive in broad ecological niches beyond the bovine gastrointestinal tract, into the environment in their feces (10, 17, 25, 28). Moreover, contacts with the farming environment and livestock density are major risk factors for human infection and disease caused by *E. coli* O157:H7. Many of the foods implicated in human disease are of bovine origin or are food or water that have come into contact with contaminated meat or bovine fecal material (14, 43).

A number of investigators have concluded that a decrease in the amount of *E. coli* O157:H7 shed as well as in the number of cattle that excrete the serotype could cause a significant reduction in the prevalence of the bacteria in cattle and the farm environment. The hypothesis that vaccination of cattle or treatment of the animals with an agent to diminish the level of colonization and shedding of *E. coli* O157:H7 could potentially lead to a decline in the incidence of human *E. coli* O157:H7-related disease (17, 22, 47) was suggested by the findings from a stochastic simulation model designed by Jordan et al. (22). Based on this idea, B. Finlay's group, our laboratory, and

others have begun to design and/or test *E. coli* O157:H7 vaccine protocols for use in cattle. Indeed, Finlay and colleagues have initiated field studies of *E. coli* O157:H7 secreted products as a subcutaneously administered bovine vaccine [B. Finlay, Abstr. 5th Int. Symp. "Shiga toxin (verocytotoxin)-producing *Escherichia coli* infections," abstr. p. 23, 2003; R. Moxley, D. Smith, T. Klopfenstein, G. Erickson, J. Folmer, C. Macken, S. Hinkley, A. Potter, and B. Finlay, Abstr. 5th Int. Symp. "Shiga toxin (verocytotoxin)-producing *Escherichia coli* infections," abstr. p. 23, 2003].

We selected a different immunogen, expression system, and route of administration for proof of concept studies to assess, in a small-animal model, the feasibility of an *E. coli* O157:H7 vaccine for cattle. For our vaccine candidate, we chose intimin, an outer membrane protein of *E. coli* O157:H7 that is required for attaching and effacing lesion formation as well as for bacterial adherence to mammalian cells and the intestinal mucosa of calves, piglets, and ferrets (8, 21, 32, 46). Intimin is the product of the *eae* (*E. coli* attach and efface) gene, which is contained within an approximately 43-kb pathogenicity island called the locus of enterocyte effacement (23, 24, 36). The carboxy-terminal portion of intimin binds the bacterium-encoded translocated intimin receptor (Tir) and a host cell receptor, nucleolin, to mediate intimate attachment of the bacteria to the eucaryotic cell surface (11, 12, 38). The reasons we consider intimin an attractive candidate for an *E. coli* O157:H7 antitransmission vaccine for cattle are based on both in vitro and in vivo studies. Specifically, members of our laboratory previously found that antibodies against the carboxy-terminal third of the molecule block adherence of wild-type *E. coli* O157:H7 to HEp-2 cells (13, 33). In addition, our laboratory, with E. Dean-Nystrom's group, showed that colostrum from pigs immunized with intimin isolated from *E. coli* O157:H7

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Reference or source
86-24	*E. coli* O157:H7, *eae* positive	15
86-24 GFP	*E. coli* O157:H7(p166)	38
86-24 Str^r	*E. coli* O157:H7, Str^r	35
86-24 *eae*Δ10 Str^r	*E. coli* O157:H7, *eae* negative, Str^r	32
DH5α	*E. coli* cloning host, *recA*	16
XL-1 Blue	*E. coli* cloning host, *recA lacI^q*	6; Stratagene
A. tumefaciens EHA105	Plant transformation strain	20

contains anti-intimin antibodies that can protect suckling piglets from colonization with *E. coli* O157:H7 (13). These tissue culture experiments and passive transfer studies suggest that antibodies specific to intimin play an important role in blocking adherence of the bacterium to host cells and can protect the host from *E. coli* O157:H7-mediated disease.

For delivery of intimin as a vaccine for cattle, we sought an oral inoculation system to facilitate induction of mucosal antibodies and for ease of administration. Therefore, we elected to use a transgenic plant cell system for intimin expression, with the ultimate goal of moving the antigen into whole-plant expression and delivery systems. Transgenic plants offer the flexibility to function as low-cost, efficient, and practical vaccine antigen oral delivery systems to stimulate mucosal immunity or to boost and shift initial immunity to a mucosal antibody response (27, 44). Indeed, transgenic plants have already been used as successful vaccine antigen production and delivery systems (18, 29, 30, 40). The vaccine antigens expressed by plants include hepatitis B surface antigen (26, 31, 42), enterotoxigenic *E. coli* heat-labile toxin B subunit (LT-B) (18, 27, 30, 40), Norwalk virus capsid protein (29, 41), cholera toxin B subunit (2, 3), and many others. For this study, we created carboxy-terminal third of intimin-expressing plant cells and evaluated the capacity of this transgenic material to induce adherence-blocking antibodies and to reduce levels and/or

time of *E. coli* O157:H7 fecal shedding in a mouse model of intimin-dependent colonization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are summarized in Tables 1 and 2, respectively. Enterohemorrhagic *E. coli* (EHEC) O157:H7 strain 86-24 (15) was isolated in 1986 from a patient in Seattle, Wash., and was kindly provided by Phil Tarr. EHEC O157:H7 strain 86-24 that expresses the green fluorescent protein arabinose-inducible plasmid p166 was used to visualize bacteria in the HEp-2 cell adherence assay, as previously described (13, 38). A streptomycin-resistant derivative of strain 86-24 (called 86-24 Str^r [35]) and its isogenic, intimin-negative mutant strain 86-24 *eae*Δ10 (called 86-24 Str^r *eae*Δ10 [32]) were used to distinguish EHEC O157:H7 from other fecal bacteria during the colonization assay by plating on sorbitol-MacConkey agar with 100 μg of streptomycin per ml. In addition, several sorbitol-negative colonies from different mice at each collection time point were tested by slide agglutination with O157 antiserum (Difco Laboratories, Sparks, Md.) to confirm that the colonies were of the O157 serogroup. All mice were free of Str^r, O157 agglutination-positive colonies prior to challenge. *Agrobacterium tumefaciens* strain EHA105, plasmids with plant-specific expression components (pBTI210.3, pBTI210.4, and pIBT210), and the binary vector pGPTV-Kan were provided by the Boyce Thompson Institute for Plant Research (Cornell University, Ithaca, N.Y.). All *E. coli* strains and clones were grown in Luria-Bertani (LB) broth or on LB agar. Prior to plant cell transformation, *Agrobacterium* strains were grown in YM broth (0.4 g of yeast extract, 10 g of mannitol, 0.1 g of NaCl, 0.2 g of MgSO$_4$ · 7H$_2$O, 0.5 g of K$_2$HPO$_4$ per liter). Antibiotics were added as needed for selection at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; carbenicillin, 100 μg/ml. Plasmid DNA was isolated by the Miniprep procedure (Qiagen, Valencia, Calif.). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.), and restriction enzyme-digested DNA fragments were purified by agarose gel electrophoresis and eluted from the gel with Geneclean spin columns and reagents (Bio 101, Carlsbad, Calif.). T4 DNA ligase was purchased from U.S. Biochemicals (Cleveland, Ohio). PCRs were done with AmpliTaq (Roche, Branchburg, N.J.) and *Pfu* Turbo (Stratagene, La Jolla, Calif.) polymerases in an MJ Research Minicycler (Watertown, Mass.). Sequencing was performed with the ABI Big Dye sequencing kit (Applied Biosystems, Inc., Foster City, Calif.). Products were separated and analyzed by the Biomedical Instrumentation Center at Uniformed Services University of the Health Sciences with Applied Biosystems sequencer model 377 or 3100. Sequence results were compared to published sequences by use of the Wisconsin Sequence Analysis package from the Genetics Computer Group, Inc. (Madison, Wis.).

Optimization of *eae* (nucleotides 1959 to 2804) for plant expression. Thirteen independent changes were made to the nucleotide sequence of the intimin gene from pMW103 with the QuikChange site-directed mutagenesis kit (Stratagene). The seven sets of oligonucleotides listed in Table 3 were designed according to the protocol listed in the QuikChange manual. The initial mutagenesis reaction was done with 50 ng of pMW103 and 125 ng of each primer from primer set 1 in a total volume of 50 μl. In addition, 1 μl of *Pfu* Turbo polymerase was added to

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics^a	Reference or source
Intimin plasmids		
pMW103	Truncated *eae* (bp 1959–2804) in pQE31 (Qiagen)	13
pNR37	*eae* from pMW103, plant optimized	This study
Plant expression vectors		
pIBT210	CaMV 35S promoter, TEV 5′ UTR, Vsp 3′ UTR	18
pBTI210.3	CaMV 35S promoter, TMVΩ 5′ UTR, Vsp 3′ UTR	Hugh Mason, while at Boyce Thompson Institute for Plant Research
pBTI210.4	CaMV 35S promoter, TEV 5′ UTR, VspB 3′ UTR, with plant-specific signal peptide sequence	Hugh Mason, while at Boyce Thompson Institute for Plant Research
pGPTV-Kan	Binary vector, Kan^r	4
Plant-specific intimin expression vectors		
pNR49	*eae* from pNR37 with signal peptide sequence from pBTI210.4	This study
pNR50	*eae* from pNR37	This study

TABLE 3. Oligonucleotide sets for *eae* (nucleotides 1959 to 2804) plant expression optimization by QuikChange site-directed mutagenesis

Oligonucleotide	Sequence[a]
1-forward	GGGTCAGCCAGTTAA**C**AA**C**CAATCCGTTACATTCTCAACAAACTTTGG
1-reverse	CCAAAGTTTGTTGAGAATGTAACGGATTG**G**TT**G**TTAACTGGCTGACCC
2-forward	GGCTGCAATATGGTCA**A**TTAAACTGAAAGCAAGCGG
2-reverse	CCGCTTGCTTTCAGTTTAAA**T**TGACCATATTGCAGCC
3-forward	GGGAAAGTCACTTTGAATGGTAAAGGC**T**CTGTCGTAATTAAAGCC
3-reverse	GGCTTTAATTACGACA**G**A**G**CCTTTACCATTCAAAGTGACTTTCCC
4-forward	GGTGATAAGCAAACAGTAAGTTACACTAT**C**AAGGCACCGTCG
4-reverse	CGACGGTGCC**T**TG**A**TAGTGTAACTTACTGTTTGCTTATCACC
5-forward	GCTATGTCCATTTGCAAAAA**C**TT**G**TTACCATCCACACAGACGG
5-reverse	CCGTCTGTGTGGATGGTAA**C**AA**G**TTTTTGCAAATGGACATAGC
6-forward	GGTATTGTCAGATAT**C**TATGACTCATGGGGGGCTGCAAA**C**AA**G**TATAGCC
6-reverse	GGCTATA**C**TT**G**TTT**G**CAGCCCCCCATGAGTCATA**G**ATATCTGACAATACC
7-forward	GGATTAAACAGACATCTAGTGAGCA**A**CGTTCTGGAGTATCAAGC
7-reverse	GCTTGATACTCCAGAACG**T**TGCTCACTAGATGTCTGTTTAATCC

[a] Sites of mutagenesis are indicated in bold.

each reaction tube immediately prior to placing the sample tubes into the Mini-cycler. The samples were subjected to a 30-s hot start at 95°C and then 12 cycles of 95°C for 30 s, 55°C for 60 s, and 68°C for 12 min. After the cycles were completed, each reaction mixture was digested with *Dpn*I, and 1 to 5 μl of the digested reaction was transformed into the supercompetent cells provided with the kit according to the protocol outlined in the QuikChange manual. The transformed cells were plated on LB agar with ampicillin. Several ampicillin-resistant colonies were selected, and 3 ml of overnight LB broth culture was prepared from each colony. Bacteria were then harvested by centrifugation, and plasmid DNA was purified from the pelleted organisms. The *eae* sequences were amplified by PCR from the plasmids, and the PCR products were then eluted from an agarose gel and sequenced. Clones for which the sequence of the mutated, PCR-amplified *eae* gene had been confirmed were used in the next round of mutagenesis with the next set of mutagenesis primers. The final plant-optimized intimin clone from pMW103 (13) that contained all 13 nucleotide changes was called pNR37. The intimin protein encoded by this gene has an amino-terminal histidine tag and consists of the carboxy-terminal 261 amino acids of intimin from *E. coli* O157:H7 (Int$_{261}$).

Plant expression plasmid construction. The nucleotide sequence for the VspA signal peptide was amplified by PCR from pHB306 (37). A fragment that contained the nucleotide sequences for the tobacco etch virus (TEV) 5′ untranslated region (UTR) and the VspA signal peptide (encodes MAMKVLVFFFVATIL VAWGA) was ligated into the *Xho*I and *Sac*I sites of pIBT210 (18) to make pBTI210.4. Plasmid pBTI210.4 codes for a plant-specific signal peptide 5′ to a *Sac*I site, a cauliflower mosaic virus (CaMV) 35S promoter, the TEV 5′ UTR, and a VspB 3′ flank. The gene for Int$_{261}$ was amplified by PCR from pNR37 to include flanking *Sac*I sites; this fragment was cloned into the *Sac*I site of pBTI210.4. The *Eco*RI-*Hin*dIII fragment of the resultant plasmid was then cloned into pGPTV-Kan (4) to create pNR49.

Plasmid pBTI210.3 was created by digestion of pHB211.1 (37) with *Nco*I. This restriction enzyme-digested DNA was 3′ end-filled with Klenow polymerase and then further digested with *Xho*I and mung bean nuclease. The resultant blunt-ended vector fragment was ligated to generate pHB211. The *Hin*dIII-*Nco*I fragment of pHB211 that contained the sequences for the CaMV 35S promoter fused to the TMV 5′ UTR was ligated into pIBT210 to create pBTI210.3. Site-directed mutagenesis was used to introduce an *Nco*I site 5′ of the histidine-tagged Int$_{261}$ gene from pNR37. The plasmid was cut with *Nco*I and *Kpn*I, and the fragment that contained the Int$_{261}$ gene was cloned into pBTI210.3. The *Eco*RI-*Hin*dIII fragment of the resultant plasmid was cloned into pGPTV-Kan to create pNR50.

NT-1 cell culture and transformation. *Nicotiana tabacum* cv. Bright Yellow 2 (NT-1) cells (1) were grown at 25°C on a rotary shaker (150 rpm) in 40 ml of NT medium [Murashige minimal organics medium (Invitrogen Life Technologies, Carlsbad, Calif.), 30 g of sucrose per liter, 3 μM thiamine, 0.58 mM myoinositol, 1.3 mM KH$_2$PO$_4$, 1 μM 2,4-dichlorophenoxyacetic acid (Sigma, St. Louis, Mo.), 2.5 mM 2(*N*-morpholino)ethanesulfonic acid, pH 5.7]. These cells were subcultured (1 part cells to 19 parts NT medium) every 7 days. To facilitate transformation, binary vectors of interest were introduced into *A. tumefaciens* EHA105 by electroporation. *Agrobacterium* strains that contained the construct of interest were used to transform NT-1 cells as previously described (1). The NT-1 cells were kindly provided by the Boyce Thompson Institute for Plant Research.

Immunoblot analysis of Int$_{261}$ expressed in plant cells. A small amount of plant cell material (~0.03 g), resuspended in an extraction buffer (25 mM sodium

phosphate [pH 6.6], 100 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 10 μg of leupeptin per ml, and 50 mM sodium ascorbate), was disrupted by sonication (25-s pulse; 5 s on, 10 s off). The lysed material was clarified by centrifugation (12,000 × *g*, 3 min, 4°C), and proteins in the extract were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins in the Tris-glycine-sodium dodecyl sulfate-polyacrylamide gels were transferred onto nitrocellulose and probed with anti-intimin polyclonal antibodies. Antigen amounts were quantified by comparing the Western blot band intensity to that of a known concentration of control purified Int$_{261}$ (~35 kDa) from pMW103 (13) by use of the NIH Image (v. 1.61) program (38).

Mouse feeding assay. NT-1 cells or transgenic NT-1 cell clones that expressed Int$_{261}$ were grown in 40-ml suspension cultures to confluence (about 7 days). The cells were pelleted by gentle centrifugation, and culture medium was removed with a pipette. Five grams of NT-1 cell material was divided into aliquots in small, individual plastic weigh dishes, and 0.5 g of sucrose was added to each sample. A 7.5-μg dose of purified cholera toxin (CT) (Sigma) was also added to appropriate samples to serve as an oral adjuvant. The addition of this dose of CT did not appear to have any detrimental effects on the mice (i.e., loose stool or diarrhea). Female BALB/c mice of 16 to 18 g were made to fast overnight before they were allowed to eat the plant material ad libitum. All of the plant material was usually consumed within 8 h. The mice were bled by a tail vein cut, and fecal pellets were collected both prior to and after feeding. Blood samples were allowed to clot at room temperature for 15 min and centrifuged at 10,000 × *g* for 10 min to pellet red blood cells. A second, 2-min centrifugation step was used to further clarify each serum sample. These clarified samples were then stored at −20°C until assayed. Fecal samples were frozen, lyophilized overnight, and then homogenized in phosphate-buffered saline (PBS), pH 7.2 (for every 15 pellets, 0.8 ml of PBS was used). The resuspended fecal material was centrifuged for 5 min, and each supernatant was then transferred to a clean tube and stored at −20°C until assayed. Mice immunized intraperitoneally (i.p.) with purified His-tagged Int$_{261}$ (from pMW103 [13]) plus TiterMax (TiterMax USA Inc., Norcross, Ga.) (20 μg on day 0 and 10 μg on days 7 and 14) served as the positive control.

ELISA. For measurement of anti-intimin antibodies, an enzyme-linked immunosorbent assay (ELISA) was developed. For that purpose, U-bottomed 96-well microtiter plates were coated with 50 ng of purified histidine-tagged Int$_{261}$ from pMW103 (13) per well. Serum samples were diluted from a titer of 1:50 to 1:781,250 and fecal pellet extracts were diluted from 1:50 to 1:3,200 and incubated overnight at 4°C. The plates were washed, and goat anti-mouse immunoglobulin G (IgG) (1:3,000) or goat anti-mouse IgA (1:3,000) conjugated to alkaline phosphatase was added to the appropriate plates. The plates were then incubated at room temperature for 1 h. The antigen-antibody reactions were detected colorimetrically by incubation with tetramethylbenzidine peroxidase substrate (Bio-Rad) for 15 min followed immediately by measurement of the absorbance at 600 nm with an ELx800 microtiter plate reader (Bio-Tek Instruments Inc., Winooski, Vt.). The titer was defined as the reciprocal of the highest dilution of serum or fecal extract that gave an absorbance reading above both background and preimmune levels. Mice were considered responders if they had detectable titers in serum or fecal material at the last collection point. Mice with no titer or that lost titer by the last collection point were called nonresponders. The geometric mean of the responders' titers was used to determine the mean response per group.
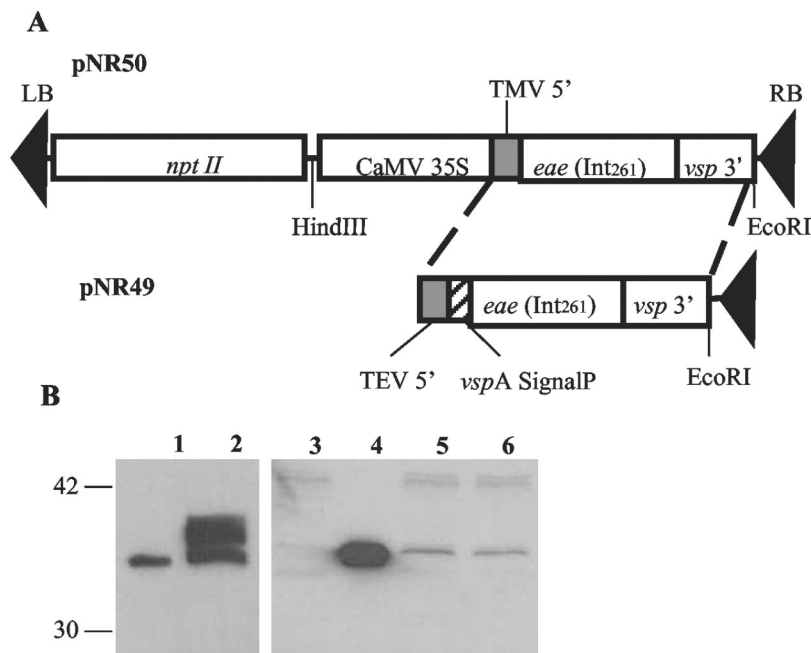
FIG. 1. Expression of Int$_{261}$ in NT-1 cells. (A) The Int$_{261}$ plant transformation vectors pNR50 and pNR49 are comprised of an Int$_{261}$ expression cassette with a CaMV 35S promoter and a neomycin phosphotransferase II expression cassette for detection of kanamycin resistance in successfully transformed clones. A VspA signal peptide was included in pNR49 to increase protein expression. (B) Immunoblot analysis of Int$_{261}$ expressed with or without the signal peptide. Int$_{261}$ (~35 kDa) purified by nickel affinity chromatography from pMW103 (13) (~75 ng) served as the positive control (lanes 1 and 4). NT-1 cells transformed with pGPTV-Kan vector only did not express Int$_{261}$ (lane 3). NT-1 cells transformed with pNR49 (CSP20) expressed several intimin-specific bands (lane 2). NT-1 cells transformed with pNR50 (C34) from both plate (lane 5) and broth (lane 6) cultures expressed a single intimin-specific band (~35 kDa).

E. coli **O157:H7 colonization of mice.** Female BALB/c mice of 16 to 18 g (Charles River Laboratories, Inc.) were made to fast overnight and fed a total inoculum of 10^8 to 10^9 CFU of *E. coli* O157:H7 strain 86-24 Str^r or 86-24 Str^r *eae*Δ10 in each of two doses administered 4 h apart. Fecal pellets were collected daily from individual mice and weighed, and serial dilutions of fecal pellet homogenates were prepared in PBS. These homogenates were plated on sorbitol-MacConkey agar (45) with 100 μg of streptomycin per ml to determine the CFU per gram of feces and the duration of colonization.

Statistical methods. Determination of the mean duration of colonization and statistical comparison of the duration of colonization among mouse groups were done with the SPSS 11.0 statistical package. The log-rank test was used to determine the statistical significance of the difference in duration of colonization among the mouse groups.

RESULTS

Construction of NT-1 plant cells that express the carboxy-terminal third of intimin. We began by generating intimin-expressing transgenic plant cells. For this purpose, we constructed two expression vectors (pNR49 and pNR50) with the *eae* gene from pMW103 (13) that we had optimized for expression in plants (Fig. 1A). The intimin protein encoded by this gene has an amino-terminal histidine tag and consists of the carboxy-terminal 261 amino acids of intimin from *E. coli* O157:H7 (Int$_{261}$). The carboxy terminus of intimin is the cell-binding domain (11, 12). *A. tumefaciens* EHA105 was used to transfer the DNA for Int$_{261}$ expression from the binary vectors into the nicotine-free tobacco cell line NT-1 (1, 20). Kanamycin-resistant calli were selected, and Int$_{261}$ expression was assayed by Western blot analysis (Fig. 1B).

Two independent transgenic NT-1 cell clones were used for the experiments described below. Clone 20 (CSP20) was cre-

ated by transformation of NT-1 cells with pNR49. This clone expressed Int$_{261}$ with a plant-specific signal peptide (~35 kDa) and produced 10 to 13 μg of Int$_{261}$ per g of total plant material. Clone 34 (C34) was generated by transformation of NT-1 cells with pNR50. This clone expressed Int$_{261}$ without a signal peptide and produced ~3 μg of Int$_{261}$ (~35 kDa) per g of total plant material (Fig. 1B).

Glycosylation of Int$_{261}$ expressed with a plant signal peptide. Although CSP20 expressed four times more antigen than C34, CSP20 accumulated higher-molecular-weight bands over time in culture (Fig. 2A). Moreover, this plant material, when fed to mice, elicited an antibody response (as assessed by ELISA) that recognized Int$_{261}$ purified from that clone, but not from bacterial cells. In addition, mice fed CSP20 were colonized at the same level and for the same duration as mice fed plant cells alone when challenged with wild-type *E. coli* O157:H7 strain 86-24 (data not shown). Based on these findings, we speculated that the higher-molecular-weight bands noted in Fig. 2A might represent a glycosylated form of Int$_{261}$ that was recognized by the murine host differently than the protein made by bacteria. To test this theory, Int$_{261}$ from CSP20 was purified by nickel affinity chromatography and then treated with trifluoromethanesulfonic acid (TFMS) (Glyco-Free deglycosylation kit from Glyco, Inc). TFMS nonselectively removes both N- and O-linked glycans from glycoproteins without altering the primary protein structure. The appearance of a band with a molecular mass consistent with that of control Int$_{261}$ (~35 kDa, purified from pMW103 [13] by nickel affinity chromatography) after treatment with TFMS
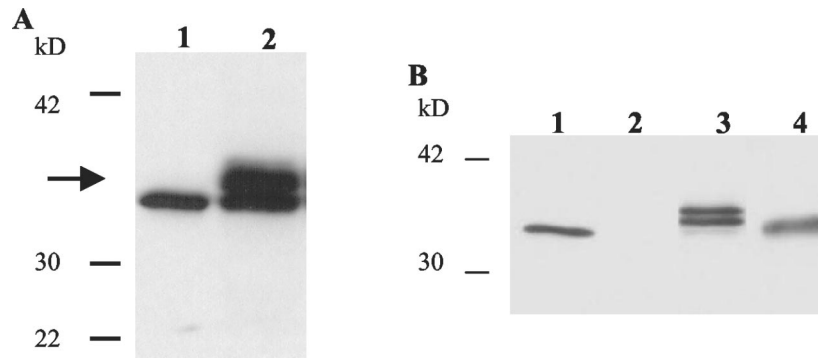
FIG. 2. Immunoblot analysis of Int_{261} from NT-1 cell clone CSP20. (A) The higher-molecular-weight, intimin-specific bands (arrow) accumulated as the culture aged. Lane 1, control Int_{261} purified from pMW103 (13); lane 2, Int_{261} from CSP20 in continuous culture for over 6 months. (B) Int_{261} from CSP20 treated with TFMS. Int_{261} purified by Ni affinity chromatography from pMW103 (13) shows the expected size of approximately 35 kDa (lane 1). Plant cell sonic extracts from NT-1 cells alone did not produce Int_{261} (lane 2). Int_{261} from CSP20 was purified by nickel affinity chromatography and showed two higher-molecular-weight intimin-specific bands compared to the control (lane 3). When material from lane 3 was treated with TFMS, a single band of approximately 35 kDa appeared that was consistent with the size of the control Int_{261} band (lane 4).

strongly suggested that Int_{261} from CSP20 was in fact glycosylated (Fig. 2B). We noted that the Int_{261} sequence contains two potential asparagine-linked glycosylation sites (NQS and NTS) that could be modified by glycosylation in this endoplasmic reticulum-targeted form. The two higher-molecular-weight bands observed for Int_{261} from CSP20 could result from glycosylation of either one or both of these N-linked sites. Since this probable glycosylation of Int_{261} in CSP20 adversely affected the immune response to this antigen, we elected to proceed with the C34 clone that expressed Int_{261} without the addition of a signal peptide and in an apparently unglycosylated state (Fig. 1B).

Generation of intimin-specific serum and fecal antibodies. We tested several different vaccination strategies with C34 in mice. The vaccine protocols and the serum (IgG) and fecal (IgA and IgG) ELISA results are summarized in Table 4. To maximize the immune response to orally administered Int_{261}, we tried a priming-boosting protocol (groups E and F) similar to that used to elicit an enhanced immune response to the B subunit pentamers of *E. coli* LT-B (27). Five of ten mice

primed by injection of purified Int_{261} from C34 and then fed nontransgenic plant material developed an intimin-specific fecal antibody response. These results suggest that even a single inoculation with Int_{261} from C34 can elicit a mucosal antibody response to intimin. Seven of ten mice primed by injection of Int_{261} from C34 and then boosted by feeding with C34 (with CT as an oral adjuvant) produced an intimin-specific fecal IgA response. The Fisher's exact test was used to compare percentages of responders, and we found that both groups E and F had a significantly higher number of fecal IgA responders than the negative control group, B ($P = 0.033$ and 0.003, respectively). There was no significant difference in percentages of responders between groups E or F and group A ($P = 0.175$ and 1.000, respectively). In addition, 3 of 10 mice developed an IgG response in serum, and serum samples from these mice blocked adherence of wild-type *E. coli* O157:H7 to HEp-2 cells compared to preimmune serum controls (data not shown).

Colonization of mice by *E. coli* O157:H7 is dependent on intimin. We determined that *E. coli* O157:H7 strain 86-24 Str[r] (35) reproducibly colonized female BALB/c mice at modest

TABLE 4. Immunogenicity in BALB/c mice of NT-1 cells that express Int_{261}

Group, immunization protocol	Vaccination method	IgG titer (no. of responders/total) in serum[a]	Titer (no. of responders/total) in feces[a]
A, i.p. Int_{261} from pMW103	20 µg of Int_{261} plus TiterMax i.p. on day 0, 10 µg of Int_{261} plus TiterMax i.p. on days 10 and 20	300,000 (10/10)[b]	560 (8/10) (IgA only)[c]
B, Fed NT-1 cells only	5 g of NT-1 cells fed on days 0, 7, and 14	<50 (0/10)	<50 (0/10)
C, Fed C34 cells only	5 g of C34 with 15 µg of Int_{261} fed on days 0, 7, and 14	<50 (0/10)	340 (4/10) (IgG and IgA)
D, Fed C34 cells with CT[d]	5 g of C34 with 15 µg of Int_{261} plus CT fed on days 0, 7, and 14	<50 (0/10)	280 (6/10) (IgG and IgA)
E, i.p. Int_{261} from C34, fed NT-1 cells only	~15 µg of Int_{261} from C34 plus TiterMax i.p. on day 0; 5 g of NT-1 cells fed on days 7 and 14	50 (1/10)	150 (5/10) (IgA only)
F, i.p. Int_{261} from C34, fed C34 cells with CT	~15 µg of Int_{261} from C34 plus TiterMax i.p. on day 0; 5 g of C34 cells with 15 µg of Int_{261} plus CT fed on days 7 and 14	80 (3/10)	180 (7/10) (IgA only)

[a] Serum and fecal pellet extracts were collected throughout the experiment and were evaluated by ELISA for the presence of antibodies against Int_{261}. The titer was defined as the geometric mean of the reciprocal of the highest dilution that gave an absorbance (A_{600}) above both preimmune and background levels. Responders were defined as mice that had a detectable titer at the final sample collection point (7 days after the final feeding or injection).

[b] Significantly higher IgG titer in serum and number of responders than other groups ($P < 0.0001$, two-sided t test; $P < 0.003$, Fisher's exact test).

[c] IgA titer in feces was significantly different than that for groups E and F ($P = 0.019$ and 0.013, respectively [two-sided t test]). The number of fecal IgA responders was significantly different from that for groups E and F ($P = 0.175$ and 1.000, respectively [Fisher's exact test]).
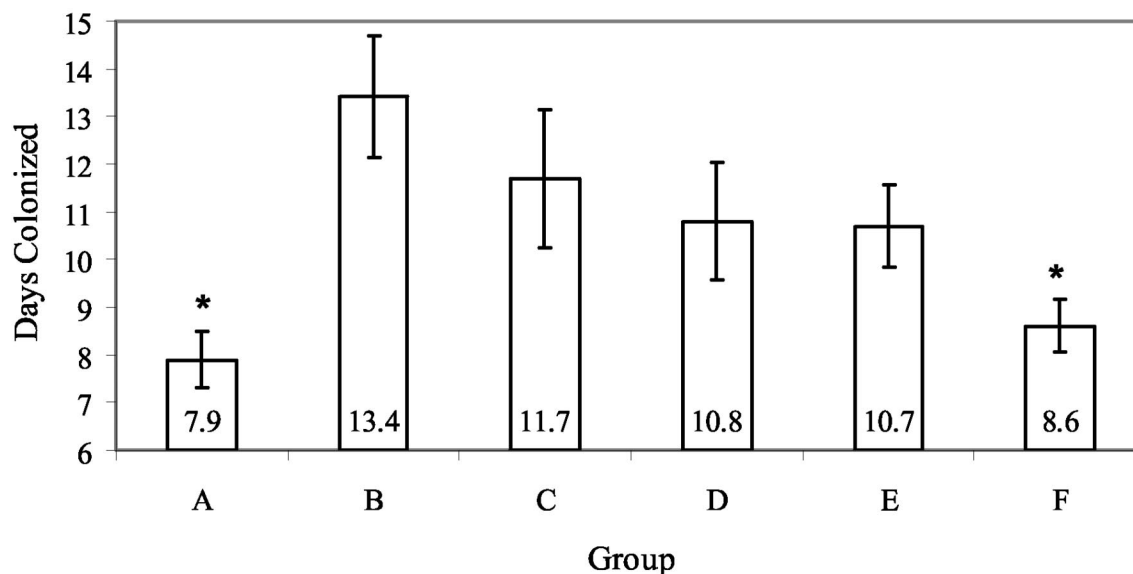
[d] CT, cholera toxin (7.5 µg; Sigma).

FIG. 3. Duration of *E. coli* O157:H7 colonization in mice immunized with Int_{261}. The same groups of mice as those shown in Table 4 were challenged with wild-type *E. coli* O157:H7. Mice from both groups A and F (∗) showed a statistically significant ($P < 0.002$; log-rank test) reduction in the duration of colonization compared to group B. No other groups demonstrated a statistically significant reduction compared to group B.

levels for approximately 10 days (mean, 6.4 to 13.4 days for unimmunized mice). Furthermore, in an experiment limited to 7 days, *E. coli* O157:H7 86-24 Str^r colonized BALB/c mice a mean of 6.4 days, whereas the intimin-negative mutant 86-24 Str^r $eae\Delta10$ showed a statistically significant reduction in the duration of colonization (mean, 3.2 days; $P < 0.005$; log-rank test).

Challenge of vaccinated mice with *E. coli* O157:H7. With this intimin-dependent mouse model of *E. coli* O157:H7 colonization, we orally infected the same groups of immunized mice described in Table 4 with strain 86-24 Str^r. We found that mice primed with an i.p. injection of Int_{261} from C34 and then fed homologous C34 plant material with CT showed a statistically significant ($P < 0.002$; log-rank test) reduction in the duration of colonization (as measured by fecal shedding) compared to the negative control mice that were fed nontransgenic NT-1 cells only (Fig. 3). The mice that were primed and boosted with Int_{261} from transgenic plant material also exhibited a statistically significant decrease in the duration of bacterial colonization compared to mice that were only immunized i.p. with Int_{261} from C34 and then boosted with nontransgenic plant material ($P = 0.033$; log-rank test). These results suggest that the feeding of transgenic, Int_{261}-expressing plant material plays a significant role in boosting the immune response to Int_{261} and in reducing the duration of bacterial colonization. The positive control mice, injected i.p. three times with highly purified Int_{261} from pMW103 (13), also displayed a statistically significant reduction in the duration of colonization versus the negative control ($P < 0.002$; log-rank test). There was no difference in the duration of shedding between the positive control immunization method and the priming-boosting protocol with oral delivery of transgenic plant material that expressed Int_{261} as a boost. This last observation suggests that immunization with a plant-based oral vaccine can induce an immune booster response sufficient to provide protection from a challenge with wild-type *E. coli* O157:H7.

DISCUSSION

Many pathogens infect or invade via mucosal surfaces, so the capacity of plant-based vaccines to induce mucosal immunity is a great advantage. Plant cells act as a natural microencapsulation system to protect the vaccine antigens from being degraded in the upper digestive tract before they can reach the gut-associated lymphoid tissue. Recent studies suggest that plant-based oral vaccines can significantly boost mucosal immune responses primed by parenteral injection (27, 44). Parenteral priming of the immune system may allow the gut-associated lymphoid tissue to react successfully to the small amounts of antigen delivered during oral immunization and thus decrease the possibility of inducing oral immunotolerance to plant-based and other orally delivered vaccine antigens. Our studies support these recent findings by demonstrating that parenteral priming of mice with intimin purified from transgenic plant cells can assist in the development of an intimin-specific fecal immune response when these mice are subsequently boosted with oral feeding of the same intimin-expressing transgenic plant material. Furthermore, in this study mice that were parenterally primed and then given an oral booster showed a statistically significant decrease in the duration of colonization by wild-type *E. coli* O157:H7 upon challenge ($P < 0.002$; log-rank test). Mice immunized entirely by oral feeding did exhibit a reduction in the duration of colonization versus unimmunized mice, but the reduction was not statistically significant. These results suggest that a combination of vaccination strategies with a vaccine antigen produced in and delivered by transgenic plants can function in inducing beneficial, specific immune responses. Further studies

are needed to determine whether an oral-only immunization route would suffice if the antigen levels in the plant material or the number of doses delivered were increased so as to induce an immune response sufficient to result in a reduction in the duration of bacterial colonization. Such approaches might eliminate the requirement for priming by parenteral injection of intimin.

Our discovery that nonglycosylated bacterial proteins can be glycosylated in transgenic plant systems highlights an issue previously not reported in other studies of vaccine antigen expression in transgenic plants. We found that the glycosylation of Int_{261} expressed in NT-1 cells adversely affected the immunogenicity of the molecule and resulted in an aberrant immune response that was unable to block adherence of *E. coli* O157:H7. Although the addition of a plant-specific signal peptide increased the vaccine antigen expression levels in the plant cells, the signal peptide may have caused the protein to be glycosylated in the plant expression system by trafficking the newly expressed protein directly into the endoplasmic reticulum, where it would be more accessible to the glycosylation pathways in the Golgi apparatus. The removal of the plant-specific signal peptide from the Int_{261} plant expression constructs resulted in decreased protein expression but appeared to produce an unglycosylated protein. Our experience emphasizes one of the challenges involved in the successful production of bacterial vaccine antigens in transgenic plant systems.

Our finding that these transgenic cells synthesize Int_{261} in a conformation that is immunologically equivalent to the native protein underscores the feasibility of transgenic plant-based systems for expression and delivery of oral vaccines. For further vaccine development, we plan to express a signal sequence-free Int_{261} construct in a whole plant, perhaps by use of recently developed chloroplast transformation and expression models (19) that maximize protein expression and minimize protein modification (such as glycosylation). The selected plant system will likely be alfalfa because it is compatible with oral administration to cattle and is amenable to both nuclear and chloroplast transformation methods. Debate still exists as to whether feedlot food type, holding pen cleanliness, or water quality management would offer the benefit of reducing EHEC O157:H7 colonization levels in cattle. One might hypothesize that removing or reducing environmental sources of EHEC O157:H7 infection, such as soiled bedding or contaminated water sources, would reduce the prevalence of EHEC O157:H7 in cattle. Unfortunately, these hypotheses have not been adequately tested. However, the notion that reduction of sources of EHEC O157:H7 infection and contamination would reduce not only the prevalence of EHEC O157:H7 in cattle, but also the risk of human infection through other environmental sources such as drinking or recreational water and fruits and vegetables, has strong intuitive appeal. We believe that the use of transgenic plants for the production and delivery of an EHEC O157:H7 vaccine for cattle, alone or in conjunction with other potential control methods (5), could lead to a significant decrease in the level of colonization or percentage of cattle infected with pathogenic *E. coli* O157:H7. Such a reduction will likely translate into a decline in beef carcass and environmental contamination by *E. coli* O157:H7 and a decrease in transmission of the bacterium to humans.

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