Enterohemorrhagic *Escherichia coli* suppresses inflammatory response to cytokines and its own toxin

Amy Bellmeyer, Cynthia Cotton, Rajani Kanteti, Athanasia Koutsouris, V. K. Viswanathan, and Gail Hecht

Department of Medicine, Section of Digestive Diseases and Nutrition, University of Illinois and Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois

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**Enterohemorrhagic Escherichia coli (EHEC)**, a noninvasive gastrointestinal pathogen, is responsible for significant morbidity and mortality, particularly in children (20). Infection with this food-borne pathogen results in watery or bloody diarrhea. EHEC elaborates several virulence factors, including Shiga toxins (Stx1 and Stx2). Translocation of the toxin across the epithelial barrier to systemic circulation can result in life-threatening conditions including hemorrhagic colitis and hemolytic uremic syndrome (29).

A number of EHEC virulence factors have been characterized. A pathogenicity island known as the locus of enterocyte effacement (LEE) encodes nearly 40 genes, including the structural components of a type III secretion system (T3SS) (21). Several proteins, including some encoded within the LEE, are translocated directly into the host cell through the T3SS. These proteins interfere with various host cell signal transduction pathways. The translocated intimin receptor (Tir) (18) is an effector protein that enters epithelial cells, inserts into host cell membranes, and serves as a receptor for the bacterial surface adhesin, intimin (3). Tir-intimin interaction results in intimate attachment of the bacteria to host cells. T3SS mutants of EHEC display impaired attachment and pathogenesis (23).

Numerous in vitro and in vivo studies have demonstrated that EHEC induces intestinal inflammation (1, 6, 7, 10, 13, 22). Production of the proinflammatory cytokine IL-8 is mediated by activation of the transcription factor NF-κB via a MAP kinase-dependent pathway (1, 6). EHEC flagellin has been established as a proinflammatory molecule although it is not known whether this protein is expressed in vivo (1). The potential of Stx, in the absence of bacteria, to induce IL-8 production by intestinal epithelial cells is controversial (31, 33). Thorpe et al. (31) demonstrated that, despite mediating a general inhibition of mRNA translation, Stx “superinduces” IL-8 mRNA translation and increases IL-8 synthesis and secretion from a human colonic epithelial cell line. Other investigators, however, have not observed an IL-8 response to Stx from intestinal epithelial cells (1). Interestingly, Hauf and Chakraborty (10) found that EHEC also suppresses cytokine- and bacteria-induced NF-κB activation in an EspB-dependent manner. EspB, a LEE-encoded protein required for type III secretion, has also been reported to be an effector protein that is translocated into host cells (30). Therefore, EspB may play a direct or indirect role in suppressing inflammation.

Stxs bind to a receptor identified as glycolipid Gb3 (16, 17). Human intestinal epithelial cells reportedly lack the Gb3 receptor (12, 25). Despite the absence of Gb3 expression, evidence suggests that Stx has direct effects on intestinal epithelial cells. These cells translocate Stxs and trigger RNA superinduction (31). Interestingly, however, Miyamoto et al. (19) have shown that Stx fails to bind to human colon epithelium in vivo, and a novel binding site for Stx has been described at the base of small intestinal crypts of Lieberkühn in Paneth cells (25). In view of the array of conflicting data regarding the effects of Stx on intestinal epithelial cells, the aim of this study was to investigate the direct effect of Stx on the inflammatory response by intestinal epithelial cells and to determine whether EHEC organisms modulate this response.

**MATERIALS AND METHODS**

**Cell culture.** T84 cells (passages 29–55) were grown in a 1:1 (vol/vol) mixture of Dulbecco-Vogt modified Eagle’s medium and Ham’s F12 medium supplemented with 6% newborn calf serum.
(Invitrogen, Carlsbad, CA). HT-29 cells were grown in high glucose DMEM supplemented with 10% FCS at 37°C in 5% CO2.

Bacterial strains, infection, and toxin treatment of host cells. Wild-type toxin-positive EHEC and an isogenic toxin-negative derivative 85-170 (32) (both generous gifts from James Kaper, Center for Vaccine Development, University of Maryland, Baltimore, MD) and EHEC toxin-positive fliC- (generous gift from Jorge Giron, Department of Immunology, University of Florida, Gainesville, FL) were used. Purified Stx1 was purchased from Toxin Technology (Sarasota, FL). For infection, bacteria were grown overnight at 37°C in Luria-Bertani broth, then diluted (1:33) in serum- and antibiotic-free tissue culture medium containing 0.5% mannose, and grown at 37°C to mid-log phase (OD 0.4). Monolayers were infected at an initial multiplicity of infection (MOI) of 10–40, unless otherwise indicated. Alternatively, cells were treated with filter-sterilized supernatants of the DMEM-grown bacterial cultures.

Cytokine or Stx challenge of EHEC-infected cells and IL-8 quantification. EHEC Stx+ and Stx− bacteria were grown overnight at 37°C in Luria-Bertani broth, then diluted (1:33) in serum- and antibiotic-free medium containing 0.5% mannose, and grown to mid-log phase (OD 0.4) at 37°C. HT-29 monolayers were then infected at an initial MOI of 10–40 or left uninfected and incubated for 1 h at 37°C with 5% CO2. Uninfected monolayers served as controls. All monolayers were then washed with 1 ml of serum- and antibiotic-free medium. Cells were subsequently treated with PBS, Stx, TNF-α, or IL-1β in serum- and antibiotic-free medium as indicated for 6 h at 37°C with 5% CO2. Supernatant was harvested for quantitating IL-8 expression. IL-8 was quantified using a dual-antibody ELISA kit (R & D Systems, Minneapolis, MN) following the manufacturer’s protocol.

Immunoblot analysis and antibodies. Control and EHEC-infected monolayers were washed with cold PBS, and proteins were extracted in 50 mM NaCl, 50 mM Tris pH 7.4, 0.5% DOC, 0.1% NP-40, 1 mM EDTA, 1 mM Na3VO4, 1 mM PMSF, 1 mM NaF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Total protein in the cell extracts was determined by Bradford assay (Bio-Rad, Hercules, CA). Samples containing 100 μg of protein were separated on a 12% SDS-PAGE gel and transferred electrothermally to 0.20-μm nitrocellulose membranes (Bio-Rad). After being blocked for 1 h at room temperature, blots were sequentially incubated for 1 h with primary antibody against NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed in TBS and incubated with appropriate dilutions of alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature. Color development was achieved by the addition of the alkaline phosphatase substrate, nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate solution (Zymed, San Francisco, CA).

EHEC Stx+ and Stx− bacteria were grown overnight in Luria-Bertani broth, then diluted (1:33) in serum- and antibiotic-free tissue culture medium, and grown at 37°C to mid-log phase to an (OD 0.4). Culture (1 ml) was centrifuged at 10,000 revolution/min for 1 min and subsequently lysed in 300 μl of Laemml buffer (Bio-Rad) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). A sample (20 μl) of extract was electrophoresed on a 12% SDS-PAGE gel and transferred to 0.20 μm nitrocellulose membrane (Bio-Rad). After being blocked for 1 h at room temperature, blots were incubated with an equal volume of DMEM. EHEC Stx+ and Stx− strains for 6 h, and IL-8 released into the medium was assessed by ELISA. EHEC Stx+ induced a more potent IL-8 response than the isogenic EHEC Stx− strain (Fig. 1A). This was not due to differences in growth, bacterial attachment (data not shown), or flagellin expression (Fig. 1B), as all of these parameters were the same for Stx+ and Stx− EHEC. In addition, whereas deletion of the fliC gene from Stx+ EHEC reduced the level of IL-8 production, a robust response following infection in the fliC mutant remained (Fig. 1C). IL-8 expression is regulated by the transcription factor NF-κB, whose activation is triggered by the degradation of

RESULTS

Stx contributes to EHEC-induced proinflammatory responses. There is controversy in the literature regarding the ability of EHEC Stx to induce IL-8 expression in intestinal epithelial cells. To determine the contribution of Stx to proinflammatory responses and define whether EHEC produces proinflammatory molecules other than flagellin, T84 monolayers were infected with EHEC Stx+ and Stx− strains, respectively, for 6 h, and the culture medium was assessed for IL-8 secretion by ELISA. EHEC Stx+ induced a more potent IL-8 response than the isogenic EHEC Stx− strain (Fig. 1A). This was not due to differences in growth, bacterial attachment (data not shown), or flagellin expression (Fig. 1B), as all of these parameters were the same for Stx+ and Stx− EHEC. In addition, whereas deletion of the fliC gene from Stx+ EHEC reduced the level of IL-8 production, a robust response following infection in the fliC mutant remained (Fig. 1C).
I-κB (5, 8, 11, 14, 26). To evaluate whether EHEC Stx\(^+\)/H\(_{11001}\) and EHEC Stx\(^-\)/H\(_{11002}\) strains activate NF-κB, protein extracts from infected cells were immunoblotted for I-κB\(\alpha\). Both EHEC Stx\(^+\)/H\(_{11001}\) and EHEC Stx\(^-\)/H\(_{11002}\) stimulated the progressive degradation of I-κB\(\alpha\) (Fig. 2). Consistent with its ability to induce a more potent IL-8 response, Stx\(^+\) strain caused a significantly more rapid decrease in I-κB\(\alpha\) levels than EHEC Stx\(^-\) strain. These results indicate that Stx contributes to EHEC-induced IL-8 expression and secretion by intestinal epithelial cells.

**Purified Stx induces I-κB\(\alpha\) degradation and dose-dependent IL-8 secretion in intestinal epithelial cells.** To confirm the effect of Stx on NF-κB activation, protein extracts from T84 cells treated with Stx1 (100 ng/ml) were immunoblotted for I-κB\(\alpha\). Purified Stx1 induced I-κB\(\alpha\) degradation as early as 60 min posttreatment (Fig. 3A). To correlate NF-κB activation with IL-8 expression, Stx dose dependence of epithelial cell IL-8 response was evaluated. HT-29 monolayers were exposed to increasing doses of purified Stx1 for 6 h, and IL-8 release into the supernatants was measured by ELISA. Stx dose-dependent IL-8 production was observed, reaching significance with concentrations as low as 50 ng/ml and saturating at 200 ng/ml toxin (Fig. 3B). Increasing toxin levels to 500 ng/ml resulted in a sharp reduction in IL-8 production coupled with lifting of cells from the plates, indicative of epithelial cell apoptosis. These data demonstrate that Stx challenge of intestinal epithelial cells triggers a proinflammatory response.

**Dose dependence of IL-8 response to bacteria and sterile supernatants.** To further characterize the dose dependence of IL-8 production in response to EHEC infection or treatment...
with filter-sterilized EHEC supernatants, HT-29 monolayers were infected with EHEC Stx\(^-\) (MOI of 10–40) or treated with filter-sterilized EHEC Stx\(^-\) supernatants (50–1,000 \mu l, in a final volume of 1 ml) for 6 h as described in MATERIALS AND METHODS. The medium was subsequently evaluated for IL-8 production by ELISA. Interestingly, there was an inverse relationship between EHEC Stx\(^-\) MOI and IL-8 release (Fig. 4A). In contrast, cells treated with Stx\(^-\) EHEC supernatant displayed a proportional dose-response relationship, and also a more robust induction of IL-8 compared with infection with bacteria (Fig. 4A). This suggests that the primary proinflammatory bacterial factors are secreted into the medium, but active infection suppresses the IL-8 response. Similar observations were made with EHEC Stx\(^+\) infections and supernatant treatment, except that the IL-8 response was more robust compared with EHEC Stx\(^-\) and supernatant treatments, respectively (Fig. 4B).

**EHEC infection reduces IL-8 production in response to host-derived cytokines.** Several enteric pathogens have been demonstrated to downregulate the host inflammatory response using a variety of different strategies. This has been reported for EHEC as well although the mechanism is not defined. To confirm that EHEC infection indeed decreases the inflammatory response in our model system, we challenged EHEC-infected and uninfected control monolayers with two important host-derived cytokines, TNF-\(\alpha\) and IL-1\(\beta\), and assessed IL-8 release. Indeed, infection of monolayers for 1 h with Stx\(^-\) EHEC reduced the IL-8 response to both TNF-\(\alpha\) (Fig. 5A) and IL-1\(\beta\) (Fig. 5B) by \(-50\%\). Pretreatment with filter-sterilized supernatant had no effect, suggesting that intact bacteria were required for suppression of the IL-8 response.

**Infection with Stx\(^-\) EHEC suppresses supernatant-induced IL-8 production.** Demonstrating the ability of EHEC to modulate the host epithelial inflammatory response to cytokines led us to question whether EHEC could also influence the response of host cells to its own secreted proinflammatory factors. Therefore, HT-29 monolayers were preinfected with EHEC Stx\(^-\) for 1 h to allow for attachment and entry of the putative inhibitory molecule(s) into host cells. Control monolayers were incubated with media alone for 1 h. The cells were then treated with equal amounts of filter-sterilized supernatants from either EHEC Stx\(^+\) or EHEC Stx\(^-\) cultures. Prior infection of monolayers with EHEC Stx\(^-\) suppressed the proinflammatory activity of both EHEC Stx\(^+\) and EHEC Stx\(^-\) supernatants, respectively, compared with prior treatment with media alone (Fig. 6) although to a greater degree for Stx\(^-\) supernatants. Induction of IL-8 production by Stx\(^+\) supernatant was inhibited by \(-40\%\), whereas the response to Stx\(^-\) supernatant was suppressed by nearly \(75\%\). These findings confirm that EHEC produces an anti-inflammatory factor(s) that suppresses the IL-8 response to its secreted proinflammatory components, but whether the Stx-induced portion of IL-8 response is attenuated could not be determined from this experiment.
**Suppression of Stx-induced IL-8 response by EHEC infection.** The partial inhibition of Stx+ supernatant-induced IL-8 response suggests that EHEC blocks proinflammatory signals specific to some but not other components in the supernatants. To directly determine whether EHEC infection attenuates the inflammatory response induced by Stx, HT-29 cells were preinfected with EHEC Stx+ for 1 h or treated with media alone and then challenged with 100 and 200 ng/ml purified Stx1. Interestingly, preinfection with Stx− EHEC significantly attenuated the expression of IL-8 stimulated by Stx (100 ng/ml) (Fig. 7). Increasing the EHEC Stx− MOI only marginally improved the suppressive effect. Furthermore, the level of IL-8 reduction by EHEC infection remained relatively consistent despite challenge with a higher concentration (200 ng/ml) of Stx. These results indicate that active EHEC infection suppresses the proinflammatory activity of a wide range of compounds, including host cytokines, secreted EHEC proinflammatory components, and, perhaps most importantly, Stx.

**DISCUSSION**

It is evident from various studies that EHEC manipulates innate immune responses in host epithelial cells (1, 2, 4, 6, 7, 9, 10, 13, 22, 31, 33, 34). EHEC flagellin plays a major role in stimulating a proinflammatory response from intestinal epithelial cells (1). Additionally, several studies using different epithelial cell lines have demonstrated a significantly higher level of IL-8 mRNA and protein production in response to Stx-positive strains compared with isogenic strains lacking the toxin (1, 13, 31). Although Berin et al. (1) made similar observations, they discounted the effects of the toxin because purified Stx2 failed to elicit an IL-8 response from Caco-2 cells. Another study in the same cell line, however, reported IL-8 mRNA and protein production following Stx1 and Stx2 (0.1–10 ng/ml) treatment for 6–48 h (33), and similar experiments using HCT-8 cells also showed induction of the IL-8 response with 10 ng/ml of purified Stx2 treatment for 8–18 h (31) but not after a 3-h exposure (13). The present study demonstrates greater NF-κB activation and IL-8 secretion in intestinal epithelial cells infected with a toxin-positive EHEC strain compared with a toxin-negative isogenic mutant. Moreover, purified Stx1 (>50 ng/ml, 6-h treatment) activated NF-κB and induced IL-8 secretion from intestinal epithelial cells. Thus the majority of published studies support a role for Stx in proinflammatory responses; the variability in the magnitude of the response likely reflects differences in experimental methodology (including toxin concentration, cell lines used, and duration of treatment).

A number of recent studies have demonstrated that A/E pathogens including enteropathogenic *Escherichia coli*, EHEC, and *Citrobacter rodentium* produce one or more proteins that limit inflammation in intestinal epithelial cells (10, 15, 24, 28). This protein(s) is likely secreted via the T3SS and functions within the host cells. Hauf and Chakrabarty (10) demonstrated the presence of such a secreted anti-inflammatory compound in EHEC. Their studies suggested that this molecule could be EspB itself, or, because EspB is also involved in effector translocation into host cells, another secreted molecule. These studies, however, failed to address the effect of EHEC on the inflammatory response of intestinal epithelial cells to Stx. Here, we show that EHEC harbors an anti-inflammatory molecule(s) that curtails the effect of host-derived cytokines as well as that of EHEC-secreted factors. Particularly interesting and important is the finding that EHEC infection inhibits Stx-induced IL-8 production. This suggests that a step in the proinflammatory pathway common to these disparate signals is being inhibited, or, alternatively, that the inhibition of the effects of Stx is mediated by a molecule(s) distinct from the one(s) involved in limiting cytokine-induced IL-8 production.

The data presented herein have important clinical ramifications, as they suggest that EHEC is able to protect the integrity of the intestinal epithelium by dampening its response to secreted proinflammatory host cytokines and bacterial factors such as flagellin and even Stx. Because the Stx Gb3 receptor is not expressed on intestinal tissues, the intestinal epithelial IL-8 response is likely Gb3 independent. However, Stx can be effectively translocated across the epithelium to sites that express Gb3 receptor such as the vascular endothelium. Thus the extraintestinal complications of EHEC are likely mediated by the disseminated toxin, as EHEC is rarely found in the systemic circulation (29). Therefore, the absence of EHEC and its anti-inflammatory factors in these tissues likely results in unimpaired toxin-mediated inflammatory responses. An additional complication of the loss of EHEC and its anti-inflammatory factors relates to antibiotic treatment. There is concern regarding the use of antibiotics to treat EHEC infections because this, in many instances, leads to the induction of the Stx-containing phage and increased toxin production (27, 29). Our studies potentially suggest that elimination of EHEC by antibiotic treatment could result in a loss of anti-inflammatory effects, and thereby result in elevated toxin-mediated inflammation.

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