Shiga toxin-producing Escherichia coli (STEC) comprises an emerging group of zoonotic enteric pathogens of animals and humans. Natural and experimental infection of calves with STEC may result in acute enteritis or subclinical infection, depending on serotype- and host-specific factors. To quantify intestinal secretory and inflammatory responses to STEC in the bovine intestine, serotypes that are associated with human disease (O103:H2 and O157:H7) were introduced into ligated mid-iletal loops in gnotobiotic and conventional calves, and fluid accumulation and recruitment of radiolabeled neutrophils were measured after 12 h. STEC serotype O103:H2, but not serotype O157:H7, elicited strong enteropathogenic responses. To determine if the inflammatory response to STEC O103:H2 in calves requires Shiga toxin 1 or intimate bacterial attachment to the intestinal epithelium, defined mutations were made in the stx1, eae, and tir genes. Our data indicate that some STEC induce intestinal inflammatory responses in calves by a mechanism that is independent of A/E-lesion formation, intimin, or Shiga toxin 1. This may have implications for strategies to reduce STEC carriage in cattle.

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Shiga toxin-producing Escherichia coli (STEC) comprises a group of attaching and effacing (A/E) enteric pathogens of animals and humans. Natural and experimental infection of calves with STEC may result in acute enteritis or subclinical infection, depending on serotype- and host-specific factors. To quantify intestinal secretory and inflammatory responses to STEC in the bovine intestine, serotypes that are associated with human disease (O103:H2 and O157:H7) were introduced into ligated mid-iletal loops in gnotobiotic and conventional calves, and fluid accumulation and recruitment of radiolabeled neutrophils were measured after 12 h. STEC serotype O103:H2, but not serotype O157:H7, elicited strong enteropathogenic responses. To determine if the inflammatory response to STEC O103:H2 in calves requires Shiga toxin 1 or intimate bacterial attachment to the intestinal epithelium, defined mutations were made in the stx1, eae, and tir genes. Our data indicate that some STEC induce intestinal inflammatory responses in calves by a mechanism that is independent of A/E-lesion formation, intimin, or Shiga toxin 1. This may have implications for strategies to reduce STEC carriage in cattle.

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a pronounced T helper cell type 1 (Th1) mucosal immune response characterized by mucosal thickening and infiltration of CD4+ T cells (30, 53). A. 

**Construction of mutations in eae, tir, and stx1.** Defined mutations were built into E. coli strain PMK5 ( intimin type e; Tir type b; stx1AB [43]). To construct an eae mutant, the PMK5 eae gene was first amplified by PCR using primers eae-sens (5’TATGATGATCTATGGCGTCTGT 3’) and eae-asens (5’TATTCTAAAAAGAATGATGTC 3’) and cloned into pCR-XL-TOPO (Invitrogen). A 3.3 kb EcoRI fragment containing the cloned eae gene was then subcloned into pUC19, resulting in plasmid pUC-eae. An apht T gene lacking a transcription terminator was excised from pB8315 using HindIII and inserted in an E. coli cloning site in pUC-eae 993 bp downstream of the eae start codon. A ca. 4.5 kb PvuII fragment containing the inactivated eae gene was then subcloned into Smal-cut pNG101, generating pNGN-eae-kan8.

To inactivate the tir gene, the PMK5 tir region was amplified using the primers orf19-sens (5’GAAAGTTGATGAGCAGTGTGG 3’) and orf19-asens (5’TATTCTAAAAAGAATGATGTC 3’) and cloned into the HindIII fragment of pB8315 containing the pshT cassette gene (the insertion is 515 bp downstream of the tir start codon). The inactivated tir gene was excised on a 2.5 kb EcoRI-AflIII fragment, the ends were filled in using T4 polymerase, and the gene was cloned into Smal-cut pNG101, generating pNGN-tir-kan8.

Approximately 3 μg of pNGN-eae-kan8 and pNGN-tir-kan8 was separately used for electroporation of PMK5 in 0.2-cm-electrode gap cuvettes at 2.5 or 12.5 kV/cm and 25 μF. Using a 5-ms pulse at 4°C and a Bio-Rad GenePulsor. After incubation in SOC medium for 1 h, merodiploids were selected on LB agar containing streptomycin and kanamycin. Double recombinants were selected by growing the merodiploids to late logarithmic phase in LB broth lacking streptomycin and plating them onto LB agar (minus NaCl) containing 5% (wt/vol) sucrose and kanamycin at 30°C. Strains resistant to sucrose and kanamycin but sensitive to streptomycin were shown to carry the mutations by PCR and Southern hybridization.

The PMK5 stx1 mutant contains a large internal deletion of the stxLA gene that also removes the start codon for the B subunit. Sequences flanking the PMK5 stx1A gene were amplified separately by PCR with Vent proofreading DNA polymerase (New England Biolabs) using the primer pairs stx3 (5’ATA TATGATGATCTATGGCGTCTGT 3’) plus stx4 (5’ATTAAATAAAGTAATATTATTATTATTATTATTATGTCG 3) plus stx5 (5’TGATGATCGTCAAGGAAATGATGTC 3’) and stx1 (5’TACATTGCTTCTTCGAAAACATTATATATGTCG 3) plus stx6 (5’ATATAGTACGCTTCAACACATCTATACGATCAG 3) (based on the sequence of the bacteriophage R93 stx gene [accession no. M19473]). The primary PCR products were gel purified and combined in an overlapping PCR (31) using the flanking primers stx1 and stx6. The secondary PCR product was then cloned into pCVD442 using Saci sites incorporated into the primers. The resulting plasmid, pCVDdetI, was introduced into a nalidixic acid-resistant derivative of PMK5 by conjugation from S17-1pir, and a merodiploid was selected on LB agar containing ampicillin. Double recombinants were selected on sucrose-containing media screen for the deletion by colony PCR, and verified by Southern hybridization. The deletion results in fusion of the region encoding the StxA N-terminal 44 amino acids to a stop codon within the stxB sequence, ensuring that a fusion protein with the B subunit cannot be made.

**Quantification of enteropathogenesis.** A Hereford-Aberdeen Angus hysterotomy-derived gnotobiotic calf was delivered into a positive-pressure isolator (10), fed sterile condensed milk (Carnation; Nestlé), and maintained aerotopically for 7 days prior to use. Conventional Friesian bull calves (25 to 30 days old) were fed on powdered milk and screened for the excretion of STEC or Salmonella by enrichment on Sorbitol MacConkey agar containing tellurite and caffeine (Oxoid, Basingstoke, United Kingdom) or Brilliant Green agar, respectively. The calves were observed twice daily for 7 days prior to surgery, and animals with diarrhea or excreting STEC were excluded from the analysis. The bovine ligated ileal loop assay has been described previously (61). Briefly, calves were anesthetized for the duration of the experiment (ca. 14 h) with pentobarbitten sodium (Sagatal; 0.44 mg/kg of body weight), and the mid ileum was flushed with intestinal wash solution (5.61 g of NaCl/liter, 0.11 g of KCl/liter, 1.09 g of KH2PO4/liter, 0.16 g of Na2HPO4/liter, 0.74 g of trisodium citrate/liter, and 5 g of N-acetyl cysteine/liter). The calves were maintained at 38.5 to 39.5°C by use of heated mats. Loops 6 cm in length with 1-cm spacers were ligated with surgical silk and inoculated with 5 ml of bacterial culture (ca. 5 x 108 CFU) or sterile medium as a negative control. Each strain was tested in triplicate in any one animal, and the experiment was repeated in a total of three calves. Approximately 70 ml of mm-diameter dish on glass coverslips and grown for 18 h at 37°C in a 5% CO2 atmosphere.

**Routine DNA manipulation.** Standard procedures were used for DNA extraction, cloning, PCR, and the verification of mutants by Southern hybridization (50).
venous blood was collected in 12 ml of acid citrate, and neutrophils were isolated according to the method of Carlson and Kaneko (4). The neutrophils were resuspended in 2 ml of Ca2+-free Tyrodes buffer, radiolabeled by mixing the suspension with 3.7 MBq of 111In-oxinate (Mallinckrodt, Petten, The Netherlands) for 5 min, washed in Tyrodes buffer, and reinserted into the cail within 2 h of inoculation of the loops with bacteria.

Twelve hours after inoculation, enteropathogenesis was assessed with respect to fluid accumulation, neutrophil infiltration, and histological changes. The animals were killed by an overdose of pentobarbitone, and 5 ml of 10% neutral buffered formaldehyde was injected into the loops to inactivate the bacteria prior to collection of the loop contents and mucosa. Fluid secretion was measured as a ratio of the volume of fluid accumulated to loop length (V/L), and the mean ± standard error of the mean (SEM) was calculated from the replicates. Radiotracer activity associated with the loop contents and mucosa was detected using a Wallac standard error of the mean (SEM) was calculated from the replicates. Radiotracer activity associated with the loop contents and mucosa was detected using a Wallac 1275 gamma counter and was corrected for differences in loop length. Neutrophil infiltration was expressed as the ratio of 111In activity in the test loops to 111In activity in the negative control loops. The mean neutrophil influx ratio (± SEM) from the three calves is shown.

FIG. 1. Enteropathogenic responses to STEC strains 84-289 (O157:H7) and PMK5 (O103:H2) and sterile brain heart infusion (BHI) broth in the intestines of ca. 28-day-old conventional calves. (A) Fluid accumulation. Each strain was tested in triplicate mid-ileal loops, and the mean V/L ratio (± SEM) from the three independent experiments is shown. (B) Neutrophil infiltration. The total 111In activity in the loop contents and mucosa was corrected for loop length, and the mean of three measurements in the mid-ileum was derived. Neutrophil influx is the ratio of the mean 111In activity in the loop contents and mucosa compared with control mucosa. A pseudomembrane containing large numbers of neutrophils was present in the lumens of inoculated ileal loops. Fluid accumulation was significantly greater in loops injected with strain PMK5 (P < 0.05) than in the control loops injected with sterile medium (Fig. 1). The enteropathogenic responses to STEC serotype O157:H7 were not significantly greater than those to the negative control (P values >0.05), and this is consistent with recent observations (48). No differences in the level of adherence of the O157:H7 and O103:H2 strains to bovine ileal mucosa could be detected by immunostaining and confocal microscopy (data not shown).

Enteropathogenic responses to E. coli PMK5 were also tested in midileal ligated loops in a 7-day-old gnotobiotic calf. Fluid accumulation was significantly greater in loops injected with PMK5 (V/L, 0.63 ± 0.04) compared to control loops (V/L, 0.40 ± 0.03; P < 0.05), and the mean neutrophil influx was 12.6-fold greater than in loops injected with sterile medium. The enteropathogenic responses to strain 84-289 were not significantly greater than those to the controls and were not improved by extending the in vivo incubation time to 18 h, by inoculation of loops with mid-logarithmic-phase bacteria grown in tissue culture medium (Dulbecco's modified Eagle's medium) or by inoculating loops constructed in the distal ileum (data not shown).

Microscopic analysis of hematoxylin- and cosin-stained sections of intestinal mucosa from ileal loops inoculated with PMK5 revealed lesions similar to those seen in natural and experimental infection of susceptible calves with STEC (Fig. 2) (7, 8, 29, 46). Subjective analysis of PMK5-infected mucosa indicated slight villus atrophy, with foci of exfoliated or rounded epithelial cells, especially on villus tips, and diffuse infiltration of neutrophils into the lamina propria and submucosa compared with control mucosa. A pseudomembrane containing large numbers of neutrophils was present in the lumen of inoculated ileal loops.

RESULTS

Bovine enteropathogenic responses to different STEC serotypes. We investigated the abilities of STEC serotypes O103:H2 and O157:H7 to elicit secretory and inflammatory responses in a bovine ligated ileal loop assay. Ligated loops were constructed in the mid-ileum in three ca. 28-day-old conventional calves, and fluid accumulation and infiltration of 111In-labeled neutrophils was quantified 12 h after inoculation of the loops. Fluid accumulation and neutrophil infiltration were significantly greater in the loops inoculated with strain PMK5 (P < 0.05) than in the control loops injected with sterile medium (Fig. 1). The enteropathogenic responses to STEC serotype O157:H7 were not significantly greater than those to the negative control (P values >0.05), and this is consistent with recent observations (48). No differences in the level of adherence of the O157:H7 and O103:H2 strains to bovine ileal mucosa could be detected by immunostaining and confocal microscopy (data not shown).

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overlying the epithelium (Fig. 2A and 3C), which is consistent with the high $^{111}$In activity detected in the loop contents.

**Enteropathogenic responses to PMK5 eae, tir, and stx1 mutants.** Defined mutations were made in the PMK5 eae, tir, and stx1 genes by allelic exchange using positive-selection suicide plasmids. Insertions in eae and tir were shown to result in a lack of intimate attachment to HeLa cells in vitro and absence of ability to nucleate F-actin under sites of bacterial adhesion in FAS tests (Fig. 3A). We were unable to detect intimin in lysates of the PMK5 eae mutant by Western blotting using antiserum raised against the conserved domain of intimin (data not shown). A deletion in the gene encoding the Stx1 catalytic A subunit was created by overlapping PCR and shown to abolish cytotoxicity of PMK5 culture supernatants for Vero cells (data not shown). Deletion of stx1 did not influence the ability of PMK5 to adhere to cultured epithelial cells or the ability to condense F-actin at sites of bacterial attachment (Fig. 3A).

Enteropathogenic responses to the PMK5 and isogenic eae, tir, and stx1 mutants were tested in triplicate ligated loops in three ca. 28-day-old conventional calves (Fig. 4). To account for interanimal variation, neutrophil influx and the volume of fluid accumulated were expressed as a percentage of the responses to the wild-type strain in the same calf. The eae, tir, and stx1 mutations did not significantly impair neutrophil infiltration or fluid secretion compared to those with wild-type PMK5 ($P$ values, $>0.05$).

**Confocal laser scanning microscopy of infected ileal mucosa.** To confirm that the eae and tir mutations impaired the ability of PMK5 to interact with the bovine intestinal epithelium, sections of ileal mucosa from ligated loops in 28-day-old calves were stained for bacteria and F-actin and examined by confocal microscopy. Relatively few bacteria could be seen in association with the mucosa, even in loops inoculated with the wild-type strain. Figure 3B shows rare microcolonies of wild-type bacteria that were detected on villus tips. No such microcolonies were detected in loops inoculated with the eae and tir mutants. Figure 3C shows typical fields, in which very few bacteria are associated with the ileal mucosa and most bacteria are associated with neutrophils in the intestinal lumen. Analysis of the luminal infiltrate at high magnification revealed that some, but not all, bacteria were located within neutrophils (data not shown).

**DISCUSSION**

We report the use of a ligated ileal loop assay to characterize enteropathogenic responses to STEC in the bovine intestine. An STEC strain isolated from a case of HUS (PMK5; serotype...
O103:H2) induced significant secretory and inflammatory responses during the 12-h assay. The elevated levels of $^{111}$In activity in the contents and mucosa of PMK5-inoculated loops correlated with the presence of an inflammatory infiltrate similar to that seen in calves infected with bovine virulent STEC (29, 46). The recruitment of neutrophils in response to PMK5 appears to be specific, since injection of similar numbers of E. coli O157:H7 cells did not induce significant enteropathogenic

FIG. 3. Confocal laser scanning microscopy showing PMK5 and isogenic eae, tir, and stx mutants interacting with HeLa cells or intestinal mucosa. (A) FAS of HeLa cells incubated with PMK5 and the isogenic mutants indicates that the eae and tir mutants are incapable of nucleating F-actin under the sites of bacterial adhesion. Magnification, ×1,000. (B and C) Sections of mid-ileal mucosa from inoculated loops stained for F-actin and bacteria. The images in column B show rare microcolonies formed by the wild type and the stx mutant. Microcolonies were not detected with the eae and tir mutants; however, small numbers of bacteria could occasionally be seen on the ileal mucosa. Column C contains typical fields showing an almost complete absence of bacteria on the intestinal epithelium and numerous bacteria and neutrophils in the gut lumen. Magnification (B and C), ×630. Green, F-actin stained with Oregon green 514-phalloidin; red, bacteria detected with rabbit anti-O103 typing serum and anti-rabbit immunoglobulin-Alexa488.
responses. Confocal microscopy of midileal mucosa from PMK5-inoculated loops revealed small foci of bacteria on the intestinal epithelium, similar to those detected by immunohistochcmical staining of ileal mucosa from *E. coli* O157:H7-infected neonatal calves (8) and O157:H7-infected ileal loop mucosa (data not shown). These data, combined with observations that STEC colonizes the bovine small intestine in vivo (7, 8, 29) and can induce A/E-lesion formation on bovine distal ileum both in vivo and in vitro (7, 8, 47), indicate that the ligated ileal loop assay is physiologically relevant and can be used to dissect bacterial and host factors influencing enteropathogenic responses.

The absence of enteropathogenic responses to *E. coli* O157:H7 may be explained by the age-related susceptibility of calves to these bacteria (5, 8). *E. coli* O157:H7 has been reported to produce colonic edema and diarrhea only in colostrum-deprived neonatal calves (<36 h old) (8). Infection of 5-day-old gnotobiotic calves with O157:H7 is asymptomatic (63). The reason PMK5 elicits stronger enteropathogenic responses than *E. coli* O157:H7 in bovine midileal ligated loops remains obscure. A human O103:H2 STEC formed extensive A/E lesions in the colons of gnotobiotic piglets with foci of neutrophils in the lamina propria and lumen (28), and O103:H2 STEC is associated with enteric disease in goats (15); however, to our knowledge the pathogenesis of O103:H2 STEC strains in calves has not been tested. Baker et al. (2) reported considerable variation in the virulence of O157:H7 strains of human and bovine origin in a gnotobiotic-piglet model, and it is possible that PMK5 possesses traits that are absent in the O157:H7 strain tested.

We sought to determine if the enteropathogenic responses to *E. coli* PMK5 required intimin and/or A/E-lesion formation by testing defined *eae* and *tir* mutants in the loop model. Intimin is required for colonization of colostrum-deprived neonatal calves and piglets by *E. coli* O157:H7, and *eae* null mutants fail to induce enteritis in such animals (7, 14, 39, 59). However in bovine ligated ileal loops, an *eae* mutant of PMK5 induced inflammatory and secretory responses similar to those induced by the wild-type strain. We have reported similar findings from an infant rabbit model of REPEC infection (37). While REPEC O103:H2 intimin is required for colonization of the rabbit intestine and the induction of diarrhea, infiltration of inflammatory cells still occurred in response to a defined *eae* mutant without destroying the brush border or general architecture. Thus, infiltration of neutrophils is probably not sufficient to induce diarrhea in the infant rabbit model. Previous studies using anti-CD18 antibody administered to STEC-infected rabbits had indicated that the host inflammatory response is implicated in the induction of diarrhea (18). Further evidence suggesting that intimin is not essential for induction of the enteropathogenic responses is provided by the finding that *eae*-negative STEC strains are associated with diarrhea in humans (16, 19) and calves (62). In addition, EPEC *eae* is not required for activation of NF-κB in epithelial cells, which in turn initiates transcription of the gene encoding IL-8 (52).

There is evidence for a direct role for intimin in stimulating mucosal inflammatory responses to *C. rodentium* in mice (30). Intimin drives pronounced Th1 immune responses in the murine gut and the purified cell-binding domain of intimin (Int280) augments mitogen-stimulated T-cell proliferation in a concentration-dependent manner (30). The Int280 domain binds not only to Tir but also to β1 integrins on T cells (23), and it is possible that this triggers signaling events that result in murine colonic hyperplasia. Our results indicate that host-and/or strain-specific differences between the animal models of A/E *E. coli* infections exist and that factors in addition to

![Graph A](image1.png)  
**A.** Fluid accumulation. To correct for interanimal variation, the mean V/L ratio for each mutant strain was expressed as a percentage of the mean V/L ratio for the wild type in the same calf. The mean percentage plus SEM from three independent experiments is shown. (B) Neutrophil influx. The total 111In activity in the contents and mucosa of test loops was corrected for loop length and expressed as a ratio to the activity in negative control loops. Neutrophil influx for the mutant strains was then calculated as a percentage of the influx for the wild type, and the mean of the three experiments plus SEM was derived.
intimin are involved in PMK5-induced intestinal inflammatory responses in calves. However, we cannot exclude the possibility that in the intact host intimin facilitates the delivery of factors chemotactic to neutrophils by enabling the bacteria to colonize the intestinal epithelium. The notion that factors encoded outside the LEE may influence STEC pathogenesis is supported by the finding that Stx-minus STEC can impair epithelial barrier function and ion transport in monolayers of T84 cells in the absence of A/E-lesion formation (35).

Mutations in the genes encoding intimin and its translocated receptor did not result in a complete lack of association of PMK5 with the bovine midileal epithelium. Intimin-independent association of STEC with bovine intestinal epithelia has been reported following oral inoculation of one calf with an O157:H7 eae mutant (7) and has also been reported in rabbits infected with a REPEC eae mutant (34). This indicates that accessory adhesins mediate the binding of STEC to intestinal mucosa. A number of loci have been implicated in the attachment of STEC to epithelial cells, including those for LEE-encoded EspA filaments (17), Efa (42), and Iha (56) and several other genes identified by transposon mutagenesis of E. coli O157:H7 (57).

We did not detect any role for Stx1 in the induction of inflammatory responses to PMK5 in calves. The finding that Stx does not influence enteropathogenesis is supported by reports that Stx-negative E. coli O157:H7 strains produce diarrhea and colonic edema in neonatal calves (60) and gnotobiotic piglets (60), with no obvious histopathological differences from the Stx-producing parent strain. Pruumboom-Brees et al. recently reported that cattle lack intestinal receptors for Shiga toxin (48) and concluded that this may explain why cattle are more resistant to the enterotoxic effects of Shiga toxin than are rabbits. Indeed, the authors reported that 10^8 to 10^9 50% more resistant to the enterotoxic effects of Shiga toxin than are piglets (60), with no obvious histopathological differences from STEC exposure to this response and to study the role of bacterial factors in this process.

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