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Bovine Immune Response to Shiga-Toxigenic *Escherichia coli* O157:H7[▽]

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Although cattle develop humoral immune responses to Shiga-toxigenic (Stx$^+$) *Escherichia coli* O157:H7, infections often result in long-term shedding of these human pathogenic bacteria. The objective of this study was to compare humoral and cellular immune responses to Stx$^+$ and Stx$^-$ *E. coli* O157:H7. Three groups of calves were inoculated intrarumenally, twice in a 3-week interval, with different strains of *E. coli*: a Stx2-producing *E. coli* O157:H7 strain (Stx2$^+$O157), a Shiga toxin-negative *E. coli* O157:H7 strain (Stx$^-$O157), or a nonpathogenic *E. coli* strain (control). Fecal shedding of Stx2$^+$O157 was significantly higher than that of Stx$^-$O157 or the control. Three weeks after the second inoculation, all calves were challenged with Stx2$^+$O157. Following the challenge, levels of fecal shedding of Stx2$^+$O157 were similar in all three groups. Both groups inoculated with an O157 strain developed antibodies to O157 LPS. Calves initially inoculated with Stx$^-$O157, but not those inoculated with Stx2$^+$O157, developed statistically significant lymphoproliferative responses to heat-killed Stx2$^+$O157. These results provide evidence that infections with STEC can suppress the development of specific cellular immune responses in cattle, a finding that will need to be addressed in designing vaccines against *E. coli* O157:H7 infections in cattle.

Enterohemorrhagic *Escherichia coli* (EHEC) is a common cause of hemorrhagic colitis (HC) in very young, elderly, and immunocompromised humans (16, 30). In the United States, the EHEC serotype most often associated with bloody diarrhea is O157:H7, but non-O157 EHEC serotypes have caused significant outbreaks in the United States and abroad (30, 35). Up to 10% of patients with EHEC-associated HC develop hemolytic uremic syndrome, with resulting renal and neurological damage and, occasionally, death (21). All EHEC strains produce one or more cytotoxins called Shiga toxins (Stx1 and Stx2) (27), which target endothelial cells, are believed to mediate much of the tissue damage during HC and hemolytic uremic syndrome (29, 37), and can influence the duration of Stx-producing *E. coli* (STEC) shedding by ruminants (7).

Ruminants are important sources of EHEC O157:H7 strains because they frequently shed STEC in their feces (26). Single STEC O157:H7 clones can be repeatedly isolated from a herd (6, 14), implying that these clones persist within that herd. This could result from frequent transmission between and reinfection of different animals (2) or from a true persistence in single animals (3, 7, 8). Experimental infections of calves revealed that several bacterial factors involved in the "attaching and effacing" adherence of *E. coli* O157:H7 to epithelial cells (e.g., intimin [10], other products of the locus of enterocyte effacement [11], and *efa-1* [32]) promote bacterial colonization of the intestinal mucosa. However, recent studies failed to link the induction of attaching and effacing to the establishment of persistent infections in animals (4, 18).

Several lines of evidence support the hypothesis that Stx can suppress the bovine immune response. Stx1 targets bovine peripheral blood (23, 25, 31) and intraepithelial lymphocytes (IEL) (22) in vitro. Stx1 binds to Stx receptor-expressing (Gb_3-/CD77-positive) lymphocytes at early activation stages (31) and blocks the proliferation of distinct lymphocyte subpopulations (CD8α^+ T cells, B cells) in vitro (25). A significant portion of bovine CD8α^+ IEL expresses Stx-receptors in situ (22). Inoculation of ligated ileal loops in 2-week-old calves with Stx1-producing STEC reduces the number of CD8α^+ IEL within 12 h (24). Direct evidence for a suppression of immune cell functions in the course of STEC infections in cattle, as observed during experimental STEC infections in pigs (5), has been lacking. Although bovine lymphocytes are sensitive to minute concentrations of Stx1 and Stx2 (1, 12, 13, 25), Stxs do not completely abolish the development of specific immune responses in naturally and experimentally infected cattle. Antibodies against O157 lipopolysaccharide (LPS), Stx1, and Stx2 are frequently detected in bovine sera and mucosal secretions (colostra) (17, 28). Since Stx1 suppresses bovine lymphocytes in vitro without inducing cell death (25), we hypothesized that in vivo, Stxs primarily reduce or delay the host's cellular immune response, thereby generating an opportunity for STEC bacterial colonization. The objectives of the present study were to monitor the levels and durations of fecal shedding of Stx-producing (Stx$^+$) and Stx-negative (Stx$^-$) *E. coli* and to examine specific cellular and humoral immune responses in calves experimentally inoculated with either human pathogenic Stx2$^+$ or Stx$^-$ *E. coli* O157:H7 bacteria.

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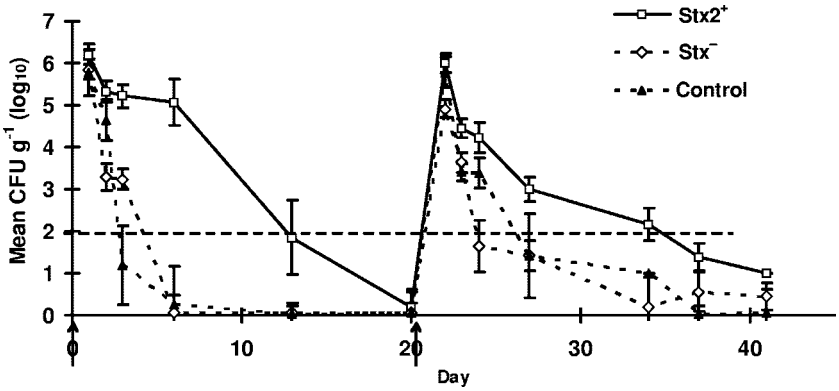

FIG. 1. Fecal shedding of *E. coli* by calves inoculated with different strains. Numbers of inoculum bacteria recovered from feces of calves inoculated with Stx2$^+$ *E. coli* O157:H7 strain 86-24 (Stx2$^+$), Stx$^-$ *E. coli* O157:H7 strain 87-23 (Stx$^-$), or control *E. coli* O43:H28 strain 123 (Control) are shown. Samples which were negative by direct culturing methods (<100 CFU g^{-1}) were tested by enrichment culturing. All values below the dashed line represent enrichment levels. Samples which were positive by enrichment analysis were given an arbitrary value of 10 CFU g^{-1}, and those which were negative were given a value of 1 CFU/g. Arrows indicate dates of inoculation. Data are presented as mean numbers (± standard errors of the means) of CFU g^{-1} for the indicated groups.

MATERIALS AND METHODS

Animals. Holstein calves 6 to 8 weeks old were purchased from local sources. Calves were allowed to acclimate to the diet and conditions at the National Animal Disease Center (NADC) for 3 weeks prior to oral inoculations. During infections, the calves were housed in BL2 containment barns and fed a diet of two-thirds grain and one-third hay. Animal care was in accordance with requirements of the NADC Animal Care and Use Committee and the Association for Assessment and Accreditation of Laboratory Animal Care. Calves were euthanized with sodium pentobarbital at the end of the study.

Inoculations. The strains used in this study were EHEC O157:H7 strain 86-24, a streptomycin-resistant mutant of an Stx2-producing strain isolated from an outbreak in Washington state (36); *E. coli* O157:H7 strain 87-23, a nalidixic-resistant mutant of a strain isolated from the same outbreak as 86-24 and lacking both Stx1 and Stx2 genes (19, 36); and, as a control strain, a nalidixic acid-resistant mutant of *E. coli* strain 123 (O43:H28), a porcine isolate which does not produce Stx and is not pathogenic in cattle (9). *Escherichia coli* cultures were prepared and stored at −80°C as previously described (9).

Calves (five calves/group) were inoculated intrarumenally, on day 0 and day 21, with approximately 10^{10} CFU of strain 86-24 (Stx2$^+$O157), 87-23 (Stx$^-$O157), or 123 (control) bacteria in 100 ml of trypticase soy broth (Becton Dickinson, Cockeysville, MD) as previously described (11, 33). Three weeks after the second inoculation (day 42), all calves were similarly inoculated with Stx2$^+$O157. This third inoculation of all calves with the same Stx$^+$O157 strain is referred to as the "challenge" to clearly differentiate it from the two initial inoculations of calves in different groups with different strains.

Bacteriology. Freshly isolated fecal samples (approximately 2 g) were diluted 1:10 and serially diluted in sterile phosphate-buffered saline. Sorbitol-negative STEC O157:H7 bacteria were quantitated on sorbitol-MacConkey agar (Oxoid, Basingstoke, United Kingdom) supplemented with streptomycin (200 μg per ml) or nalidixic acid (20 μg per ml) to select for strains 86-24 or 87-23, respectively. Randomly selected antibiotic-resistant, sorbitol-negative colonies were tested for O157 LPS antigen by slide agglutination (Oxoid). *E. coli* strain 123 bacteria were quantitated on MacConkey agar (Becton Dickinson, Cockeysville, MD) containing 20 μg per ml of nalidixic acid. Enrichment cultures were performed by adding up to 10 g of feces to trypticase soy broth containing 0.01% bile salts 3 (Difco, Detroit, MI) to a final volume of 100 ml. These cultures were incubated overnight, without shaking, at 37°C. Samples (100 μl) from enrichment cultures of feces that were negative by direct plating were incubated on sorbitol-MacConkey agar with the appropriate antibiotic. Enrichment plates were scored as either positive or negative and were not quantitated. Positive enrichment cultures were arbitrarily assigned a value of 10 CFU g^{-1}, and those which were negative were given a value of 1 CFU/g. Student's *t* test was used to determine statistical significance.

Detection of plasma antibodies to O157 LPS and Stx2. Titers against O157 LPS were determined by a blocking enzyme-linked immunosorbent assay (20), using monoclonal antibody MARC 13B3 (38) and highly purified LPS from *E. coli* O157:H7 (strain CDC EDL 933). MARC 13B3 lacks cross-reactivity with other bacteria, such as *Brucella* spp. and *Yersinia enterocolitica*, a problem often found with polyclonal antisera against O157. Endpoints were defined using receiver-operator-characteristic analysis of 1:4 dilutions of known positive and negative serum samples (20).

Vero cell cytotoxicity neutralization assays were performed as previously described (15). Briefly, dilutions of plasma were added to samples containing Stx2. After incubation for 1 h at 37°C, the plasma-toxin mixtures were added to Vero cells and incubated at 37°C for 48 h. The cells were then stained for viability with trypan blue. Antisera obtained from four calves hyperimmunized by subcutaneous injections with 100 μg of a noncytotoxic amino acid substitution mutant of Stx2e (15) were pooled and used as a positive anti-Stx control serum. Antibodies against Stx2e cross-reacted with and neutralized Stx2 in our system.

Preparation of antigen for peripheral blood mononuclear cell (PBMC) stimulation. Heat-killed Stx2$^+$ *E. coli* O157:H7 bacteria (HK-O157) were used for in vitro stimulation. One milliliter containing 10^{10} CFU of *E. coli* O157:H7 strain 86-24 (harvested from a fresh overnight culture) was boiled for 15 min. The cells were pelleted by centrifugation and resuspended in 1 ml of complete media (RPMI; 25 mM HEPES and L-glutamine [Gibco, Carlsbad, CA], 10% fetal calf serum, and 50 μg gentamicin per ml [Sigma, St. Louis, MO]). Aliquots of the cells were tested for sterility by plating them on trypticase soy agar plates and stored at −80°C until needed.

PBMC stimulation. PBMCs were isolated from whole blood collected at weekly intervals. The blood was centrifuged for 25 min at 1,170 × *g*. Plasma samples were removed and stored at −80°C for further analysis. Buffy coat cells were subjected to two rounds of hypotonic red blood cell lysis. PBMCs were then isolated by Percoll (Pharmacia, Piscataway, NJ) density gradient (P = 1.08) centrifugation at 450 × *g* for 40 min. Cells were washed twice in complete media. Cells were then diluted to 6.6×10^6 cells per ml and aliquoted into 96-well plates at 150 μl of cells (10^6 cells) per well. Stimulants (HK-O157, equivalent to 5×10^4 CFU *E. coli* O157:H7 cells, or 1 μg per ml ConA), 50 μl per well, were added to each well. Plates were incubated for 72 h in 5% CO_2 at 39°C. PBMCs were then pulsed with media containing 50 μCi of [^{3}H]thymidine and incubated for an additional 18 h. Cells were harvested with a Harvester 96 (Tomtech, Orange, CN) and analyzed using a 1450 Microbeta Plus scintillation counter (Wallac, Gaithersberg, MD). The stimulation index (SI) is the mean for three wells of cells incubated with stimulant divided by the mean for three wells incubated with media alone. Cell preparations in which the SI for ConA was less than 10 times that for cells incubated with media alone were considered poor and were excluded from further analysis. Statistical analysis was performed using the SAS software package and the analysis of variance technique.

RESULTS

All of the calves remained healthy throughout the study. None of the calves shed detectable levels of *E. coli* O157:H7 prior to the first inoculation. Only one of the calves in the

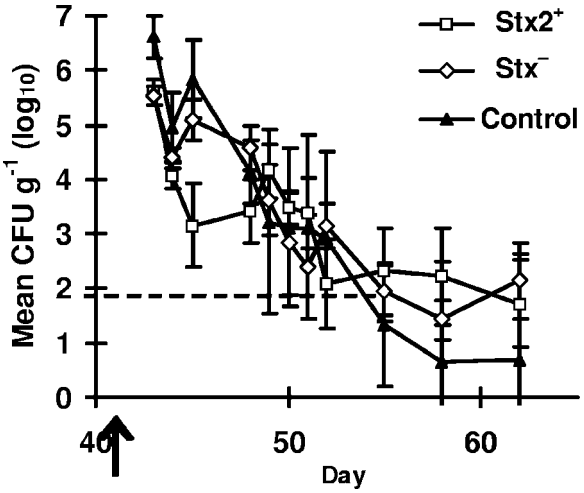

FIG. 2. Fecal shedding of the challenge strain by three groups of calves which were challenged with Stx2$^+$ O157:H7 strain 86-24 following prior inoculations with different strains of *E. coli*. The arrow indicates the date of the challenge. Data are presented as mean numbers (± standard errors of the means) of CFU g^{-1} of sorbitol-negative Str^r O157:H7 bacteria in the feces of the indicated groups. Group designations are same as those in the legend to Fig. 1.

study (one calf in the negative-control group) had detectable antibodies against O157 LPS prior to the first inoculation.

The inoculum strain was recovered in higher numbers and for a longer time from calves initially inoculated with Stx2$^+$ O157:H7 strain 86-24 than from calves inoculated with either of the other two *E. coli* strains (Fig. 1). More than 10^2 CFU of Stx2$^+$O157 bacteria/g of feces were recovered for 9 and 14 days after the first and second inoculations, respectively. Fecal shedding of Stx$^-$O157 decreased at a rate similar to that of the control bacteria, with both strains at $<10^2$ CFU g^{-1} of feces by the fifth day after each inoculation. The differences in fecal shedding between the Stx2$^+$ and Stx$^-$O157 strains were statistically significant ($P < 0.05$ by Student's t test) between days 2 and 13 after the first inoculation and days 22 and 27 after the second inoculation.

The fecal shedding of Stx2$^+$O157 bacteria was similar in all of the calves after they were challenged with Stx2$^+$O157 during week 7 (3 weeks after their second inoculation with Stx2$^+$O157, Stx$^-$O157, or control *E. coli* bacteria) (Fig. 2). Thus, prior exposure to high doses of STEC O157:H7 strain 86-24, Stx$^-$ *E. coli* O157:H7 strain 87-23, or control strain 123 did not reduce the duration or extent of fecal shedding of STEC O157:H7 bacteria after the challenge.

Following the initial two inoculations, all calves inoculated with either Stx2$^+$O157 or Stx$^-$O157 developed antibodies to the O antigen of *E. coli* O157:H7 (Fig. 3). The rates of seroconversion were similar in both groups. Three of the calves inoculated with control bacteria had low titers (≤16) of anti-O157 LPS, and the highest titer in this group was seen at week 4 in the calf which had serum antibodies to LPS O157 prior to the first inoculation. Calves in all three groups developed antibodies to O157 LPS following challenge with Stx2$^+$O157 (Fig. 3). None of the calves in any of the three groups developed antibodies against Stx2.

The in vitro PBMC proliferation assay was used to monitor the lymphoproliferative responses of infected calves to HK-O157 antigens (Fig. 4). Four of the five calves initially inoculated with Stx$^-$O157 developed a high proliferative immune response to HK-O157. This response became apparent at week 3 and reached its strongest level after the challenge. Three of the five calves in this group had SIs greater than 10 for 2 or more weeks. In contrast, calves initially inoculated with either Stx2$^+$O157 or control bacteria did not develop consistent PBMC proliferative responses to HK-O157 at any point during the study. Two of the five calves inoculated with Stx2$^+$O157, and one of the five inoculated with control bacteria, had SIs of >10 in a single assay (at week 4 or 8 for Stx2$^+$O157 calves and at week 10 for the control calf). Trend analysis using the sum of squares technique showed that the cumulative PBMC proliferative response for the calves inoculated with the Stx$^-$ strain was significantly higher than that for the calves which received Stx2$^+$O157 ($P = 0.0001$) or control bacteria ($P = 0.0009$). There were no consistent differences in overall ConA activation (Fig. 4).

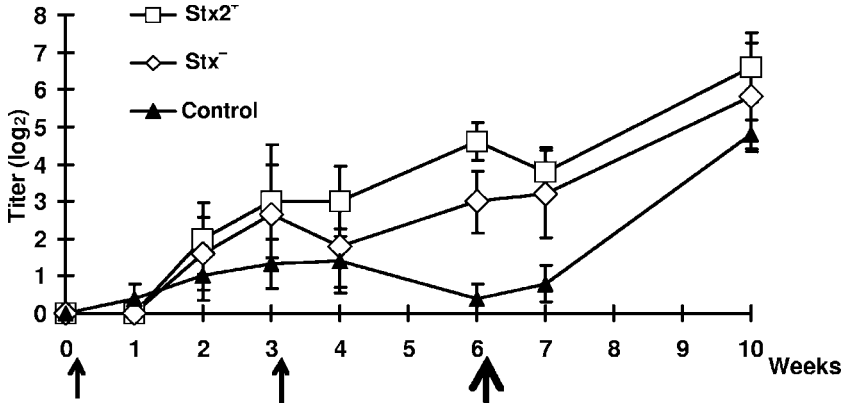

FIG. 3. Development of antibody titers against O157 LPS in calves inoculated with different *E. coli* strains. Data are shown as the means (± standard errors of the means) of $\log_2$ titers of plasma antibodies. For the designations of calf groups, see the legend to Fig. 1. Arrows indicate the dates of the initial inoculations (small arrows) and challenge (large arrow).

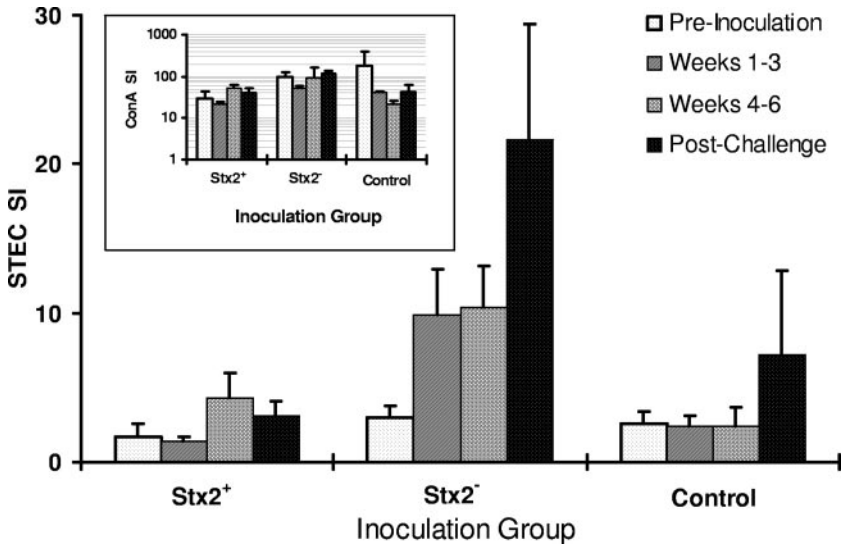

FIG. 4. Comparison over time of the lymphoproliferative responses to heat-killed Stx2$^+$ *E. coli* O157:H7 bacteria (HK-O157) in calves inoculated with different *E. coli* strains. Calves were inoculated twice (on days 0 and 21) with Stx2$^+$ *E. coli* O157:H7 strain 86-24 (Stx2$^+$), non-Shiga-toxigenic *E. coli* O157:H7 strain 87-23 (Stx$^-$), or control *E. coli* O43:H28 strain 123 (Control) prior to a final challenge (at 7 weeks postinoculation) with Stx2$^+$ *E. coli* O157:H7 strain 86-24. Results are shown as the means (± standard errors of the means) of the SIs obtained with PBMCs from five calves per group. The index for an individual animal was determined by dividing the mean counts per minute for three wells stimulated with HK-O157 by the mean counts per minute for three wells of nonstimulated cells. Lymphoproliferative responses to ConA stimulation are shown in the insert. Data obtained with blood samples during weeks 1, 2, and 3, during weeks 4 and 6, and postchallenge (during weeks 7, 8 and 10) were combined within those respective groups.

DISCUSSION

This study provides the first evidence that STEC infections can suppress the development of an antigen-specific cellular immune response in cattle. Following the initial inoculations, calves inoculated with Stx2$^+$ *E. coli* O157:H7 strain 86-24 shed the inoculum strain at higher levels and for longer periods than did calves inoculated with Stx$^-$O157:H7 strain 87-23. Despite this increased colonization, the lymphoproliferative responses to HK-O157 in calves inoculated with Stx2$^+$O157 were significantly lower than the responses in calves inoculated with Stx$^-$O157 and were similar to the responses in calves that received control bacteria. This reduced cellular immune response in Stx2$^+$O157-inoculated calves extends evidence that STEC possesses virulence traits that actively suppress cellular elements of the host immune response (1, 22, 23, 32). The differences observed in the lymphoproliferative responses in calves inoculated with the Stx2$^+$O157 and Stx$^-$O157 strains used in this study are consistent with the suppressive effects of Stxs on bovine peripheral and intraepithelial lymphocytes observed in vitro and in vivo (1, 12, 13, 22–25, 31).

Our studies were not designed to examine the mechanism underlying the observed immunosuppression. However, the observation that the PBMCs obtained from the different inoculation groups did not significantly differ in their proliferative responses to ConA provides preliminary evidence that the effect of STEC infection on the bovine immune system was not generalized. Furthermore, the observed immunosuppressive effects of STEC infections were restricted to antigen-specific in vitro PBMC proliferative responses. This contrasts with a generalized suppression of T cells described for mice and piglets, which is characterized by a drop in the reactivities of lymphocytes to ConA following injection with purified Stx2 and inoculation with Stx1$^+$ *E. coli* strain O111:NM, respectively (5, 34). Differences in levels of systemic Stx may be responsible for the observed differences in the effects of Stx on the immune system in the different studies. The mouse and piglet studies were more likely to involve high systemic concentrations of Stx. Mice were intravenously injected and piglets were orally inoculated with high doses of Stx$^+$ bacteria at 24 h of age, when gut closure may not have been complete and the likelihood of toxin absorption from the intestine was increased. Consequently, the effects of Stx on the immune system in these models may have been high enough to affect "general" functions, like mitogenic responsiveness. The effects of Stx in calves infected at 6 to 8 weeks of age (notwithstanding the high inoculum doses) may better reflect the effects of "physiologic" infections that occur in the field.

Thus, we interpret the present findings as support for our hypothesis that Stxs are immunosuppressive factors which play a role in promoting STEC colonization in the bovine host primarily by reducing or delaying the host's antigen-specific cellular immune response against the bacteria.

As previously postulated (31), the immunosuppressive effects of STEC infections appeared to result from a prevention of the onset of an immune response rather than the downregulation of an established one. The development of an in vitro PBMC response was prevented when Stx2 was present at the time that the naïve O157:H7 *E. coli*-specific immune cells were first stimulated by the initial inoculations (i.e., in calves initially inoculated with Stx2$^+$O157). In contrast, the Stx2$^+$O157 strain did not exert an immunosuppressive effect after the immune response was efficiently induced by two inoculations with Stx$^-$O157. Calves initially inoculated with Stx$^-$O157 showed a continued proliferative response that, similar to an anamnestic

response, was even more pronounced after they were challenged with Stx2$^+$O157.

Remarkably, inoculation of calves with Stx2$^+$O157, on the contrary, did not hinder the development of a humoral response against O157 LPS (17; this study). B-cell responses in calves apparently were less sensitive to STEC-induced suppression than in vitro PBMC proliferative responses (25), particularly when the B cells were directly activated in vivo by T-cell independent B-cell antigens, like LPS. The absence of antibodies against Stx2 in our calves, which is consistent with previous observations after experimental STEC O157:H7 inoculations in cattle (17), can therefore not be explained by a suppression of B cells.

Despite the different immune responses in the three groups of calves following the initial inoculations, the shedding patterns for Stx2$^+$O157 bacteria were similar in all groups in the first weeks after the calves were challenged with the Stx2$^+$O157 strain. Neither the induction of O157 antibodies in the Stx2$^+$O157 group nor the induction of O157 antibodies and HK-O157-specific lymphocytes in the Stx$^-$O157 group reduced the duration or magnitude of shedding after the challenge compared to what was found for the control group during the observation period. Further experiments will be necessary to determine how O157:H7 *E. coli*-specific cellular immune responses affect long-term and intermittent STEC shedding (3, 8).

The effects of STEC infections on the development of specific cellular immune responses must be addressed in designing vaccines aimed at reducing STEC O157:H7 infections in cattle, especially vaccines directed against Stx, which is the only virulence factor shared by all STEC strains.

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Disclaimer: the use of names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of any product, and the use of a name by the USDA implies no approval of the product to the exclusion of others that may also be available.

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