Role of Shiga toxin dissemination and inflammation in the pathogenesis of Shiga toxin-producing *Escherichia coli* infection

by

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General Introduction

Rational and experimental goals

Hemolytic uremic syndrome (HUS), a systemic complication of Shiga toxin-

producing *Escherichia coli* (STEC) infection, is the number one cause of renal failure in

children in the United States [1-3]. At present, there is no known method to predict

which patients with STEC infection will develop HUS and no effective treatment for the

disorder once it is diagnosed. One of the primary barriers inhibiting development of an

adequate therapy and/or preventative is an insufficient understanding of the intricate

pathogenic cascade. It is known that Shiga toxin (Stx) is produced locally within the

gastrointestinal tract, is absorbed into the systemic circulation by a yet undefined

mechanism, binds to vascular endothelial cells expressing the Stx receptor [4-6]. Stx is

then internalized within the endothelial cell and interrupts protein synthesis, causing cell

death.   This endothelial cell injury causes microthrombi formation in key target organs

(kidney, brain, colon) leading to the characteristic clinical triad of HUS:

microangiopathic hemolytic anemia, thrombocytopenia, and acute renal damage [7].

Although Stx is widely presumed to be the principal mediator of HUS, proinflammatory

cytokines have been repeatedly implicated as substantial contributors to disease

development. Stx induces cytokine production in a variety of cell types *in vitro,* and

cytokines, in turn, greatly increase the sensitivity of endothelial cells to Stx [8-11].

Several clinical studies have identified increased proinflammatory cytokines in patients

with HUS compared to those with STEC-induced diarrhea alone [12-15]. A more complete understanding of both toxin transport mechanisms and cytokine responses during HUS would aid in the development of treatment modalities; however, both the primary mode for toxin transport in vivo and the stimulus for cytokine alterations seen in HUS remain uncharacterized. Specifically, the role of Stx production and dissemination in proinflammatory cytokine alterations observed in clinically-affected patients has not been established. The goals of the studies outlined in this thesis were to: 1) clarify the role of Stx in modulating inflammation during STEC infection 2) identify specific interactions between Stx and neutrophils to assess the potential role of this leukocyte in Stx transport during STEC infection and 3) assess the efficacy of an oral Stx-binding agent at preventing systemic disease following STEC inoculation. To accomplish these goals, in vitro experiments, as well as in vivo experiments in an animal model of STEC infection, were used. The animal model chosen for the in vivo experiments was edema disease of swine, a naturally occurring STEC disease of weaned swine caused by host-adapted strains of *E. coli*. Experimentally infected swine are colonized in the intestinal tract and approximately 30% of individuals develop clinical edema disease manifested by neurological symptoms due to systemic Shiga toxemia [16-18]. Edema disease has been used as an animal model to study HUS based on the similar pathogenesis between the two diseases [18].

Dissertation organization

This dissertation is composed of an introduction and review of the literature (Chapter 1) followed by three separate manuscripts to be submitted to peer reviewed journals (Chapters 2-4). A general conclusion is given in Chapter 5. References are cited at the conclusion of each chapter. The following is a summary of author's roles for each of the three manuscripts included in Chapters 2-4:

Chapter 2:

Shannon J. Hostetter: Graduate student, primary researched and author.
Amy Helgerson: Research associate and contributing researcher.
James Paton: Collaborator.
Adrienne Paton: Collaborator.
Nancy A. Cornick: Associate professor and corresponding author.

Chapter 3:

Shannon J. Hostetter: Graduate student, primary researcher and author.
Jack M. Gallup: Contributed to RT-PCR design and analysis.
Nancy A. Cornick: Associate professor and corresponding author.

Chapter 4:

Shannon J. Hostetter: Graduate student, primary researcher and author.
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Chapter 1: Literature review

Escherichia coli are Gram negative facultative anaerobes of the family Enterobacteriaceae and are a major component of the normal intestinal flora in many species. Although most *E. coli* are non-pathogenic, some have acquired virulence attributes and are capable of causing severe disease in a variety of hosts. STEC (Shiga toxin-producing *Escherichia coli*) produce a potent cytotoxin, known as Shiga toxin (a.k.a. verocytotoxin, Shiga-like toxin), that inhibits protein synthesis in susceptible host cells (105). Serotyping is a common way to characterize STEC strains, and is based on the O antigen (somatic antigen) and H antigen (flagellar antigen). The most common serotype associated with human disease is O157:H7 (55, 144).

STEC infection in human beings is primarily a zoonotic disease, and ruminants are considered the major animal reservoir, although pigs and other animals also may serve as carriers (55). The primary site of intestinal colonization of STEC in cattle is the terminal rectal mucosa and rectoanal junction (23, 106, 128). Some cattle are known to yield far greater numbers of O157:H7 than most, and are referred to as "super-shedders." They are defined as shedding greater than or equal to 10^4 colony forming units (CFU) per gram of feces, and epidemiologic evidence suggests they are critical sources of both parallel transmission within the reservoir, as well as zoonotic transmission (21). Human exposure to STEC can come from multiple sources, including ingestion of contaminated fruits and vegetables, undercooked ground beef, unpasteurized apple cider or milk, salami, contact with livestock, and parallel transmission from infected individuals (21).

The infectious dose of STEC is remarkably low (50 CFU or less), and this contributes to the virulence of the organism (144).

Shiga toxin-producing *Escherichia coli* infections in human beings.

Shiga-toxin producing strains of *Escherichia coli* (STEC) are responsible for approximately 110,000 cases of food borne illness annually in the U.S. and are a major cause of hemorrhaghic colitis (95). STEC strains capable of causing severe disease in human beings are also classified as enterohemorrhagic *Escherichia coli* (EHEC), one of the pathotypes of *E. coli* that cause enteric disease. A small percentage of patients (3-15%) with diarrhea secondary to STEC infection develop a systemic and potentially fatal complication known as hemolytic uremic syndrome (HUS), characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal damage (68, 122). HUS is primarily mediated through Shiga toxin (Stx) dissemination and is reported to be the most common cause of acute renal failure in children (95, 133). Although renal lesions (microvascular thrombopathy in glomeruli) are the histologic hallmark of HUS, death of HUS patients is usually attributed to similar vascular lesions in the brain rather than the kidney (137, 138). HUS can be broadly classified into two main types: diarrhea associated (D+HUS) and diarrhea negative HUS (D-HUS). D-HUS is less common than D+HUS, making up approximately 10% of total reported cases of HUS. D-HUS can occur in association with a variety of conditions, including complement and coagulation

abnormalities, antiphospholipid syndrome, pregnancy, and human immunodeficiency virus infection (7, 89). The vast majority of D+HUS cases are caused by STEC infection; however, *Shigella dysentariae* type 1 infection is the primary cause of HUS in some developing countries, and HUS also occurs as a rare sequelae to *Streptococcus pneumoniae* infection in children (27, 89).

STEC were initially implicated as the cause of diarrhea-associated HUS by Karmali and colleagues back in the mid-1980's (68). STEC that produce Shiga toxin (Stx) 1, Stx2, or both toxins have been associated with HUS development; however, both epidemiologic evidence and work with animal models show that strains producing Stx2 only are more likely to cause severe disease (34, 43, 124, 151). Characterization of STEC isolates from patients with HUS suggest that *E. coli* O157:H7 is the predominant strain recovered in the United States (4, 9). HUS is more prevalent in children under the age of 10 and the elderly, and it remains the leading cause of renal failure in children in the U.S. (39, 52, 67). At present, there is no known method to predict which patients with diarrhea caused by STEC will develop HUS, and no known treatment for the disorder once it is diagnosed. Clinically, HUS secondary to STEC infection is preceded by a prodromal diarrheal phase that typically lasts approximately 7 days. In most patients, the diarrhea progresses to bloody diarrhea within a few days (144). Although it is based somewhat on both bacterial serotype and patient age, up to 15% of individuals with STEC infection will develop HUS (data for serotype O157:H7) (144, 172). Patients who develop HUS are currently managed with supportive therapy only (160). Although recent research on HUS has identified some elements in the pathogenesis of the disease

and certain risk factors for disease development, many details of the pathogenic cascade beginning with STEC ingestion and culminating in HUS remain unknown.

Virulence attributes of STEC causing human disease

Locus of enterocyte effacement and intimin

The locus of enterocytes effacement (LEE) is a chromosomally associated pathogenicity island encoding the proteins responsible for attaching and effacing lesions (A/E) on the intestinal mucosa (93). A/E lesions are characterized by the loss of apical villi on enterocytes with rearrangement of the cytoskeleton leading to the formation of an intimate association between bacterium and enterocyte forming a classic pedestal appearance (105). The formation of A/E lesions is prototypical of another *E. coli* pathotype, the enteropathogenic *E. coli* (EPEC); however, many STEC strains responsible for human disease also possess LEE and form A/E lesions experimentally (105). Five polycistronic operons make up LEE, designated LEE1-LEE5. The products of LEE include a Type III secretion system, intimin, and secreted effector proteins such as Tir, EspF, EspG, and EspH (55). Intimin, an outer membrane protein adhesin, is encoded by *eae* and is primarily responsible for the intimate attachment of EPEC and STEC to the enterocyte. Multiple subtypes of intimin have been described, and this heterogeneity may contribute to some variability in binding to various eukaryotic cell receptors (46). Regardless of subtype, however, the primary host cell receptor for intimin,

Tir, is actually produced by the bacterium and translocated into the host cell through the Type III secretion system also encoded on LEE. Additionally, there is evidence that some intimin subtypes bind to native host receptors. Intimin-α of EPEC binding to β1 integrins has been identified, and several intimin subtypes have the ability to bind nucleolin (44, 140). Moreover, Tir, nucleolin, and β1 integrins were all identified in areas of O157:H7 adherence to enterocytes using immunohistochemistry in bovine and porcine intestine (140).

The majority of STEC strains pathogenic to human beings are *eae*-positive, and *eae* has been identified as a risk factor for HUS development (169). Regulation of expression of LEE in EPEC is mediated through LEE encoded regulators Per and Ler, as well as more global regulators such as H-NS and quorum sensing regulators (72, 139). STEC do not appear to possess a Per homologue; however, it is suggested that Ler plays a role in gene regulation of LEE in O157 STEC, and may be involved in intimin-independent adherence (113). Despite the presumed importance of LEE in the pathogenesis of STEC infection, a number of *eae*-negative STEC strains have been associated with outbreaks of hemorrhagic colitis and HUS, most notably STEC O113:H21 (108). Currently, little is known about how *eae*-negative STEC strains mediate specific adherence to enterocytes during colonization.

Toxins

    Shiga toxin

Shiga toxin is the principal mediator of diarrhea-associated HUS. Stx of *E. coli* were first described in the late 1970's when it was noted that filtrates from certain *E. coli* strains were toxic to Vero cells (derived from the kidney of African green monkeys) in vitro (77). They were originally designated verocytotoxins, and were subsequently recognized for their structural and functional similarity to Shiga toxin of *Shigella dysenteriae* type I (111). Two main subgroups of STEC Stx are recognized, Stx1 and Stx2 (112, 152). There are several variants described for Stx2, including Stx2e, the toxin principal of porcine edema disease. Stx1 is 99% similar to Shiga toxin of *Shigella dysenteriae* type I, yet Stx2 shares only ~56% amino acid homology with Stx1 (143, 161). Stx1 and Stx2 are phage-encoded, although Stx1 may also be chromosomally encoded (110, 142). Stx are classic AB_5 toxins, composed of a single enzymatic A subunit non-covalently associated with 5 glycolipid-binding B subunits (122). Both Stx1 and Stx2, as well as most Stx2 variants, preferentially recognize globotriaosylceramide (Gb_3) on the surface of cells; however, differences in receptor affinity between Stx1 and Stx2 have been described (104). Following specific binding of Stx to Gb_3, the complex is internalized via clathrin-coated pit mediated endocytosis. The toxin then travels via retrograde transport to the Golgi and then the endoplasmic reticulum, during which time the A subunit is cleaved to form an active *N*-glycosidase. This active enzyme functions in the cytosol to remove an adenine residue on the 28S rRNA, thereby rendering it inactive and interrupting protein synthesis (174).

Cytolethal distending toxin

Cytolethal distending toxin (CDT) was named for the observation that it induced cell swelling with subsequent death in certain cell lines (65). It was later discovered that CDT induced cell cycle arrest at the G_1 or G_2 phase. CDT is not unique to *E. coli*, and has been described in numerous other pathogens including *Helicobacter* spp. and *Shigella* spp. (125). The CDT holotoxin is composed of three different proteins encoded by adjacent genes (*cdtA, cdtB, cdtC*). Of these proteins, CdtB appears to be enzymatically active and cleaves host DNA to promote the cell cycle effects. Multiple CDT alleles exist for *E. coli* (63). There is some debate as to the significance of CDT in STEC pathogens causing human disease, especially since many of the human isolates producing Stx are negative for CDT, and the vast majority of O157:H7 strains analyzed likewise lack CDT (10). However, many non-motile O157 strains, as well as non-O157 STEC, possess *cdt*. Additionally, one of the more recently described CDT proteins, CDT-V, was found to cause death in multiple endothelial cell lines in vitro, raising the possibility that CDT might contribute to vascular pathology in STEC infection (12).

Subtilase toxin

Subtilase toxin (SubAB) of *Escherichia coli* was initially described in serotype O113:H21 as the prototype of a novel family of AB_5 toxins in 2004 (119). The toxin

was more cytotoxic to Vero cells than Shiga toxin, and the enzymatic A subunit contained a "catalytic triad" denoting its similarity to subtilase serine proteases. The B binding pentamer showed affinity for GM2 ganglioside. Subtilase toxin was found to be plasmid encoded by two genes (*subA* and *subB*) which were identified in approximately 50% of the 60 additional STEC strains tested. Parenteral administration of 200 ng of purified toxin was lethal for mice. The mice exhibited neurological signs before death and had histologic lesions consisting of microvascular thrombosis and necrosis in multiple organs (brain, kidney, and liver). The authors proposed that these histologic lesions were suggestive of endothelial cell injury, and that Subtilase toxin may contribute to the characteristic vascular lesions of HUS, particularly in cases involving *stx*-negative *E. coli* strains.

In an eloquent follow up study, the authors determined the mechanism of action for SubAB using proteomic analysis of Vero cells exposed to either SubAB or an inactive SubAB mutant (116). Using this analysis, the cellular target of SubAB was determined to be the endoplasmic reticulum (ER) chaperone BiP. BiP has multiple functions that are essential for eukaryotic cell survival, including serving as a master ER regulator, directing misfolded proteins to the proteosome and assisting proper folding of secreted proteins. Cleavage by SubAB renders BiP inactive, thereby inducing cell death. The specificity of SubAB for BiP was also confirmed in vivo using the mouse model of SubAB toxicity and assessing BiP integrity in liver tissue with SDS-PAGE and western blot analysis (116). Intracellular trafficking of SubAB to the ER was clathrin-dependent and appeared to be similar to other AB_5 toxins (31).

Proteases

StcE

In 2002, Welch et. al. described the function of a previously undescribed protein encoded on the virulence plasmid pO157. Having noted that O157:H7 was capable of aggregating Jurkat cells in vitro, and that this property was conferred on pO157, the investigators used site directed mutagenesis to identify the specific gene responsible for this cytopathic effect. When the protein was expressed in vitro, it was found to be a zinc metalloprotease that specifically cleaved the serine protease inhibitor C1 esterase inhibitor (C1-INH). C1-INH plays a critical role in both inflammation and coagulation by inhibiting the classical and mannose-binding complement cascades, as well Factor XIIa and XIa. The protease was named StcE, was speculated to be secreted by type II secretion system, and analysis of several O157:H7 isolates revealed the presence of *stcE*. Additionally, StcE secretion was enhanced by expression of the LEE-regulator Ler (173). Interestingly, a later study identified that StcE actively recruited C1-INH to the cell membrane. Although StcE specifically cleaved C1-INH, the functional ability of C1-INH to cleave its substrate and thereby inhibit the classical complement was maintained, leading the authors to speculate that StcE may function to actively reduce inflammation at the site of colonization (80). Later studies identified a role for StcE in the intimate adherence of O157:H7 to HEp-2 cells, putatively via the active cleavage of glycoproteins on the host cell surface (53). Finally, an additional substrate for StcE was recently

identified, mucin MUC7, and its activity as a mucinase in addition to protease was confirmed (53).

EspP

Escherichia coli secreted protein P (EspP) is a plasmid-encoded serine protease autotransporter found in both virulent and non-virulent STEC strains. Recently, Brockmeyer et. al. identified several different subtypes of EspP in STEC, denoted EspPα, β, γ, and δ. These subtypes possessed differing proteolytic activity and distribution amongst the STEC strains examined (17). More recently, the prevalence of *espP* amongst strains associated with human disease was assessed. Twelve strains from HUS patients were examined, five of these were *espP* positive, and all five were subtype α. Additional analysis of STEC strains causing bloody diarrhea also revealed the majority were *espP* positive, and most of these were subtype α (73). Although the exact role of EspP in STEC pathogenesis remains to be determined, these studies suggest further investigations into its role as a putative STEC virulence factor are warranted.

Fimbrial adhesins

Although intimin is by far the most researched and best characterized adherence factor for STEC, clearly other adhesins play a role in some, if not all, STEC strains,

particularly since numerous *eae*-negative strains have been associated with human disease. Intimin-independent adherence could potentially be mediated by fimbrial adhesins, non-fimbrial adhesins, or a combination thereof. Several fimbrial adhesins have been described in STEC and some are suspected to have a substantial role in STEC pathogenesis (85). Long polar fimbriae (Lpf) similar to that of *Salmonella enterica* serovar *Typhimurium* have been identified in STEC (155). Several Lpf variants have been identified, and some appear to facilitate adhesion of STEC strain O113:H21 to epithelial cells in vitro (35). In animal models, Lpf contributes to colonization of O157:H7 in both sheep and pigs (66). Additionally, the gene encoding LpfA has been identified in numerous *eae*-negative STEC, and is theorized to be an key adhesin in these strains (114). An additional fimbrial adhesin that may play a role in STEC infection is Sfp, which has been identified in some non-motile O157 and O165:H25 strains (11). These fimbriae were involved in adherence of O157:NM to intestinal epithelial cells in vitro and were identified as a putative virulence attribute for these strains (103). Finally, a newly identified fimbrial adherence factor, *E. coli* common pilus (ECP), is expressed in most EHEC strains studied thus far. Isogenic *ecpA* mutants had decreased adherence to cultured epithelial cells, and ultrastructural data suggests these adhesins form numerous attachments to epithelial cells, suggesting ECP plays a role in EHEC adherence to host epithelium (127).

Non-fimbrial adhesins

Additionally, a number of non-fimbrial adhesins have received attention as putative virulence factors in STEC, particularly those strains that are LEE-negative. EHEC factor for adherence (Efa1) adhesin is important in colonization of bovine intestine by O111 STEC and mediates adherence to Chinese hamster ovary cells in vitro (79, 141). A homologue of Efa1, ToxB, is another purported non-fimbrial adhesin for STEC. ToxB is encoded on pO157 virulence plasmid, and *toxB* mutants exhibit decreased adherence to epithelial cells in vitro. ToxB's role in adherence may not be direct, however, as it is known to modulate effectors of LEE and can contribute to adherence indirectly in this manner (145). STEC autoagglutinating adhesin (Saa) is a plasmid-encoded non-fimbiral adhesin that was first described by Paton et. al. in LEE-negative STEC isolates pathogenic to humans (120). Saa helped mediate attachment of STEC to HEp-2 cells in vitro, and a comparison between bovine and human STEC strains found a higher prevalence in bovine strains, leading the authors to theorize that it may be an important factor in colonization of cattle (64, 120). Some additional proteins that may be involved in adherence include OmpA, Iha, and Cah (155).

Flagellin

Flagella are complex organelles which function primarily in motility, although they also function as adhesins and are important antigens in host immune responses. They appear to be important in the pathogenesis of STEC infections for several reasons. Firstly, flagella contribute to STEC adherence and colonization of the intestinal epithelium. In a chick model of EHEC infection, flagella are important for persistent colonization (8, 78). The importance of H7 flagellum in intestinal adherence in the bovine, the primary reservoir host for STEC, was further exemplified recently. Colonization of rectal epithelial cells with either a $fliC_{H7}$ mutant or wild type blocked with specific anti-H7 antibody was significantly less than that of wild type (3). Furthermore, colonization of O157:H7 in the bovine was inhibited by immunization with H7 flagellin (94). Secondly, flagellins are potent inducers of chemokine expression in vitro, and appear to play a role in host immune responses to STEC. IL-8, a chemokine that promotes neutrophil recruitment and chemotaxis, and CCL20 (MIP3A), a chemokine promoting chemotaxis of lymphocytes and dendritic cells, are both upregulated and secreted by cultured intestinal epithelial cells in response to H flagellin (49). Finally, signaling through TLR5 in colonic epithelial cells in response to H7 flagellin induced chemokine expression (176).

Lipopolysaccharide

Lipopolysaccharide (LPS) is a major structural component of the cell wall of Gram negative bacteria. In the host, LPS from pathogenic bacteria is a well know trigger of innate and adaptive immune responses and a major inducer of inflammation. LPS is a polyclonal mitogen, inducing proliferation of both B and T lymphocytes. Although bacteremia is not considered a feature of STEC infection in human beings, LPS antigens can circulate bound to plasma LPS-binding protein (LBP), leukocyte receptors such as CD-14, and have even been shown to bind platelets. LPS bound to LBP and CD14 forms a complex capable of triggering innate immune responses through TLR-4 signaling, including production of proinflammatory cytokines like TNF-α. Antibodies against O157 LPS were consistently identified in HUS patients, and thus LPS is likely to contribute to the proinflammatory cytokine response observed in HUS patients (20). Additionally, LPS may promote intestinal colonization of STEC. LPS-specific antibodies were able to prevent adherence of O157 and O111 to the human intestinal epithelial cell line Henle 407 in vitro (121). O antigen also may promote colonization of the bovine terminal rectal mucosa by O157:H7 (134).

Shiga toxin dissemination in the pathogenesis of the hemolytic uremic syndrome.

Following colonization of STEC, Stx are produced locally within the intestine, then cross the intestinal mucosa and vascular endothelium via a yet undefined mechanism to gain access to the bloodstream. It has been shown in tissue culture systems that biologically active Stx can cross intestinal epithelial cells, and that neutrophil transmigration across epithelial monolayers enhances Stx translocation (1, 58). Once the toxin reaches the blood, it binds globotriaosylceramide (Gb_3) on the surface of vascular endothelial cells in key target organs (kidney, brain) and is internalized (62). Toxin internalization within endothelial cells results in inhibition of protein synthesis, leading to cell death (45). How Stx is transported in the bloodstream has been debated, and although Stx1 and/or Stx2 binding to erythrocytes, platelets, and monocytes has been described, it is currently presumed that neutrophils serve as the primary carriers of the toxins in the bloodstream.

Role of neutrophils

Several years ago, the first in a series of publications appeared implicating peripheral blood neutrophils in the dissemination of Shiga toxin in vivo. In this study, Stx1 was incubated with human whole blood and bound exclusively to polymorphonuclear leukocytes. Neutrophil-bound Stx1 was readily transferred to cultured human microvascular endothelial cells and led to cell death via inhibition of

protein synthesis. Using radiolabeled Stx1, binding studies showed the neutrophil receptor bound Stx1 less avidly (approximately 100-fold) than the native Stx receptor on vascular endothelial cells, globotriaosylceramide. The authors theorized that this was the mechanism for Stx reaching the renal endothelium from the site of production in the colon (147). Subsequently, the same group was able to detect Stx2 in the systemic circulation of patients with HUS. In this study, 9 out of 10 HUS patients examined had Stx2 bound to neutrophils that was detected using indirect immunofluorescence and flow cytometry (148). These same techniques were then used to screen the peripheral blood of other members in the households of HUS patients for subclinical STEC infection. The sensitivity of this assay was then compared to fecal culture and serologic testing for *E. coli* LPS for diagnosis of STEC infection. Stx was detected bound to neutrophils in 82% of household members screened, versus 10% of household members being positive for either fecal culture or serology. The authors concluded, based on their findings, that traditional methods for detection of STEC infection likely underestimate the number of infected individuals (150). Stx binding to human neutrophils in HUS patients was later corroborated using a similar flow cytometry technique (146). This same group also found a positive correlation between fecal Stx and neutrophil-bound Stx in the systemic circulation of HUS patients (16). More recently, Griener et. al. compared binding of Stx2 to human versus mouse neutrophils. They found that while Stx2 bound to murine neutrophils via the classical Stx receptor Gb3, binding of Stx2 to human neutrophils was independent of Gb3 (51).

In contrast, several studies have refuted the finding that Stx binds to human neutrophils. Flagler et. al. examined the effects of Stx1 and Stx2 on neutrophil apoptosis and binding. Neutrophil apoptosis was not affected by Stx, nor was significant binding of either toxin to neutrophils observed. The authors concluded that Stx had no direct effect on human neutrophils (40). Finally, in 2007, the same group that published the original series of papers describing Stx binding to PMN refuted their original findings in a new study. They re-examined the specificity of their anti-Stx2 antibody used to detect neutrophil-bound Stx, using neutrophils from healthy individuals, hemodialysis patients, and peritoneal dialysis patients as controls. The antibody bound non-specifically to neutrophils from hemodialysis patients, and the authors concluded that the antibody likewise bound non-specifically in HUS patients. They were unable to detect any significant binding of Stx1 to human neutrophils in vitro. The authors concluded that neutrophils were not serving as the mechanism for dissemination of Stx in vivo (47).

Role of platelets in the pathogenesis of HUS

Since HUS is a thrombotic disorder, with microthrombi formation in multiple organs, platelets play an integral part in the pathogenesis. Peripheral thrombocytopenia develops secondary to thrombi formation, and is one of the three key clinical criteria for HUS. Widespread endothelial cell injury leads to exposure of subendothelial collagen and platelet activation. Additionally, several studies have identified a more direct

interaction between Shiga-like toxin (Stx) and platelets. Supernatants from STEC culture were shown to activate platelets in vitro (175). Platelets also express surface Gb_3, the preferred receptor of Stx1 and Stx2. An early study identified Stx (derived from *Shigella dysenteriae* only) binding to human platelets via both flow cytometry and thin layer chromatography. Binding was mediated through both Gb_3 and a newly described glycosphingolipid, identified as band 0.03 (26). Another study noted that Stx1 and its isolated binding subunit, Stx1B, bound to platelets, were internalized, and induced aggregation. Stx1 also enhanced fibrinogen binding to platelets, platelet retention, and induced platelet binding to cultured umbilical vein endothelial cells in vitro (69).

The activation status of platelets apparently influences their ability to bind Stx directly. Ghosh et. al. demonstrated that platelets activated via high speed centrifugation, EDTA or thrombin exposure had higher concentrations of Gb_3 on their surface and therefore bound greater amounts of Stx than resting platelets. Platelet activation status was assessed via P-selectin exposure (48). Moreover, virulence factors of STEC have the ability to directly activate platelets. Both human monocytes and the human monocytic cell line THP-1 were shown to produce chemokines such as IL-8 and RANTES following exposure to Stx or LPS. The supernatants from cultured monocytes exposed to Stx and/or LPS were then added to isolated human platelets, and activation status was assessed via aggregation studies, P-selectin exposure, and serotonin release (54).

Role of inflammation and proinflammatory cytokines in the hemolytic uremic syndrome.

Although the production and dissemination of Stx is essential for HUS development, there is compelling evidence that the interaction of Stx with endothelium alone is not sufficient to induce HUS. A growing body of research exists to implicate proinflammatory cytokines as contributing factors in the pathogenesis of HUS. Several studies have discovered an increase in the concentration of proinflammatory cytokines in patients who develop HUS over those with STEC-induced diarrhea alone (60, 82, 170). Decreased levels of the anti-inflammatory cytokine IL-10 have also been associated with HUS development (170, 171). In several *in vitro* studies, TNF-α and IL-1β greatly enhance the cytotoxicity of Stx. The direct cytotoxicity of Stx on cultured human vascular endothelial cells is minimal; however, when endothelial cells are exposed to TNF-α and IL-1β, they are profoundly sensitized to the cytotoxic effects of Stx. This enhancement of Stx cytotoxicity is presumably mediated by the up-regulation of Gb_3 on endothelial cells (37, 83, 84, 126, 153, 162-164). One study in particular looked at the potential *in vitro* cytokine production in patients who had experienced an episode of HUS using a whole blood stimulation model. Their findings indicate that HUS patients have a propensity to produce greater amounts of several proinflammatory cytokines including IL-1, and reduced amounts of the anti-inflammatory cytokine IL-10 (171). Further support for the role of cytokines and inflammation in HUS development is that certain indicators of systemic inflammation have been identified as risk factors for HUS

development in patients with bloody diarrhea, including leukocytosis and neutrophilia

(18).

Increases in proinflammatory cytokine production, particularly TNF-α and IL-1β,

following exposure to Stx occurs in human monocytes, murine macrophages, and human

cerebral microvascular endothelial cells (38, 57, 153). The precise signaling pathway

involved in Stx induction of proinflammatory cytokines has not been fully characterized.

The increase in cytokine production by monocytes appears to involve transcriptional

activation via NF-κB and activator protein-1. Recent work has shown that functional

tyrosine kinase activity also is required for TNF-α activation by Stx1 (41, 130). More

specifically, transcription factors (e.g. c-Jun, NF-κB) and p38 MAPK have been linked in

the STEC induced signally cascade (19, 42, 130, 149). Stx 1 and 2 can increase p38

MAPK activity leading to enhanced TNF-α expression in peripheral blood monocytes

(19). NF-κB and c-Jun are also activated by STEC infection and these pathways can lead

to proinflammatory cytokine expression of TNF-α and IL-1 (42, 130, 149). Stx exposure

also increases the stability of cytokine transcripts in human monocytes (57). TNF-α has

been shown to enhance clinical disease and depletion of hepatic and splenic macrophages

(a primary source of systemic cytokine production) decreases lethality in mouse models

of STEC infection (61, 115). Based on these studies, hepatic and splenic macrophages

are implicated as the source of systemic proinflammatory cytokine production in STEC

infection *in vivo.* Chemokine expression is also induced in both macrophages and

intestinal epithelial cells following exposure to Stx1 and LPS, thereby promoting further

inflammation via leukocyte recruitment and activation (56, 154). Although these studies

suggest Stx can contribute to proinflammatory cytokine production and inflammation, it is not known to what extent this systemic increase in cytokines *in vivo* is due to circulating Stx versus other STEC virulence factors such as LPS.

Experimental therapeutics in Hemolytic Uremic Syndrome

Shiga-like toxin Receptor Mimics

One potential therapeutic approach in STEC infection is to mitigate the effects of Stx via the use of Gb_3 receptor mimetics, exogenous ligands which interact specifically with Stx thereby preventing its interaction with Gb_3 on host cells. Since the onset of systemic disease is preceded in most cases by a prodromal diarrheal phase that lasts several days, the opportunity exists to intervene to prevent systemic disease. There are two major classes of receptor mimics that have been produced thus far: oral binding agents designed to trap Stx at the site of formation within the gut, and parenterally administered compounds that neutralize Stx after it has left the intestinal tract (13, 118).

Two main classes of orally administered Stx binding agents have been developed to prevent Stx absorption into the peripheral bloodstream. These are probiotics that express a receptor mimic of Gb_3 on their surface and entirely synthetic oligosaccharide moieties. Due to the inherent low affinity between the protein binding subunit of Stx and

its carbohydrate receptor, the interaction of the five binding subunits with its ligand is complex, and requires engagement of multiple B subunits. Therefore, in order to be effective, these receptor analogues require complex three dimensional structures capable of binding multiple binding subunits of the toxin in order to be effective (30, 74-76, 99, 107, 122). One of the earliest oral binding agents to be developed, SYNSORB Pk, consisted of the oligosaccharide portion of Gb_3 covalently bound to a particle of insoluble silicon dioxide (2). SYNSORB Pk was efficacious at binding and neutralizing Stx in vitro, and had no negative side effects in Phase I clinical trials (74, 102). Its ability to neutralize toxin in vitro, however, did not translate to success at preventing serious disease in vivo. The results of a clinical trial in which children with diarrhea-associated HUS were treated with either SYNSORB Pk or placebo yielded no significant difference between treatment groups in the incidence of either death or significant involvement of additional organ systems (156). The authors proposed several potential explanations for the failure of the oral binding agent in this study: 1) oral therapy was initiated too late in the course of the disease (e.g. following the clinical diagnosis of HUS) 2) reduced gastrointestinal motility in patients limited interaction of the binding agent with intraluminal toxin in the colon 3) intimate association of STEC with the GI mucosa impaired the ability of the binding agent to adsorb toxin prior to systemic absorption.

More advanced carbohydrate inhibitors of Stx have since been developed that are able to engage more of the 15 binding sites per Stx molecule. STARFISH is a pentameric structure of P^k trisaccharide dimers attached to a core consisting of a single glucose molecule. This multivalent inhibitor is able to engage the five B subunits of Stx

in much the same way as its natural ligand, Gb_3. As a result, STARFISH demonstrated profound inhibitory activity against Stx1 and Stx2, far surpassing the activity of its predecessors (74). Similarly, another group developed a multivalent analogue of the Gb_3 ligand attached to an acrylamide polymer denoted "SUPER TWIG" (33). This compound was highly efficacious at neutralizing Stx1 and Stx2 in vitro. Additionally, in a mouse model of fatal STEC infection, SUPER TWIG prevented death when administered intravenously during the early stages of infection (109). Oral administration of the analogue was similarly effective at preventing brain lesions and death in the same mouse model of STEC infection. SUPER TWIG was efficacious at reducing Stx levels in circulation and reducing histologic lesions in the brain (167).

More recently, a probiotic that expresses Stx receptor mimics on its surface was developed for treatment of STEC infection (118). The probiotic is a recombinant *E. coli* RI strain (CWG308) that contains a plasmid (pJCP-Gb3) encoding several *Neisseria sp.* genes for oligosaccharides that when expressed, create a cell surface mimic of the Stx receptor. The binding capacity of this recombinant strain for either Stx1 or Stx2 is approximately 10,000x greater than that of SYNSORB Pk, and this efficacy has been corroborated by several *in vivo* protection studies in mice (117, 118, 123). This particular construct was effective at neutralizing most of the Stx2 variants, Stx2c and Stx2d; however, it was less efficacious against Stx2e, which binds preferentially to Gb_4. As a result, a new recombinant strain that expressed surface Gb_4 was created as a potential probiotic strain for use in the prevention of edema disease of swine (117).

One of the main barriers to success for orally administered Stx adsorptive compounds is that they have no ability to neutralize toxin once it is absorbed systemically. This is a complicating factor for several reasons. Firstly, due to the reported delay that exists between the start of STEC infection and diagnosis, many patients may have already had some systemic Shiga toxemia. Secondly, despite the success of oral binding agents at preventing serious systemic disease in certain animal models, this may not correlate to success in a clinical setting. Based in part on the findings of the SYNSORB Pk study, it is plausible that some systemic absorption of toxin will occur, even in the presence of an oral Stx binding agent (156). Therefore, parenterally administered compounds may prove more effective at preventing HUS, whether used alone or in conjunction with an oral binding agent. Additionally, because of their ability to neutralize toxin within the bloodstream and potentially within the organs developing lesions (e.g. brain, kidney, colon), parenterally administered compounds may be useful in treatment of HUS.

One of the most traditional options for a parenterally administered Stx neutralizing compound is specific antibody. Numerous studies in several animal models of STEC infection have shown protection from serious negative outcomes using parenterally administerd specific antibody to Shiga toxin (passive immunization)(34, 90, 101, 132, 135, 136). Active immunization is also successful at preventing systemic vascular disease in numerous animal models (14, 50, 177). The practicality of using active immunization is low, considering the relative low risk of HUS development in the general population. Passive immunization as therapy in STEC infection may show

promise, and phase one clinical trials have already been completed for a humanized monoclonal anti-Stx2 antibody (36). Due to the lack of cross reactivity between antibody to Stx1 and Stx2, specific antibody for each toxin must be used if strain phenotyping is not performed in each clinical case.

Edema disease of swine

Edema disease (ED) is a naturally occurring disease of weaned swine caused by host-adapted strains of *E. coli* that produce a variant of Stx2, the edema disease toxin (Stx2e). Edema disease is the only other naturally occurring STEC infection other than HUS caused by vascular damage in multiple organs induced by Shiga toxemia. Susceptibility to edema disease is age-dependent owing to developmentally regulated expression of the intestinal bacterial receptors necessary for colonization. Colonization porcine intestine by edema disease strains is mediated by F18 fimbriae, which bind to glycoconjugates on the surface of enterocytes in the distal small intestine whose expression is induced at weaning (59). The most common serogroups of isolates from clinical outbreaks are O138, O139, and O141. Edema disease causes sporadic losses for swine herds with variable morbidity and mortality. Interventions for control of naturally occurring disease include antimicrobials, feed changes, and vaccination. Experimental reproduction of ED can be induced either by intravenous administration of Stx2e or by oral inoculation with STEC (28). In an experimental setting, the incidence of clinical ED

can be reduced by a variety of measures, including the use of vaccination, antimicrobials, probiotics, and administration of specific Stx2e antibody (14, 90, 157).

Virulence attributes of edema disease strains

Stx2e

Stx2e is the toxin principal of edema disease, causing vascular necrosis in the ileum, brainstem, and subcutis thereby resulting in the characteristic clinical signs. Stx2e was initially described as the edema disease principal, an *E. coli* verocytoxin produced by strains associated with edema disease of swine (81). The similarity between the edema disease principal and Stx2 was subsequently revealed when it was discovered that it was neutralized by Stx2, but not Stx1, specific antibody. Stx2e is approximately 89% and 50% homologous to Stx2 and Stx1, respectively, based on amino acid sequencing (168). Despite the structural similarity between Stx2 and Stx2e, differences in the cytotoxicity for cultured cell lines exist between these two toxins. While both are toxic to Vero cells, Stx2e shows reduced toxicity for HeLa cells compared to Stx2. The reason for this disparity is related to differences in ligand preference between the two toxins. The glycolipid receptor preference of Stx2e was identified by thin layer chromatography as globotetraosylceramide (Gb_4), in contrast to Stx2 which binds Gb_3. Vero cells express both Gb_4 and Gb_3, while HeLa cells only express Gb_3. Stx2e also binds to Gb_3 and Gb_5 to a lesser extent (32, 71, 131).

Edema disease was experimentally reproduced initially by intravenous administration of partially purified Stx2e, and later purified Stx2e, to piglets (86, 88). Clinical signs of edema disease were induced using as little as 3ng/kg Stx2e intravenously, and parenterally-administered purified toxin was also lethal for mice (86). Later studies used site-directed mutagenesis to identify which amino acids in the B subunit of Stx2e were responsible for the preference of Gb_4 over Gb_3. Substitution of the Gln_{64} and Lys_{66} of Stx2e to the Glu and Gln of Stx2 altered its glycolipid binding preference to mimic that of Stx2, binding only Gb3 (159). This mutant, termed GT3, exhibited differing biological behavior in vivo than wildtype Stx2e. In a pig model, the tissue distribution of radiolabeled GT3 was compared to that of wildtype Stx2e and Stx1 following intravenous administration. The GT3 mutant behaved in a manner similar to Stx1, and showed increased localization to neural tissues (cerebrum, brainstem, and cerebellum) and less affinity for the gastrointestinal tract than wildtype. Wildtype Stx2e was found to bind extensively to erythrocytes, which express high levels of its preferred ligand, Gb_4. Because of the interaction of Stx2e and erythrocytes, the distribution of Stx2e in vivo was affected by tissue blood flow, unlike Stx1 or the GT3 mutant, which did not bind significantly to erythrocytes (15).

F18 fimbriae

F18 fimbriae are filamentous bacterial surface antigens composed of 15kDa units that mediate colonization of ED STEC in the lower small intestine of swine by binding to host enterocyte receptors (59). Two antigenic variants of F18 are recognized, F18ab and F18ac, and are most commonly identified in STEC associated with ED and ETEC associated with post-weaning diarrhea, respectively. F18ab and F18ac fimbriae were formally known as F107 and 2134P, respectively (22, 129). F18 fimbriae recognize the F18 receptor (F18R) on the surface of enterocytes, expression of which is developmentally regulated and corresponds to weaning; therefore, ED and post-weaning diarrhea are age-related disease entities. Genetic resistance to ED occurs and is mediated through activity of the *FUT1* gene, which encodes alpha (1,2)-fucosyltransferase (FUT1) and thereby indirectly affects expression of F18R (6, 96, 97). Genetically resistant pigs possess a single DNA polymorphism on both alleles resulting in an amino acid switch rendering them F18R$^-$ (97). Similar to F18R expression, certain histo-blood group antigens (HBGAs), exhibit age-related changes in expression rates and are expressed by certain endothelial and epithelial cells in addition to erythrocytes. Recently, both H-2 HBGAs and A-2 HBGAs have been implicated in F18-mediated adherence of *E. coli* in porcine intestine (24, 25).

Enterotoxins

Three plasmid-encoded enterotoxins, heat-labile toxin (LT), heat-stabile enterotoxin A (STa) and heat-stabile enterotoxin B (STb), are associated with pathogenic *E. coli* in pigs, and STEC strains causing ED are frequently positive for one or more these enterotoxins. Expression of and response to these toxins in vivo causes the prodromal diarrhea associated with many ED outbreaks. STa and STb are low molecular weight polypeptides induce ion secretion into the intestinal lumen. STa activates guanylate cyclase within the cell resulting in increased cGMP levels and subsequent chloride and water secretion, while STb induces bicarbonate secretion (59). LT, like Stx2e, is a complex AB_5 toxin. LT has a similar mechanism of action to cholera toxin of *Vibrio cholerae*. Once internalized within the enterocyte, the active A subunit activates adenylate cyclase to raise cAMP levels leading to chloride and water secretion from the cell (59). Stx2e itself is mildly enterotoxigenic under certain experimental conditions; however, it does not appear to cause fluid secretion within the intestinal tract of pigs and therefore is unlikely to directly contribute to diarrhea in ED (87).

Hemolysin

Hemolysin is an RTX pore-forming toxin, expression of which results in β-hemolysis on blood agar plates. Many STEC and ETEC strains that cause disease in

piglets are hemolysin-positive, although it is not considered a putative virulence gene in these strains. Hemolysin is plasmid encoded (*hlyA*), and an isogenic deletion mutant of an ETEC strain in gnotobiotic piglets did not affect strain pathogenicity in experimental inoculation studies(100).

Clinical edema disease

ED causes sporadic outbreaks and typically occurs in the 1-2 week period following weaning. Susceptibility is multifactorial, with genetic resistance, declining secretory IgA levels from the sow, and diet changes all purported as important factors in disease development. ED outbreaks typically show low morbidity with higher mortality (range 15-90%), and traditionally affect the stronger animals in the litter (59). Outbreaks in litters last three days or less, and although recurrence within the same litter is not typically observed, recurrence on the same farm is common.

Following oral exposure, preferential colonization of ED STEC occurs within the lower small intestine (distal jejunum and ileum) (5). Bacterial adherence is mediated by F18 fimbriae, is morphologically evident by 2 days post-inoculation, and occurs mainly at the villous tips of enterocytes. Significant morphologic changes to enterocytes are not reported to occur (98). ED strains that elaborate one or more enterotoxins may cause diarrhea prior to clinical ED.

Local production of Stx2e within the intestinal lumen occurs as early as 1 day post-inoculation (29). Despite the lack of visible changes in enterocytes following STEC colonization, systemic Stx2e absorption occurs soon after colonization, and vascular lesions characteristic of Stx2e are evident by two days post-inoculation (98). The route of intestinal absorption of Stx2e remains elusive. Both Gb_3 and Gb_4 are expressed in porcine jejunum and ileum, although the precise role of these glycolipids in Stx2e absorption has yet to be characterized (166). Under experimental conditions, Stx2e fecal titers peak at 4-6 days post-inoculation. Although fecal Stx2e is a risk factor for clinical disease development, fecal Stx2e titers alone are not predictive of clinical outcome for individual pigs (29). Oral administration of Stx2e in pigs does not result in clinical ED; however, if intestinal absorption of toxin is experimentally increased with sodium deoxycholate, signs of ED are reproduced. Sodium deoxycholate is an unconjugated bile salt that causes increased permeability of the intestinal mucosa to macromolecules (165). These findings suggest that increased intestinal permeability may be a factor in Stx2e absorption and subsequent disease development.

Following intestinal absorption, Stx2e is presumed to travel in the systemic circulation bound to the surface of erythrocytes. In an experimental setting, significant amounts of erythrocyte-bound Stx2e were detected two hours following intravenous Stx2e injection. The erythrocyte receptor for Stx2e is presumed to be Gb_4, the preferred ligand for Stx2e, since erythrocyte membranes express significant amounts of Gb_4 (15). Erythrocyte-bound Stx2e is readily transferred to sensitive Vero cells in vitro, thereby inducing cell death (29, 91). Additionally, Stx2e could be detected in the peripheral

blood of STEC challenged swine and the presence of erythrocyte-bound Stx2e was strongly associated with an increased risk of clinical edema disease. Stx2e was not detected in plasma. In this study, two different Stx2e erythrocyte-binding types were described. The majority of pigs consistently bound high levels of Stx2e (termed the "high-binding phenotype"), while a small number of pigs had erythrocytes that consistently bound lower levels of Stx2e ("low-binding phenotype"). It was proposed that the binding phenotype could affect the risk for edema disease development by affecting the amount of Stx2e delivered to critical organs (91). Presumably, variations in membrane expression of Gb_4 would account for the differences in Stx2e binding between the two phenotypes; however, this has not been experimentally verified.

The clinical manifestations of ED are caused by systemic effects of Stx2e. A spectrum of clinical signs is reported. Milder signs are characterized by subcutaneous edema which is typically most pronounced on the eyelids, but may also be noted in the submandibular, thoracic, and abdominal areas (59). The more severe clinical manifestations of ED are caused by vascular changes within the brain. Neurological signs in ED include incoordination, changes in gait, ataxia, tremors, paralysis, altered squeal, and paddling movements of the limbs (88, 158). Lateral recumbency and sudden death are the most severe manifestations of ED. Stx2e localization within tissues is dependent upon both receptor expression and blood flow (15). The key target organs affected in ED are the brain and intestinal tract. Gross lesions of ED are typically mild and may be absent in some clinical animals (92). When present, they consist of edema of the stomach submucosa, mesentery, mesenteric lymph nodes, and cerebellum. Serous

effusion of the abdominal and/or pleural cavities also has been reported (5). Once the toxin is internalized, it disrupts protein synthesis within the cell. Although apoptosis secondary to Stx can be induced experimentally in several cell types, ultrastructural examination of vascular lesions from pigs with ED determined that necrosis of endothelial cells and myocytes within the tunica media was the predominant change in vivo (92). Despite a range of described microscopic vascular lesions that occur in ED, the classical lesion is vascular necrosis of arterioles, commonly of the brain (brainstem, meninges, cerebrum) and intestinal tract (submucosa of stomach, intestine, and mesentery). Rarely, vascular lesions can be identified in other organs including pancreas, liver, and kidney (70). Acutely affected pigs may have minimal microscopic lesions, consisting of mild perivascular edema and/or karryorhectic debris within myocytes. Some acutely affected pigs have more severe vascular lesions, consisting of microvascular thrombosis and/or fibrinoid necrosis (98). Subclinical pigs also have vascular lesions, and they are often more pronounced than those of pigs that die acutely. Proliferative arteriolar changes are more common in chronic subclinical cases as well. A recent study by Tsukahara et. al. suggests that additional microscopic lesions of ED may include eosinophilic enteritis and renal thrombosis; however, the specificity of these lesions for STEC infection is questionable (157).

Edema disease of swine as a model for STEC infection in human beings.

The edema disease animal model has been used to study HUS (29, 88).   In approximately 30% of ED-STEC challenged swine, clinical signs (subcutaneous edema, neurological signs, sudden death) of edema disease develop 3-10 days post inoculation (5, 29). The edema disease model has several advantages over other models of HUS, the most outstanding of which is that it is a naturally occurring disease. Edema disease and HUS are presumed to share similar early events in their pathogeneses, including localized production of toxin in the gut, toxin translocation across gut epithelium, and toxin dissemination to target organs via the bloodstream (29, 34). Additionally, as in human STEC infections, not all STEC inoculated swine develop systemic disease; the majority of pigs undergo an uncomplicated recovery from the diarrhea phase. Other advantages include a similar time course of pathogenic events including a prodromal diarrhea phase with delayed development of systemic complications (29). A comparison of the pathogenesis between HUS and edema disease is made in Table 1.

Table 1: Comparison of pathogenesis between HUS and edema disease. Unknowns highlighted with italics are to be addressed by the research outlined in this thesis.		
Element of Disease Pathogenesis	**Hemolytic Uremic Syndrome**	**Edema Disease**
Anatomic location of bacterial colonization	Colon	Lower small intestine
Primary virulence factor	Stx1 and Stx2	Stx2e
Primary toxin receptor on target cells (vascular endothelium)	Gb3	Gb4
Proposed blood carrier responsible for toxin dissemination	Neutrophil (?)	Erythrocyte
Target organs	Kidney, brain, colon	Ileum, brainstem
Monocytes and/or macrophages produce pro-inflammatory cytokines following exposure to Shiga toxin	Stx1 and Stx2: Yes	Stx2e: Unknown
Circulating Shiga toxin detected in individuals that develop systemic clinical disease	Yes	Yes
Circulating Shiga toxin detected in individuals that fail to develop systemic clinical disease	Yes	Yes
Increased proinflammatory cytokines in individuals with clinical disease	Yes	Unknown
Increased cytokines secondary to Shiga toxin dissemination in clinical individuals	Presumed, but unclear	Unknown

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Chapter 2: Treatment with a receptor mimic probiotic reduced intestinal Shiga toxin but did not prevent systemic toxemia in Shiga toxin-producing *Escherichia coli* inoculated piglets.

A paper to be submitted to the *Journal of Medical Microbiology*

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Abstract

Hemolytic uremic syndrome is a systemic and potentially fatal complication of Shiga toxin-producing *Escherichia coli* (STEC) in human beings, and is a leading cause of renal failure in children. Edema disease, an infection of swine caused by host adapted STEC, shares a similar pathogenesis to hemolytic uremic syndrome and is used as an animal model to study this disease. Previously, we have prevented edema disease in STEC inoculated pigs with parenteral administration of antibody to Shiga toxin. This study describes the effect of treatment with a probiotic expressing surface globotetraose, the preferred oligosaccharide receptor for Shiga toxin 2e (Stx2e), on clinical edema disease. STEC inoculated pigs were treated with the probiotic (5×10^{11} CFU) twice daily from days 1-10 post inoculation. Treatment with the probiotic significantly reduced intestinal Stx2e, as reflected by decreased fecal toxin titers on days 1-10 post-inoculation

(p<0.05). Despite this reduction of intestinal toxin, treatment failed to reduce vascular necrosis in target organs and had no effect on the incidence of clinical disease in STEC inoculated pigs. These findings suggest that treatment with an oral Stx binding agent may not be as efficacious at preventing systemic disease secondary to Shiga toxemia as parenterally administered specific antibody in a naturally occurring STEC infection.

Introduction

A small percentage of patients (5-15%) with diarrhea secondary to Shiga toxin producing *Escherichia coli* (STEC) infection develop a systemic and potentially fatal complication known as hemolytic uremic syndrome (HUS), characterized by the triad of hemolytic anemia, thrombocytopenia, and acute renal damage. HUS is more prevalent in children under the age of 10 and the elderly, and it remains the leading cause of renal failure in children in the United States (Paton and Paton 1998; Siegler and Oakes 2005; Tarr, Gordon et al. 2005). At present, there is no known method to predict which patients with diarrhea caused by STEC will develop HUS, and no known treatment for the disorder once it is diagnosed.

One potential therapeutic approach is to neutralize the activity of Shiga toxin (Stx), the principle mediator of HUS development in STEC patients. The preferred receptor for Stx1, Stx2, and most of its variants is globotriaosylceramide (Gb_3); however, globotetraosylceramide (Gb_4) is the preferred receptor of Stx2e, the Stx2 variant which is primarily associated with edema disease of swine (Boyd, Tyrrell et al. 1993). Following STEC colonization, Stxs are produced locally within the intestine, then cross the intestinal mucosa and vascular endothelium to gain access to the bloodstream. Once the

toxin reaches the blood, it binds the receptor on the surface of vascular endothelial cells in key target organs (kidney, brain) and is internalized (Jacewicz, Clausen et al. 1986). Toxin internalization within endothelial cells results in inhibition of protein synthesis, leading to cell death (Paton and Paton 1998). Recent studies in animal models of STEC infection have had success in preventing systemic complications associated with Stx through the use of specific antibody that binds and neutralizes Stx (Matise, Cornick et al. 2001; Mukherjee, Chios et al. 2002; Sheoran, Chapman et al. 2003; Sheoran, Chapman-Bonofiglio et al. 2005). Several other synthetic compounds have been developed to bind and/or neutralize Stx in the gut prior to systemic absorption. One such compound, SYNSORB Pk, was created by attaching synthetic carbohydrate mimics of the Stx receptor, Gb3, onto the surface of diatomaceous earth (Takeda, Yoshino et al. 1999). Synsorb PK was designed as an oral therapeutic compound that would theoretically trap Stx at the site of production within the intestinal lumen, thereby preventing systemic Stx absorption. Despite promising data from *in vitro* studies looking at toxin neutralization, Synsorb Pk failed to reduce the incidence of HUS in clinical trials.

More recently, a probiotic that expresses Stx receptor mimics on its surface was developed for treatment of STEC infection (Paton, Morona et al. 2000). The probiotic is a recombinant *E. coli* RI strain (CWG308) that contains a plasmid (pJCP-Gb3) encoding several *Neisseria sp.* genes for oligosaccharides that when expressed, create a cell surface mimic of the Stx receptor. The binding capacity of this recombinant strain for either Stx1 or Stx2 is approximately 10,000x greater than that of Synsorb Pk, and this efficacy has been collaborated by several *in vivo* protection studies in mice (Paton, Morona et al. 2000; Paton, Morona et al. 2001; Paton, Rogers et al. 2001). This particular construct

was effective at neutralizing most of the Stx2 variants, Stx2c and Stx2d; however, it was less efficacious against Stx2e, which binds preferentially to Gb4. As a result, a new recombinant strain that expressed surface Gb4 was created as a potential probiotic strain for use in the prevention of edema disease of swine(Paton, Morona et al. 2001).

Edema disease, an infection of weaned swine caused by host-adapted strains of *E. coli*, has been used as an animal model of systemic STEC disease in human beings (Cornick, Matise et al. 2000) . In this model, approximately 30% of STEC challenged swine succumb to clinical disease secondary to Shiga toxemia, characterized by subcutaneous edema, neurological signs and/or sudden death (MacLeod, Gyles et al. 1991). The edema disease model has several advantages over other models of HUS, the most outstanding of which is that it is a naturally occurring disease. Edema disease and HUS share similar early events in their pathogeneses, including localized production of toxin in the gut, toxin translocation across gut epithelium, and dissemination to target organs via the bloodstream (Donohue-Rolfe, Kondova et al. 1999; Cornick, Matise et al. 2000). Additionally, as in human STEC infections, not all STEC inoculated swine develop systemic disease; the majority of pigs undergo an uncomplicated recovery from the diarrhea phase. Other advantages include a similar time course of pathogenic events manifested by a prodromal diarrhea phase with delayed development of systemic complications (Cornick, Matise et al. 2000).

We hypothesized that treatment with the probiotic expressing surface globotetraose would bind and trap Stx2e within the intestinal lumen, thereby reducing systemic toxin absorption and preventing clinical edema disease. The purpose of this

study was to investigate whether this probiotic would decrease the incidence of clinical edema disease in STEC-challenged swine.

A preliminary report of this work was made at the Annual Meeting of the American Society for Microbiology, May 2006, Orlando, FL

Materials and Methods

Bacterial strains. S1191 is an STEC strain isolated from a pig with edema disease. This strain belongs to serogroup O139, is resistant to chloramphenicol, and produces Stx2e, hemolysin, F18ab fimbriae and heat-stable enterotoxin B (L.R.M. Marques 1987). Strain 123 is a non-pathogenic *E. coli*, serogroup O43, isolated from a healthy pig. Inocula were prepared as described previously (Sarmiento, Casey et al. 1988; Cornick, Matise et al. 2000).

Creation of the probiotic strain, an *E. coli* R1 derivative expressing surface Gal*N*Acβ(1→3)Galα(1→4)Galβ(1→4)Glc (globotetraose), has been described in detail previously (Paton, Morona et al. 2001). Briefly, three *Neisseria sp.* glycosyltransferase genes (*lgtC, lgtE,* and *lgtD*) and a UDP-Gal*N*Ac-4-epimerase gene (*gne*) were cloned into the plasmid vector pK184 to create plasmid pGb_4 and inserted into a derivative of *E. coli* R1 (CWG308). This strain has a *waaO* mutation in the outer core lipopolysaccharide (LPS) biosynthesis locus that results in production of a truncated LPS core terminating in glucose. Expression of the plasmid-encoded glycosyltransferase genes results in linkage of Gal*N*Acβ(1→3)Galα(1→4)Galβ(1→4) onto the terminal glucose. The sham strain is the *E. coli* derivative R1 (CWG308) with the plasmid vector pK184 only. The treatment

and sham strains were grown overnight in tryptic soy broth plus kanamycin (30 μg/ml) and IPTG (20 μg/ml), concentrated 100× and resuspended in TSB containing 10% $NaCO_3$, 20% sucrose and 21% glycerol. Treatments were stored at -80C and thawed just prior to use.

Reproduction of clinical edema disease. Animal experiments were carried out in accordance with the Iowa State University Animal Care and Use Committee. The study design proposed here to reproduce clinical edema disease follows a previously described protocol and is outlined in Table 1 (Cornick, Matise et al. 2000). Briefly, two week-old crossbred pigs were acclimated to a high soy protein diet for one week prior to challenge (Bosworth 2002). Sixty pigs (twenty pigs per trial, three separate trials) were inoculated orally with 10^{10} CFU of STEC strain S1191 eight days after weaning. Control pigs (ten pigs) received 10^{10} CFU of the non-pathogenic *E. coli* strain 123. Pigs were monitored twice daily for clinical signs of edema disease (subcutaneous edema, recumbency or neurological disturbances such as ataxia, circling or sudden death). Pigs that developed neurological signs were euthanized by an intravenous overdose with barbiturate. Pigs that failed to develop neurological signs were euthanized at the termination of the study (14 days post-inoculation). All pigs underwent a complete necropsy, with inspection of major organs for gross lesions and collection of tissue samples from ileum and brainstem in neutral buffered formalin for histology. Hematoxylin and eosin stained sections of ileum and brainstem were examined by a pathologist blinded to the study for evidence of vascular necrosis (positive if two or more necrotic vessels were identified) (Matise, Sirinarumitr et al. 2000).

Treatment with the probiotic. Pigs inoculated with STEC strain S1191 were treated with either *E. coli* CWG308 containing the plasmid pGb_4 (probiotic; thirty pigs in total) or CWG308 containing the vector pK184 only (sham; thirty pigs in total). Individual pigs were orally dosed with 5×10^{11} CFU of either strain twice daily for 10 days beginning day 1 post-inoculation.

Determination of Stx2e in feces. Feces were collected daily from control and STEC-inoculated pigs, on days 1-10 post-inoculation, and stored at $4^{o}C$ until processing. Stx2e concentrations were assessed using a Vero cell assay as described previously (Cornick, Matise et al. 2000). Briefly, samples were diluted two-fold in PBS and added to monolayers of Vero cells in microtiter plates. Toxin titers were expressed as the log of the reciprocal of the greatest dilution that resulted in $\geq 50\%$ Vero cell death. Specificity of the titers for Stx2e was confirmed by neutralization with anti-Stx2e antibody.

Statistical analysis. Incidences of vascular lesions and clinical disease between probiotic and sham groups were compared using Fisher's exact test. Fecal toxin titers were compared between treatment groups using repeated measures ANOVA.

Results

Bacterial colonization. STEC strain 1191 was recovered from all of the challenged pigs (probiotic and sham groups) at both 2 and 5 days post inoculation. The probiotic *E. coli* strain CWF308 ($pJCPGb_4$) was recovered from all pigs in the probiotic treated group at

both 2 and 5 days post inoculation.   None of the negative control pigs shed STEC at either time-point.

Clinical disease. Seven out of sixty pigs inoculated with the STEC strain 1191 developed clinical edema disease over three replicates of the trial (Table 2). During the first experimental replicate, two of the pigs in the sham treated group and none of the pigs in the probiotic treated group developed clinical disease. One of the STEC inoculated pigs within the sham treated group and one within the probiotic treated group developed clinical disease during the second replicate, and one in the sham and two in the probiotic developed edema disease during the third replicate. Of the pigs with clinical edema disease, three exhibited neurological signs prior to euthanasia and four were found dead. None of the ten pigs inoculated with the non-pathogenic *E. coli* strain 123 developed clinical signs of edema disease or died. In summary, three of the seven clinically affected pigs were in the probiotic treatment group, and four were in the sham group resulting in an overall disease incidence of 10% in the probiotic group and 13% in the sham group.

Vascular lesions. Brainstem and ileum were assessed for vascular evidence of Shiga toxemia microscopically. Histologic evidence of edema disease (vascular necrosis in two or more vessels per tissue) was not detected in any of the ten pigs inoculated with the non-pathogenic *E. coli* strain.   Vascular lesions consistent with edema disease (Figure 1) were identified in the ilea of 26/60 and in the brainstem of 24/60 STEC inoculated pigs

(Table 2). Although the incidence of vascular lesions was slightly lower in the probiotic treated group, the results were not statistically significant (p>0.05, Chi Square test).

Fecal and blood toxin titers.   Stx titers were measured in both feces and blood to assess the efficacy of the probiotic at adsorbing Stx within the intestinal lumen. Increases in fecal Stx titers in both of the STEC inoculated groups were detectable beginning day 1 post inoculation, and peaked on days 5-7 post inoculation (Figure 2A). Stx2e titers in the sham group were significantly higher than the negative control group on all days that samples were collected (days 1-10 post inoculation, p<0.05, ANOVA). Treatment with the probiotic reduced the fecal Stx2e levels from 2-20 times compared to the sham treated group (p<0.05, Student's t-test). None of the control pigs had detectable Stx2e in their feces at any time point throughout the study. Stx2e was not detected in the blood of the 4 pigs that exhibited neurological signs typical of clinical edema disease prior to euthanasia (data not shown).

Comparison of fecal toxin titers between clinical and non-clinical pigs. Fecal toxin titers in clinical pigs were compared with titers in non-clinical pigs in both the sham and probiotic treatment groups. Since all pigs that developed edema disease fell ill prior to day 8 post inoculation, fecal titers are not available for these pigs beyond day 7 post inoculation. Although treatment with the probiotic reduced fecal toxin titers overall, those pigs treated with the probiotic that developed clinical disease had, on average, toxin titers similar to the sham treated group and were significantly different than probiotic treated subclinical pigs on days 3-5 post inoculation (Figure 2B).

Discussion

      The probiotic strain expressing globotetraose used in this study was designed specifically to bind and neutralize Stx2e, the toxic principal of piglet edema disease. This construct was effective at neutralizing 98.4% of Stx2e cytotoxicity in vitro (Paton, Morona et al. 2001). The dose of probiotic and frequency of administration used here were extrapolated from previous studies with a similar probiotic expressing a surface Gb_3 mimic in a mouse model of STEC infection. In STEC inoculated piglets, treatment twice daily with the probiotic was very effective at reducing the intestinal concentration of Stx2e, as reflected in the reduced fecal toxin titers in the probiotic group. However, treatment with the probiotic failed to reduce the incidence of vascular lesions and clinical disease in STEC inoculated swine, indicating systemic absorption of Shiga toxin still occurred. Interestingly, those pigs that developed clinical disease within the probiotic group had significantly higher fecal toxin titers than subclinical pigs within this group, with the average toxin titers of clinical pigs approaching those of the sham treated groups prior to clinical disease onset. This could indicate a failure of the probiotic to reduce toxin titers in those pigs that broke with clinical disease, despite an overall reduction in toxin titers within this treatment group.

      In mice, treatment with a probiotic expressing a Gb_3 mimic was protective against fatal systemic complications of infection with human-derived STEC (Paton, Morona et al. 2000). There are several differences between the mouse studies and the current study that may have influenced treatment outcome. The affinity of the Gb_4 mimic bacterium for Stx2e is not quite as high as that of the previously described Gb_3 mimic for other

members of the Stx family that was used in the mouse studies (Paton, Morona et al. 2001). This reduced affinity may have resulted in sub-optimal retention of Stx2e in the gut lumen. Another disparity between these studies is the timing of probiotic treatment. In mice, treatment with the probiotic strain was initiated at the time of STEC challenge. Our experimental design delayed treatment for 24 hours in an attempt to more closely mimic post-exposure therapy. As a result, pigs in this study were exposed to intestinal Stx2e prior to treatment with the probiotic. Pigs are known to be extremely susceptible to intravenous Shiga toxin (LD_{50} 3 ng/kg) (MacLeod, Gyles et al. 1991). It is likely that the early and continuous exposure to systemic toxin absorbed from the gut initiates an irreversible cascade of events leading to clinical disease development in some pigs. This may represent an obstacle for post-exposure therapy in human beings because of the delay between the onset of diarrhea and diagnosis in STEC infections (Tarr, Gordon et al. 2005). Similarly, when probiotic treatment was delayed in the mouse model for 24 or 48 hrs, mice were not protected from death when challenged with the most virulent STEC strain (Paton, Rogers et al. 2001). Finally, the lack of normal gastrointestinal flora in the streptomycin-treated mouse model could potentially facilitate physical interaction between the STEC and probiotic strains, thereby enhancing its efficacy. The commensal flora of the ileum in pigs could impede the distribution and/or interaction of the probiotic strain. Based on the outcome of this study, probiotic therapy may be less effective at preventing systemic complications in a naturally occurring STEC infection than parenteral antibody administration. Previously we have shown that a single dose of parentally delivered anti-Stx2e antibody given up to 4 days post-inoculation protects pigs against clinical edema disease(Matise, Cornick et al. 2001). Similarly, Sheoran et al.

reported that human anti-Stx2 administered 48 hours after the onset of diarrhea protected gnotobiotic piglets from fatal complications of Shiga toxemia (Sheoran, Chapman-Bonofiglio et al. 2005).

The unexpected results obtained here are similar to those from previous studies that employed a different oral Stx binding agent, SYNSORB Pk, which did not protect children with HUS from developing extrarenal complications or death, despite the compounds' ability to bind and neutralize Stx in vitro (Takeda, Yoshino et al. 1999; Trachtman, Cnaan et al. 2003). Several potential explanations for SYNSORB Pk's lack of efficacy in clinical trials were proposed: 1) therapy was initiated too late in the disease process after systemic absorption of Stx had already occurred; 2) altered gastrointestinal motility seen in STEC patients affected delivery to the colon, thereby diminishing the drugs effectiveness; 3) the intimate association of STEC with the intestinal mucosa may have limited the ability of the compound to interact with Stx within the intestinal lumen (Trachtman, Cnaan et al. 2003). Although no studies have been published to specifically document altered gastrointestinal motility in pigs with edema disease, pigs often have a prodromal diarrhea phase, and GI motility is likely altered in edema disease as well. This may have affected transit time of the probiotic, and could have affected its ability to bind Stx2e within the intestinal lumen, thereby diminishing its effectiveness. Finally, although attaching and effacing lesions are not formed by the STEC strains responsible for edema disease, the bacteria form intimate attachments to the intestinal mucosa. This intimate association between pathogen and mucosa may allow Stx2e to reach and cross the mucosa before the toxin has a chance to interact with the probiotic within the intestinal lumen.

In conclusion, the results of this study in the edema disease model show that treatment with a Stx2e-receptor mimic probiotic significantly reduced fecal Stx2e titers; however, the probiotic failed to prevent vascular necrosis in target organs and provided no protection against clinical disease.Our findings suggest that treatment with an oral Stx binding agent is less effective than specific antibody at preventing systemic complications during naturally occurring STEC infection.

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Figures and Tables

Figure 1. Histological confirmation of vascular injury secondary to Shiga toxemia in ilea (submucosa). Arterioles in control pigs inoculated with non-pathogenic *Escherichia coli* lacked lesions (**A**). Arteriole from pig inoculated with Shiga toxin producing *E. coli* (STEC) strain S1191 and treated with the sham strain (**B**). Segmental changes within the tunica media characterized by the presence of karyorrhectic nuclear debris from a myocyte (arrow). Arteriole from pig inoculated with STEC strain S1191 and treated with the receptor mimic probiotic (**C**). Note nuclear remnants of myocytes within the tunica media (arrows), as well as vacuolization of the sarcoplasm (arrowhead). These findings indicate that treatment with the receptor mimic probiotic failed to prevent Shiga toxemia. Hematoxylin and eosin, ×1000 magnification.
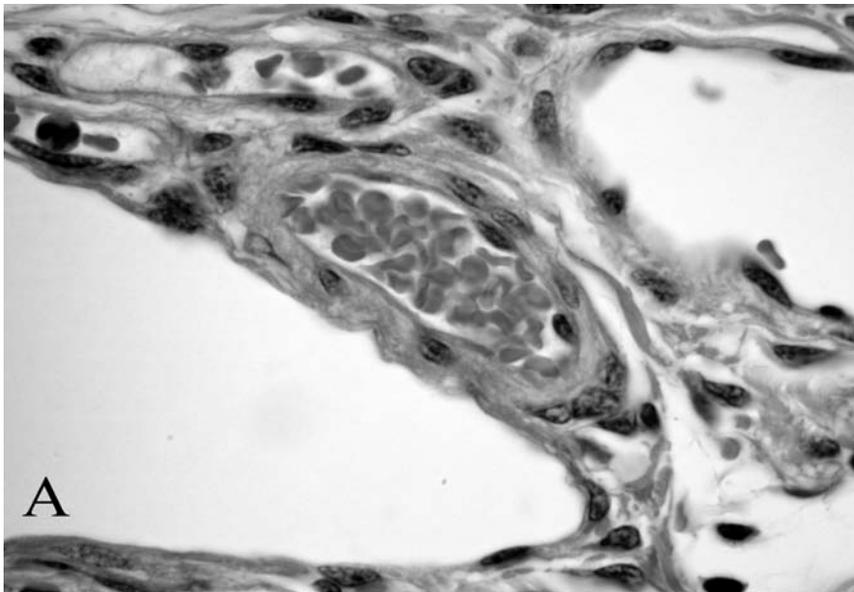
Figure 1 (continued)
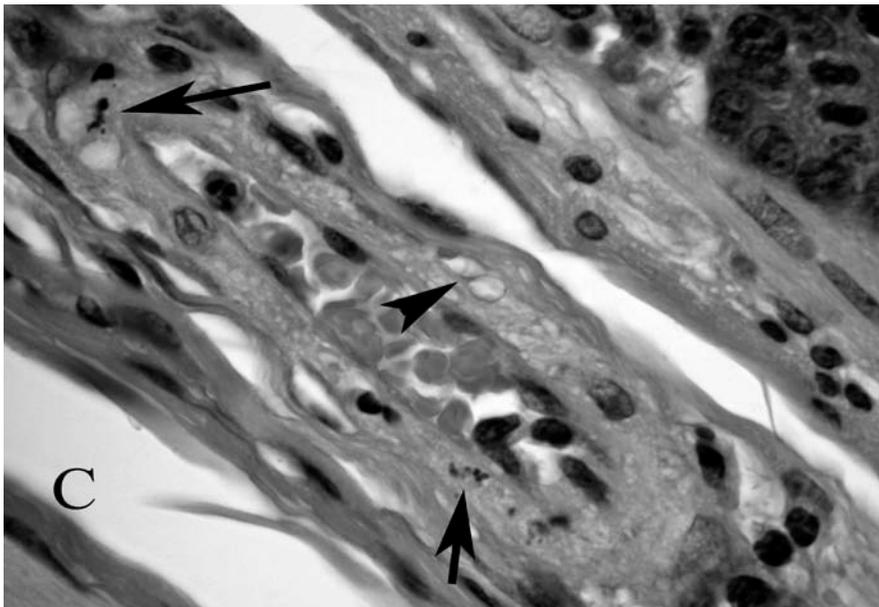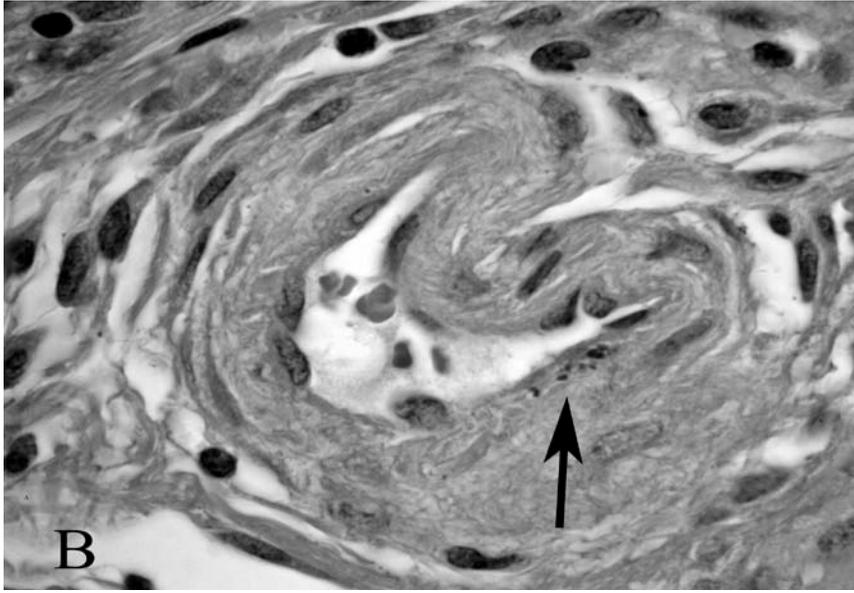
Figure 2. Effect of treatment with a probiotic expressing globotetraose on mean fecal
Shiga toxin 2e titers in Shiga toxin-producing *Escherichia coli* inoculated pigs. A.
Treatment with the probiotic significantly reduced titers compared to sham treated pigs
on all days post inoculation (*, p<0.05, repeated measures ANOVA). B. Although fecal
Shiga toxin levels were reduced overall within the probiotic group, pigs that developed
clinical edema disease had higher levels of fecal Shiga toxin, similar to those of the sham
treated group (*, p<0.05).

A.
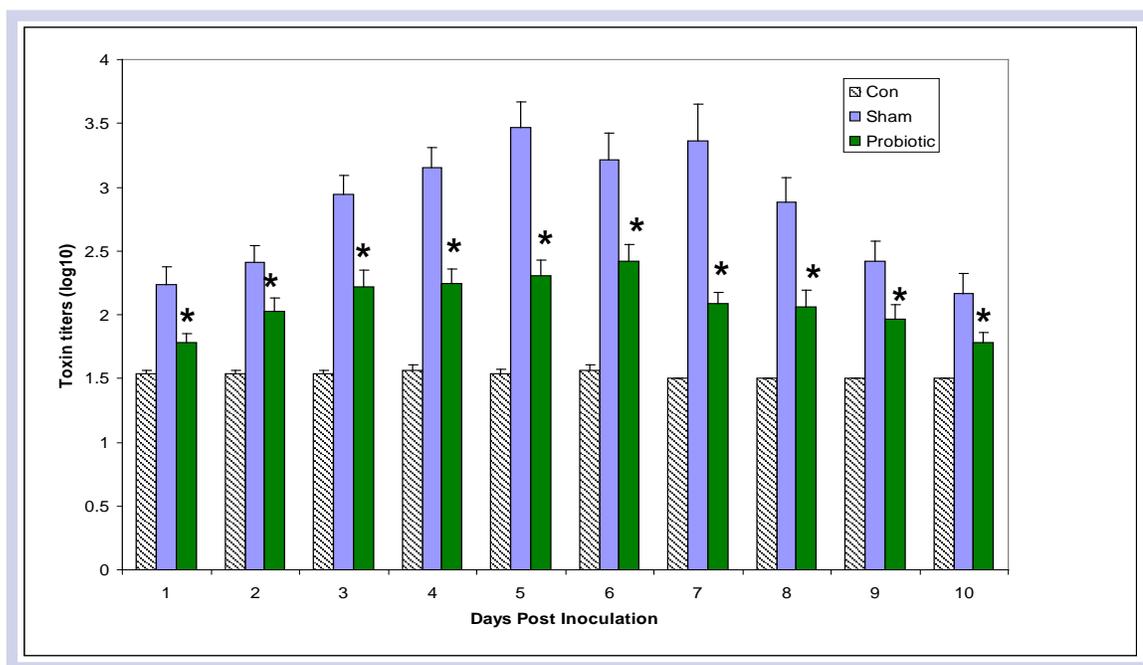
Figure 2 (continued)

B.
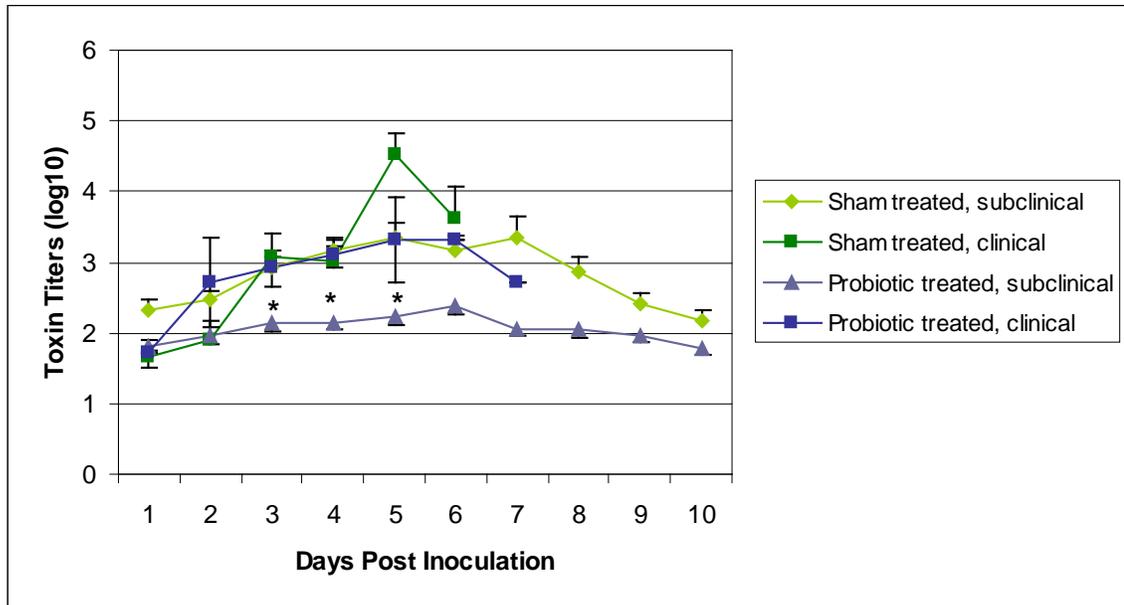
Table 1. Experimental design to assess the effect of a probiotic expressing Gb_4 on the incidence of edema disease.

Experimental group	Number of pigs[a]	Pig age at inoculation	Inoculation strain	Treatment
Probiotic	30	24-26 days	S1191[c]	CWF308(pJCPGb$_4$)
Sham	30	24-26 days	S1191[c]	CWF308
Negative control	10	24-26 days	123[b]	None
[a] Each group represents the cumulative number of pigs from three separate experimental replications [b] Non-pathogenic *E. coli* strain [c] STEC strain				

Table 2. Incidences of clinical disease and vascular lesions in pigs inoculated with STEC 1191 and treated with a Gb4 expressing probiotic vs. a sham strain lacking Gb4. No significant differences between groups were identified ($p > 0.05$, Chi square test).

Experimental group	Number of pigs[d]	Incidence of clinical disease	Incidence of vascular lesions in brainstem	Incidence of vascular lesions in ileum
Probiotic[a]	30	3/30	10/30	11/30
Sham[b]	30	4/30	14/30	15/30
Negative control[c]	10	0/10	0/10	0/10
[a] Inoculated with STEC strain, treated with probiotic [b] Inoculated with STEC strain, treated with sham [c] Inoculated with non-pathogenic *E. coli* strain, no treatment [d] Sum of three experimental replicates				

Chapter 3: Proinflammatory cytokine responses in naturally occurring Shiga-toxin producing *Escherichia coli* infection in piglets.

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Abstract

Hemolytic uremic syndrome (HUS), a systemic and potentially fatal complication of Shiga toxin-producing *Escherichia coli* (STEC) infection, is a leading cause of renal failure in children. The goal of this study was to characterize the proinflammatory cytokine changes during STEC infection in experimental porcine edema disease, an animal model of HUS. Piglets were inoculated with either a Stx2e-producing host adapted (STEC group) or non-pathogenic (control) *E. coli* strain. Clinical edema disease was observed between days 5 and 10 post-inoculation with a clinical disease incidence of 35% in the STEC group. Clinical pigs had significantly higher fecal Stx concentrations than subclinical pigs on days 2 and 5 post-inoculation. At day 2 post inoculation, plasma TNF-α was significantly higher in STEC inoculated pigs that went on to develop clinical disease. Liver TNF-α and IL-1β protein levels were significantly higher in clinical STEC pigs compared to either subclinical STEC pigs or controls. Ileal IL-10 levels in subclinical STEC pigs were significantly increased compared to both clinical STEC pigs and controls, and brainstem IL-10 was higher in subclinical STEC pigs than controls. In

an attempt to further clarify the contribution of Stx2e to the enhanced TNF-α levels observed in livers of clinical pigs, porcine macrophages were stimulated with either Stx2e or erythrocyte-bound Stx2e. Neither Stx2e nor erythrocyte-bound Stx2e were potent stimulators of TNF-α production by porcine macrophages. In conclusion, higher levels of liver IL-1β and TNF-α were detected in clinical pigs than subclinical pigs. This could indicate a greater systemic inflammatory response in STEC inoculated pigs that develop clinical disease. Importantly, clinical pigs had higher levels of plasma TNF-α prior to the onset of clinical disease, making plasma TNF-α a potential candidate for systemic disease risk assessment in STEC infected individuals.

Introduction

Hemolytic uremic syndrome (HUS), a systemic complication of Shiga toxin-producing *Escherichia coli* (STEC) infection, is the number one cause of renal failure in children in the United States (Karmali, 1989, 2004; Paton and Paton, 1998). STEC are a common cause of food-borne illness in the United States, and ruminants are the primary reservoir. Individuals are typically exposed through ingestion of either undercooked ground beef or raw fruits and vegetables contaminated with STEC or person-person transmission (Karmali, 2004) . Following colonization, Shiga toxins (Stx) are produced locally within the intestine, then presumably cross the intestinal mucosa and vascular endothelium to gain access to the bloodstream. It has been shown in tissue culture systems that biologically active Stx can cross intestinal epithelial cells, and that

neutrophil transmigration across epithelial monolayers enhances Stx translocation (Acheson et al., 1996; Hurley et al., 2001). Once the toxin reaches the blood, it binds globotriasoyl ceramide (Gb$_3$) on the surface of vascular endothelial cells in key target organs (kidney, brain) and is internalized (Jacewicz et al., 1986). Toxin internalization within endothelial cells results in inhibition of protein synthesis, leading to cell death (Furutani et al., 1990). The resultant vascular necrosis can lead to the formation of thrombi within affected vessels in susceptible individuals, and subsequently produce the characteristic clinical triad of HUS (microangiopathic anemia, thrombocytopenia, and acute renal damage (Mark Taylor, 2008).

Although the production and dissemination of Stx is essential for HUS development, there is compelling evidence that the interaction of Stx with endothelium alone is not sufficient to induce HUS. A growing body of research exists that implicates proinflammatory cytokines as contributing factors in the pathogenesis. Several studies have discovered an increase in the concentration of proinflammatory cytokines in patients who develop HUS over those with STEC-induced diarrhea alone (Inward et al., 1997; Lopez et al., 1995; Westerholt et al., 2000). Decreased levels of the anti-inflammatory cytokine IL-10 have also been associated with HUS development (Westerholt et al., 2000; Westerholt et al., 2003). One study in particular looked at the potential *in vitro* cytokine production in patients who had experienced an episode of HUS using a whole blood stimulation model. Their findings indicate that HUS patients have a propensity to produce greater amounts of several proinflammatory cytokines including IL-1, and reduced amounts of the anti-inflammatory cytokine IL-10 (Westerholt et al., 2003). In several *in vitro* studies, TNF-α and IL-1β greatly enhance the cytotoxicity of Stx. The

direct cytotoxicity of Stx on cultured human vascular endothelial cells is minimal; however, when endothelial cells are exposed to TNF-α and IL-1β, they are profoundly sensitized to the cytotoxic effects of Stx. This enhancement of Stx cytotoxicity is presumably mediated by the up-regulation of Gb3 on endothelial cells (Eisenhauer et al., 2001; Louise and Obrig, 1991; Louise et al., 1997; Ramegowda et al., 1999; Tesh et al., 1994; Van de Kar et al., 1993; van de Kar et al., 1995; van Setten et al., 1997).   More recently, a study in a rabbit model revealed that inflammation secondary to Stx2-induced vascular damage within the central nervous tissue exacerbated degenerative changes within the tissue (Takahashi et al., 2008). Further support for the role of cytokines and inflammation in HUS development is that certain indicators of systemic inflammation have been identified as risk factors for HUS development in patients with bloody diarrhea, including leukocytosis and neutrophilia (Buteau et al., 2000).   Finally, increases in proinflammatory cytokine production, particularly TNF-α and IL-1β, following exposure to Stx occurs in human monocytes, murine macrophages, and human cerebral microvascular endothelial cells (Eisenhauer et al., 2004; Harrison et al., 2004; Tesh et al., 1994). Although these studies suggest Stx can contribute to proinflammatory cytokine production and inflammation, it is not known to what extent this systemic increase in cytokines *in vivo* is due to circulating cell-bound Stx versus other STEC virulence factors, such as LPS.

The goals of this study were to: a) characterize and compare the proinflammatory cytokine response between clinical versus subclinical piglets with systemic STEC infection b) assess the potential of Stx itself to promote proinflammatory changes in STEC infection and c) identify predictors of systemic disease during the initial stages of

STEC infection. To accomplish these goals, we compared local and systemic Shiga toxin levels, as well as TNF-α, IL-1β and IL-10 levels, and compared clinical versus subclinical individuals in our animal model of systemic STEC infection, edema disease of swine. We also evaluated the ability of cell-bound Stx to induce cytokine production in porcine macrophages in vitro, and compared the effects to cell-bound LPS. We hypothesized that clinical pigs would have a greater proinflammatory response than subclinical pigs, and that this response was due to greater Stx production and dissemination in these pigs. Additionally, we proposed that clinical pigs would have evidence of a greater proinflammatory response prior to the onset of clinical signs. We chose edema disease of swine as the animal model to test these hypotheses because of the similar pathogenesis to HUS. Edema disease is a naturally occurring STEC disease of weanling swine caused by host-adapted strains of *E. coli* that produce a Stx2 variant, Stx2e. Experimentally infected swine are colonized predominantly in the ileum, suffer a prodromal diarrhea phase, and approximately 30% of individuals develop clinical edema disease manifested by neurological symptoms due to systemic Shiga toxemia (Cornick et al., 1999, 2000; MacLeod et al., 1991). Stx2e, the primary virulence factor in edema disease, is detected in peripheral blood bound to porcine erythrocytes, and the presence of erythrocyte-bound Stx2e is strongly associated with the development of clinical disease (Matise et al., 2003). Our findings in this model support a role of a proinflammatory cytokine response in clinical disease development in STEC infection, and show that this response begins prior to the onset of clinical disease.

Materials and Methods

Bacterial strains: Strain S1191, an STEC strain isolated from a pig with clinical edema disease, belongs to serogroup O139 and produces Stx2e, hemolysin, F18ab fimbriae, and heat-stable enterotoxin B (L.R.M. Marques, 1987; Sarmiento et al., 1988). S1191 is hemolytic on sheep blood agar and is resistant to chloramphenicol. Non-pathogenic *E. coli* strain 123 belongs to serogroup O43 and was isolated from a healthy pig. Inocula were prepared as described elsewhere (Sarmiento et al., 1988).

Reproduction of clinical edema disease: Animal experiments were carried out in accordance with a protocol approved by the Iowa State University Animal Care and Use Committee. Two week-old crossbred swine were purchased from a commercial facility and housed in a biosafety level II animal facility at Iowa State University. The study design to experimentally reproduce edema disease follows a previously described protocol (Cornick et al., 2000; Matise et al., 2003). Briefly, swine (20) were inoculated orally with 10^{10} colony forming units (CFU) of STEC strain S1191 8-10 days after weaning. Control pigs (5) received 10^{10} CFU orally of non-pathogenic *E. coli* strain 123. Pigs were fed a custom antibiotic-free soy protein diet and monitored twice daily for evidence of edema disease (subcutaneous edema, recumbency, or neurological disturbances such as ataxia and circling). For the purposes of this study, piglets were considered to manifest clinical systemic disease only when neurologic signs or sudden

death were observed, since some piglets with mild signs (e.g. subcutaneous edema) recover uneventfully. Pigs that developed neurological signs (clinical pigs) were euthanized by intravenous overdose with barbiturate. Those pigs that failed to develop neurological signs (subclinical pigs) were euthanized in a similar manner at the termination of the study (14 days post-inoculation). Pigs inoculated with the non-pathogenic *E. coli* strain were also euthanized at day 14 post-inoculation. Feces (collected daily) and blood (collected every other day in EDTA) were collected from all pigs for analysis of Stx levels (feces and packed red blood cells) or cytokine protein levels (plasma). All pigs underwent a complete necropsy, with inspection of major organs for gross lesions, and collection of samples from ileum and brainstem (tissues known to develop vascular lesions in edema disease), as well as liver and kidney (tissues chosen for evaluation of more systemic responses) for cytokine protein levels and qRT-PCR.

Toxin and reagents: Crude Stx2e was prepared from whole cell lysate of *E. coli* strain DH5α containing a plasmid encoding the Stx2e gene as described previously (Matise et al., 2003; Samuel et al., 1990). Stx2e was purified using an affinity column containing a carbohydrate analogue of the Stx receptor, Gb_3 (Nakajima et al., 2001). The final Stx2e concentration in the purified preparation was approximately 65 μg/ml, Vero cell titer 10^8. As an additional control in porcine macrophage experiments, unpurified whole cell lysates from the same *E. coli* DH5α strain lacking the plasmid encoding Stx2e were prepared. The endotoxin concentrations of both the purified Stx2e preparation and

crude lysate were determined by LAL assay as described previously (Kreeftenberg et al., 1977). Porcine ELISA kits for measurement of IL-1β, TNF-α, and IL-10 were purchased from commercial sources (Biosource/Invitrogen, Carlsbad, CA; R&D Systems, Inc., Minneapolis, MN). Antibodies for magnetic cell sorting were purchased from Miltenyi Biotech, Inc. (Auburn, CA). Primers and probes for qPCR were purchased from IDT Technologies (Corallville, IA) and the one-step qPCR master mix was purchased from Invitrogen (SuperScript™ III Platinum® One-Step quantitative RT-PCR System with ROX kit; Carlsbad, CA).

Vero cell assay for determination of Stx2e in blood and feces: Feces were collected daily from control and STEC-inoculated pigs and stored at 4° C until assayed. Fecal samples were prepared by high speed centrifugation with subsequent filter sterilization of the supernatant. Blood samples were collected in EDTA from all pigs every other day beginning 2 days post-inoculation for Stx2e analysis. Additionally, blood was drawn prior to euthanasia in pigs that developed neurological impairment. All blood samples were centrifuged, the plasma and buffy coats removed, and RBC fractions were stored at 4° C until the assay was performed (within 3 days). Stx2e concentrations were assessed in feces and RBC fractions using a Vero cell assay as described previously (Cornick et al., 2000; Gordon et al., 1992). Briefly, samples were diluted two-fold in PBS and added to monolayers of Vero cells in microtiter plates. Toxin titers were expressed as the log of the reciprocal of the greatest dilution that resulted in ≥ 50% Vero

cell death. Specificity of the titers for Stx2e were confirmed by neutralization using a bovine polyclonal antibody to Stx2 as previously described (Karch et al., 1992).

Cells: Porcine monocytes were either separated from whole blood via magnetic cell sorting (AutoMACS) or derived from porcine peripheral blood mononuclear cells separated from whole blood via density centrifugation with subsequent positive selection of monocytes by adherence. For both techniques, whole blood was collected from pigs in sodium heparin and used within two hours of collection. Blood was diluted 1:2 in sterile phosphate buffered saline (PBS) and layered over Ficoll-Paque PLUS (25 ml diluted blood to 20 ml Ficoll; GE Healthcare, Piscataway, NJ) and centrifuged at room temperature for 30 minutes at 500 x g. The mononuclear cell fraction was removed, placed in a sterile 50 ml conical tube, and washed in sterile PBS. Cells were resuspended in PBS and counted with differential analysis using the ADVIA 120™ automated hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, IL). Cells selected via adherence were diluted in complete media (DMEM base, 10% pig serum, 2mM L-glutamine, and antibiotic) and added to 24 well plates at a concentration of approximately 500,000 monocytes per well. After 24 hours incubation at $37°$C plus 5% CO_2, non-adherent cells (lymphocytes) were removed, and complete media supplemented with 30% L929 cell media to drive differentiation into macrophages was added. Cells were used on the 4th day following isolation. For cells selected via magnetic cell sorting, mononuclear cells were diluted in AutoMACS buffer (Miltenyi Biotech Inc., Auburn, CA) at a concentration of 80×10^6 cells per ml following counting on the ADVIA 120™.

Mononuclear cells were incubated with anti-SWC3a antibody (BD Biosciences, San Jose, CA) at a concentration of 4 μg/ ml for 30 minutes at 4°C on a platform rocker. Cells were washed twice in cold AutoMACS buffer and resuspended in 500 μl cold AutoMACS buffer. Goat anti-mouse IgG MicroBeads (Miltenyi Biotech Inc.) were added at a concentration of 20 μl beads per 10^7 monocytes (based on counts from ADVIA 120™) and incubated at 4°C for 15 minutes. Cells were washed in cold AutoMACS buffer and diluted to a concentration of 500 μl buffer for each 10^8 cells and filtered through a 40 micron cell strainer (BD Biosciences). Labeled monocytes were then positively selected on the AutoMACS analyzer (Miltenyi Biotech Inc.) using the "Possel-S" program. Monocytes were then diluted in complete DMEM at added to 24 well plates at a concentration of 500,000 per well and differentiated into macrophages as described above.

Porcine erythrocytes were collected from whole blood anticoagulated with EDTA and centrifuged at 1000 x g for 6 minutes at room temperature within 2 hours of collection. The plasma and buffy coats were removed aseptically, and packed erythrocytes were washed in Hank's balance salt solution lacking calcium, magnesium, and Phenol red (HBSS, Invitrogen) and re-centrifuged for 10 minutes at 1000 x g. Packed, washed erythrocytes were stored at 4°C for up to 3 days prior to use in experiments.

Transfer of erythrocyte-bound Stx2e to porcine macrophages: Porcine erythrocytes (50 μl packed RBCs) were incubated with either HBSS only (negative control, 50 μl) or purified Stx2e (high dose 325 ng; low dose 32.5 ng) for 1 hour at room

temperature. RBCs were subsequently washed twice in HBSS and resuspended in 500 µl

HBSS prior to addition to macrophages.   Purified Stx2e (approximately 32.5 ng) or LPS

(approximately 3.5 ng) was added directly to some wells. After one hour of co-incubation

with macrophages at 37°C/ 5% CO_2, cell culture supernatants were collected and

macrophages were washed with HBSS to remove any remaining RBC, and fresh

complete media was added to each well. Supernatants were collected again at 24 hours

and stored at -80°C prior to measurement of TNF-α levels via ELISA.

Measurement of cytokine levels in plasma, tissues, and cell culture

supernatants: Plasma was obtained from whole blood within one hour of collection and

stored at -80°C until analysis. Fresh tissues from ileum, brainstem, kidney, and liver

were collected aseptically at necropsy and flash frozen in liquid nitrogen, with

subsequent storage at -80°C. Tissue homogenates were prepared by suspending thawed

tissues in PBS (2 grams tissue per ml of PBS) and homogenizing using a high speed

blade homogenizer (Omni TH homogenizer (Omni International, Marietta, GA)).

Homogenates were clarified via low speed (500 x g for 10 minutes) followed by high

speed (3000 x g for 5 minutes) centrifugation and supernatants were likewise stored at -

80°C prior to analysis. Cell culture supernatants were collected from porcine

macrophage cultures at 24 hours post treatment. Supernatants were stored at -80°C and

centrifuged prior to analysis to remove any cellular debris.   Levels of TNF-α, IL-1β, and

IL-10 were measured using commercially available ELISA kits according to the

manufacturer's instructions.

RNA Isolation: Tissues (ileum, brainstem, liver, and kidney) were collected aseptically at necropsy and flash frozen in liquid nitrogen. These samples were stored at -80°C until RNA isolation. RNA was isolated as described previously (Sow et al., 2009). Briefly, tissue samples were partially thawed, weighed, and homogenized in 3 ml TRIzol reagent (Invitrogen) using an Omni TH homogenizer (Omni International, Marietta, GA). The samples were then further diluted in TRIzol, based on tissue weight, to obtain a uniform 0.0909 g tissue/ ml homogenate. Chloroform (200 μl) was added to homogenates, and they were centrifuged at 12,000 x g for 10 minutes. The aqueous layers were then transferred into 500 μl isopropanol, mixed, and centrifuged with subsequent removal of the top aqueous layer. Pellets were washed twice in ice-cold ethanol, air dried, and resuspended in nuclease-free 0.1 mM EDTA. Nucleic acid concentration and purity was evaluated by absorbance readings at 260 and 280 nm (Nanodrop™), and samples were DNase treated (TURBO DNA-free kit, Ambion. Austin, TX) and diluted to the same RNA concentration with nuclease-free water and RNaseOUT (Invitrogen) for use in one-step qPCR.

One step quantitative real-time (qRT)-PCR: Primer and probe sequences for the three targets (IL-1β, TNF-α, IL-10) were taken from USDA's Porcine Immunology and Nutrition database (http://199.133.11.115/fmi/iwp/cgi?-db=PINdb&-loadframes). One-step qRT-PCR was performed as described previously (Gallup et al., 2005). The final 25 μl reaction volumes for samples included one-step master mix (SuperScript™ III Platinum® One-Step quantitative RT-PCR System with ROX kit, Invitrogen), primers

and probe (750 nM for primers and 150 nM for probes), and diluted RNA sample (6 µl).

Thermocycling was performed with all samples run in duplicate on a GeneAMP 5700

sequence analyzer (Applied Biosystems, Forest City, CA) under the following conditions:

15 minutes at 55°C, 2 minutes at 95°C, and 55 cycles of 15 seconds at 95°C followed by

30 seconds at 58°C. Output data was then analyzed using custom Excel files

(PREXCEL-Q™ software) with relative quantitation based on target-specific fluorescent

signals generated during qPCR of total RNA from the whole tissue homogenates for the

study. Levels for each target were normalized to the total amount of mRNA per well for

final analysis as described in detail previously (Sow et al., 2009).

Statistical analysis: JMP software (SAS) was used for statistical analysis. The

distribution of all data sets were initially assessed, and data were normalized via log

transformation when necessary. Data were analyzed with ANOVA followed by

Student's t test. Results were considered significant at a value of $p<0.05$.

Results

Clinical disease development in STEC inoculated pigs: Seven out of the

twenty piglets inoculated with the STEC strain developed clinical edema disease (clinical

disease incidence of 35%). Clinical disease was observed between days 5-10 post

inoculation (two on day 5, one on day 6, two on day 8, and two on day 10). Six of the

clinical pigs were discovered to have neurological signs during observation periods and

were euthanized, the seventh pig was found dead (data not shown). None of the piglets

inoculated with the non-pathogenic strain developed any disease.

Local and systemic Shiga toxin levels: To correlate clinical signs and

proinflammatory cytokine changes to Shiga toxin production and dissemination, local

and systemic Stx levels were measured throughout the study. Fecal Stx concentrations

peaked at day 4 post inoculation in the STEC group. Clinical pigs had higher levels of

fecal Stx than subclinical pigs on all days post inoculation; the difference was statistically

significant at days 2 and 5 post inoculation (Figure 1, $p<0.05$). Five of the eight pigs

with the highest fecal Stx concentration (Vero cell titer >65,000) developed clinical

edema disease. The calculated relative risk of clinical disease for pigs with the highest

fecal Stx levels was 3.125. Shiga toxemia was detected in 11/20 STEC inoculated pigs

and 0/5 control pigs via Vero cell assay. Four of the eleven pigs with detectable Shiga

toxemia developed clinical disease. Blood Vero cell titers ranged from 1:64 to 1:128 and

were neutralized by specific antibody. The relative risk of clinical disease for pigs with

positive blood Stx titers was 1.09; therefore, the risk of clinical disease development was

greater in pigs with high fecal Stx titers compared to those with positive blood Stx titers.

Free fecal Shiga toxin was not detected in control pigs.

Levels of IL-1β, IL-10 and TNF-α in plasma: To assess systemic changes in

the pro-inflammatory/anti-inflammatory responses during STEC infection, plasma TNF-

α, IL-1β, and IL-10 were measured every other day using ELISA. Plasma TNF-α levels were significantly higher in clinical pigs than subclinical pigs within the STEC group on day 2 post inoculation ($p<0.05$, Figure 2A). This timepoint preceded clinical disease development by at least three days. Levels of TNF-α and IL-1β in plasma increased over time in all groups, peaking at day 6-8 post inoculation (Figure 2B and 2C). IL-10 levels in plasma were minimal at the time of inoculation, and were at or below detection limits in all three groups by day 8. A mild rebound back to initial levels was seen at day 10 in both the subclinical and control groups. There was a spike in plasma IL-10 on day 10 in the sole remaining clinical pig. No significant differences in either IL-1β or IL-10 levels were observed between groups at any timepoint (Figure 2b and 2c).

IL-1β, IL-10, and TNF-α levels in tissues: Protein levels of TNF-α, IL-1β, and IL-10 were measured in brainstem, ileum, liver, and kidney by ELISA to evaluate the local and systemic cytokine response. Brainstem and ileum were chosen as they are the sites of pathologic significance in porcine edema disease, whereas liver and kidney were chosen to assess a more systemic inflammatory response. TNF-α protein levels in liver from clinical pigs within the STEC group were significantly higher than either control or subclinical pigs ($p<0.05$, Figure 3A). Significant differences between groups were not observed in TNF-α protein levels from ileum, kidney, or brainstem. Liver IL-1β levels were likewise significantly higher in clinical pigs than either subclinical or control pigs, but a similar increase was not observed in other tissues ($p<0.05$, Figure 3B). IL-10 levels in liver of clinical pigs were significantly higher than controls (Fig. 3C). In contrast, IL-10 levels in subclinical pigs were higher than either control pigs only (brainstem) or in

either control or clinical pigs (ileum) ($p<0.05$). No significant differences between groups were noted in IL-10 levels in kidney.

mRNA expression of TNF-α, IL-1β, and IL-10 was determined by one-step qRT-PCR. For final analysis, data were normalized to total mRNA per well. In the liver, IL-10 mRNA expression was significantly lower in clinical pigs than controls (Figure 4, $p<0.05$). Significant differences in IL-1β or TNF-α mRNA expression were not observed in liver. No significant differences between groups were noted in mRNA expression from brainstem, ileum, or kidney as determined by qRT-PCR for any of the three targets examined (Figure 4).

Cytokine production by porcine macrophages secondary to cell-bound Stx:
To better assess the ability of Stx2e to induce proinflammatory cytokine production in macrophages, we compared the capacity of Stx2e and erythrocyte-bound Stx2e to induce TNF-α production by porcine macrophages as determined by ELISA. Direct addition of purified Stx2e to macrophage cultures resulted in a slight but insignificant difference ($p >.05$) in TNF-α at 24 hours (Figure 5). In contrast, addition of LPS (approximately 3.5 ng) as a positive control led to a marked and statistically significant ($p<0.01$) increase in TNF-α production at 24 hours. Additional experiments evaluated the effect of erythrocyte-bound Stx2e on TNF-α production by isolate porcine macrophages to more closely mimic in vivo conditions. Likewise, erythrocyte-bound Stx2e did not generate significant TNF-α production by porcine macrophage compared to negative control (Figure 5).

Discussion:

The main objectives of this study were to further clarify the role of the proinflammatory cytokine response in clinical disease development during STEC infection, and to help identify the primary stimulus for any identified cytokine changes. A further goal was to measure systemic levels of several classical proinflammatory and anti-inflammatory cytokines following STEC infection to identify potential risk factors prior to clinical disease development. Some of our major findings supported our hypothesis that clinical pigs would have a greater proinflammatory cytokine response. Specifically, we found that those pigs that later develop clinical disease have increased plasma TNF-α during the prodromal phase of STEC infection. Additionally, clinical pigs had evidence of a greater systemic proinflammatory cytokine response than subclinical pigs, and subclinical pigs had a more substantial anti-inflammatory response in ileum and brainstem, tissues known to develop Stx2e-induced histologic lesions. We also hypothesized that greater Stx production and dissemination would be a direct cause of this enhanced proinflammatory cytokine response in clinical pigs. Interestingly, clinical pigs had both greater local Stx production and plasma TNF-α levels on day 2, suggesting a possible correlation. To assess a more direct link between the two events, the in vitro studies with porcine macrophages were performed. Based on the latter findings, however, a direct cause and effect between Stx production with subsequent dissemination and a systemic increase in TNF-α production is considered less likely. For the in vitro studies, we were particularly interested in more closely mimicking in vivo conditions; therefore, we looked at the influence of erythrocyte-bound Stx2e on cytokine production by porcine macrophages. Our results suggest that Stx2e is a less potent stimulator of

TNF-α production in porcine macrophages than LPS. Previous work in the human monocytic cell line, THP-1, showed that Stx1 induced significant proinflammatory cytokine production (Harrison et al., 2004; Ramegowda and Tesh, 1996; Sakiri et al., 1998). The results illustrated in this study with pig macrophages suggest less of a role for Stx2e in proinflammatory cytokine production. Future studies are warranted to assess the effects of other STEC antigens, such as flagellin, in this in vitro system.

The plasma cytokine changes in the STEC group that occurred during the prodromal diarrhea phase were of particular interest, since identification of risk factors in the animal model might help better identify which STEC infected individuals are at greatest risk for HUS development. Some risk factors, such as neutrophilia, have already been identified in human beings; however, additional parameters may impart greater sensitivity and specificity. The findings in this study suggest that plasma TNF-α increases in clinical individuals prior to the onset of clinical signs; therefore, measurement of plasma TNF-α during the prodromal diarrhea phase of STEC infection could serve as a good indicator of HUS risk in human beings. Surprisingly, plasma levels of both IL-1β and TNF-α increased over time in all groups, including the non-pathogenic control group. One potential explanation for these findings is that the cytokine changes reflected in plasma are secondary to a stress of handling response. Although pigs were acclimated for one week prior to inoculation, the pigs were handled more after inoculation, including daily collection of feces from individual pigs and blood draws every other day, during days 0-10 post inoculation.

The liver findings were of particular interest, since Kupffer cells in sinusoids are initially exposed to antigens within the intestine. In our pig model, it is known that Stx2e

is found in the circulation bound exclusively to erythrocytes, and that erythrocyte-bound toxin is a risk factor for clinical disease development (Matise et al., 2003). Kupffer cells are also exposed to various other STEC antigens, such as LPS, which can circulate bound to plasma proteins (LPS binding protein) or attached to the surface of erythrocytes and leukocytes. Additionally, it has been shown previously that depletion of hepatic and splenic macrophages in a mouse model is protective against the systemic effects of STEC infection (Palermo et al., 1999). Our data provide further support for a role of the liver in the proinflammatory cytokine response in STEC infection. The greater anti-inflammatory response observed in subclinical animals could represent either a rebound anti-inflammatory effect following STEC infection, or alternatively, a protective effect of a greater anti-inflammatory response on clinical outcome. Future studies that further clarify the activation status of the systemic inflammatory response antemortem, such as gene microarray analysis of serially collected PBMC during STEC infection, may be useful in determining which explanation is more accurate.

One consideration in this data is that clinical pigs died earlier than subclinical pigs or controls. Since clinical pigs were euthanized and necropsied as neurological symptoms arose, between 5 and 10 days post inoculation, tissues from these pigs were assessed at a different timepoint than subclinical and control pigs, which were euthanized and necropsied at 14 days post inoculation. Thus, the argument can be made that the changes seen are potentially an artifact of timepoint. It is possible that the proinflammatory changes seen in liver from clinical pigs are a result of the general mild systemic changes reflected in the plasma findings and attributed to stress of handling. Similarly, the anti-inflammatory changes seen in ileum and brainstem in subclinical pigs

may just be a general recovery response following STEC infection, and may not reflect a protective effect of a greater anti-inflammatory response in subclinical pigs. We feel, however, that this is a less likely explanation for the following reasons. Firstly, two of the clinical pigs were necropsied at day 10, a timepoint at which plasma cytokine levels had returned to baseline (fig. 2). Additionally, two pigs fell ill on day 5, and although plasma was not taken at this timepoint, it is likely that the cytokine levels fall in between the day 4 and 6 values. Consequently, only three of the seven clinical pigs were necropsied during the plasma TNF-α and IL-1β peak. Finally, two of these three remaining pigs were necropsied at day 8, at which point plasma TNF-α and IL-1β were already falling in the clinical group, with no statistically significant differences between levels on that day and day 0. Therefore, we conclude that the changes in TNF-α and IL-1β protein levels in liver observed in clinical pigs are more likely a reflection of an increased proinflammatory response in clinical individuals.

In conclusion, the findings of this study provide further evidence for a role of the systemic inflammatory response in the pathogenesis of STEC infection. Although Stx itself has been found to stimulate proinflammatory cytokine production in vitro in a variety of cell types, the findings of this study indicate Stx2e is less efficient at eliciting TNF-α production by porcine macrophages than LPS. Therefore, we conclude that Stx2e is likely to have a more secondary role in proinflammatory cytokine responses in porcine edema disease. Finally, measurement of plasma TNF-α during the prodromal diarrhea phase of STEC infection may be a useful indicator of risk for systemic disease development.

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Figures

Figure 1. Comparison of intestinal Shiga toxin (Stx) production between clinical and subclinical Shiga toxin-producing *Escherichia coli* (STEC) inoculated pigs. Fecal samples were collected daily and Stx levels were analyzed by Vero cell assay. Specificity of titers was confirmed using specific anti-Stx antibody. Clinical pigs had significantly greater Stx production than subclinical pigs on days 2 and 5 post inoculation ($p < 0.05$).
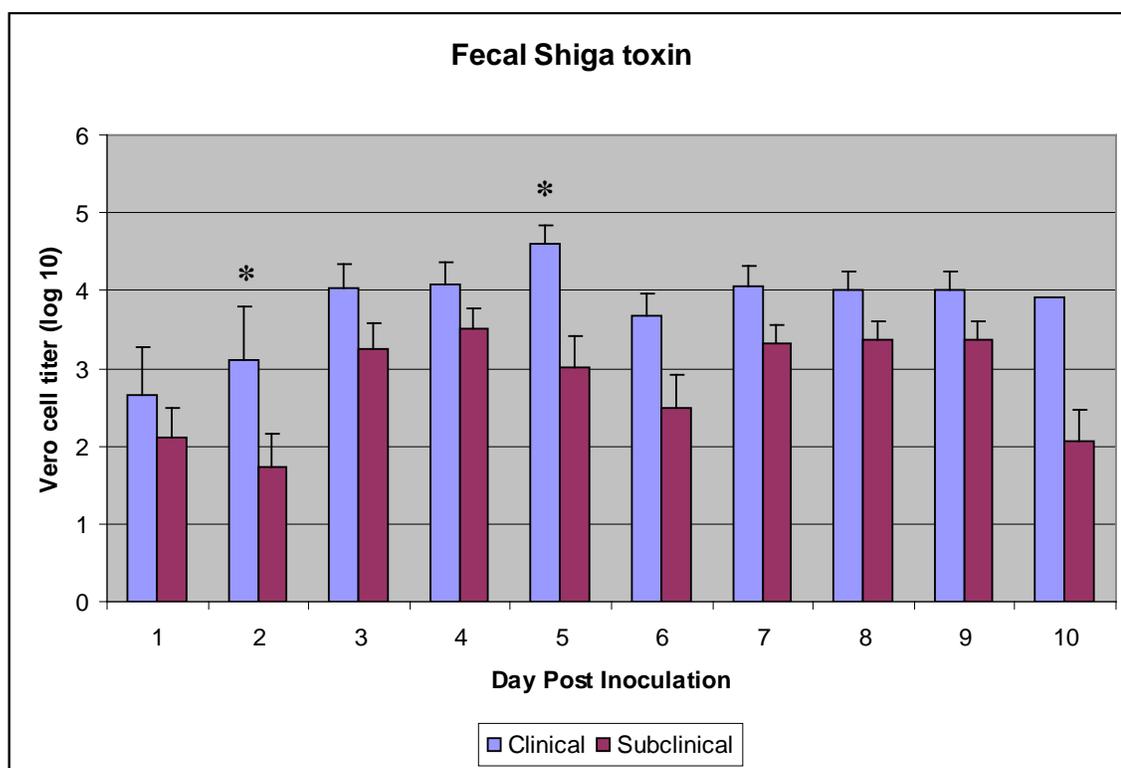
Figure 2. Plasma cytokine (TNF-α, IL-1β and IL-10) levels were determined by ELISA in both Shiga toxin-producing *Escherichia coli* (STEC) inoculated and control pigs to assess systemic proinflammatory cytokine changes. A. Clinical pigs within the STEC group had significantly higher levels of plasma TNF-α at day 2 post-inoculation (p < 0.05). This preceded the onset of clinical disease in pigs by 3 days. B and C. Significant differences between groups were not noted for IL-1β (B) or IL-10 (C).

A.
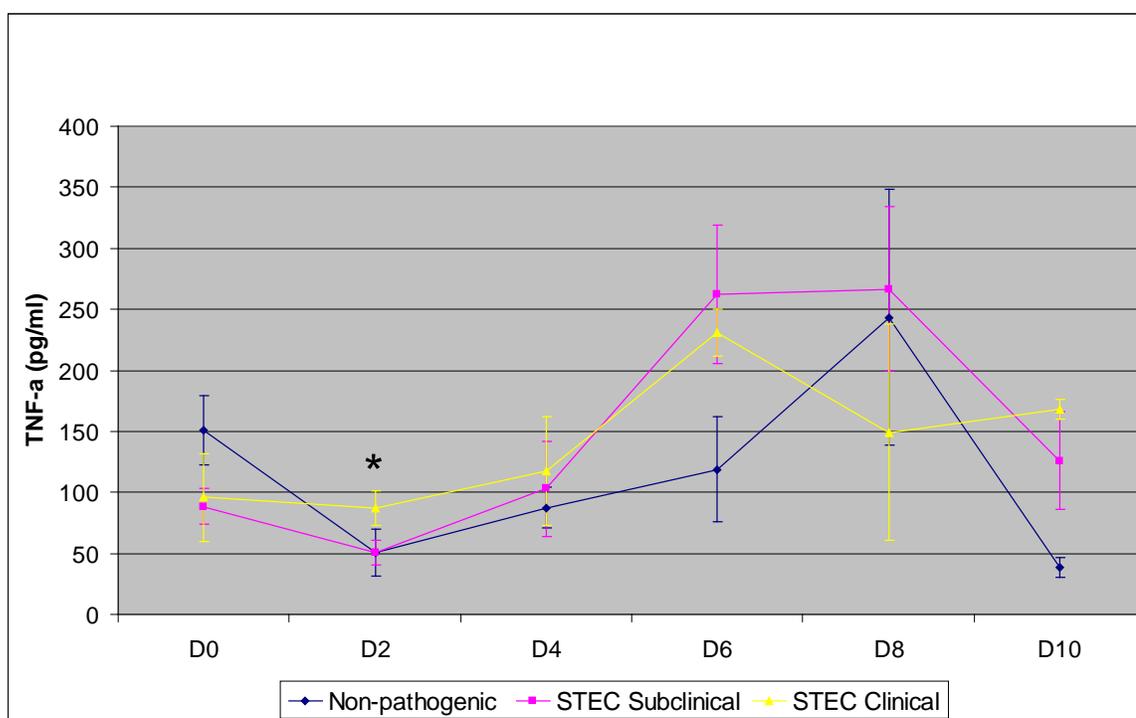
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B.
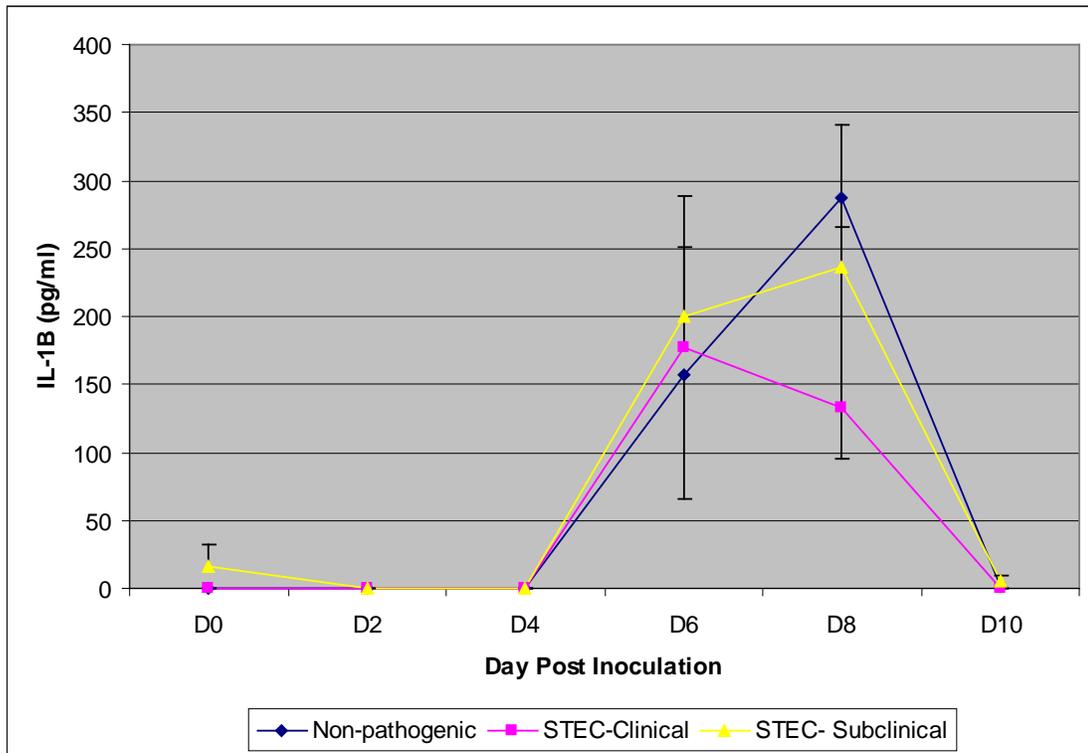
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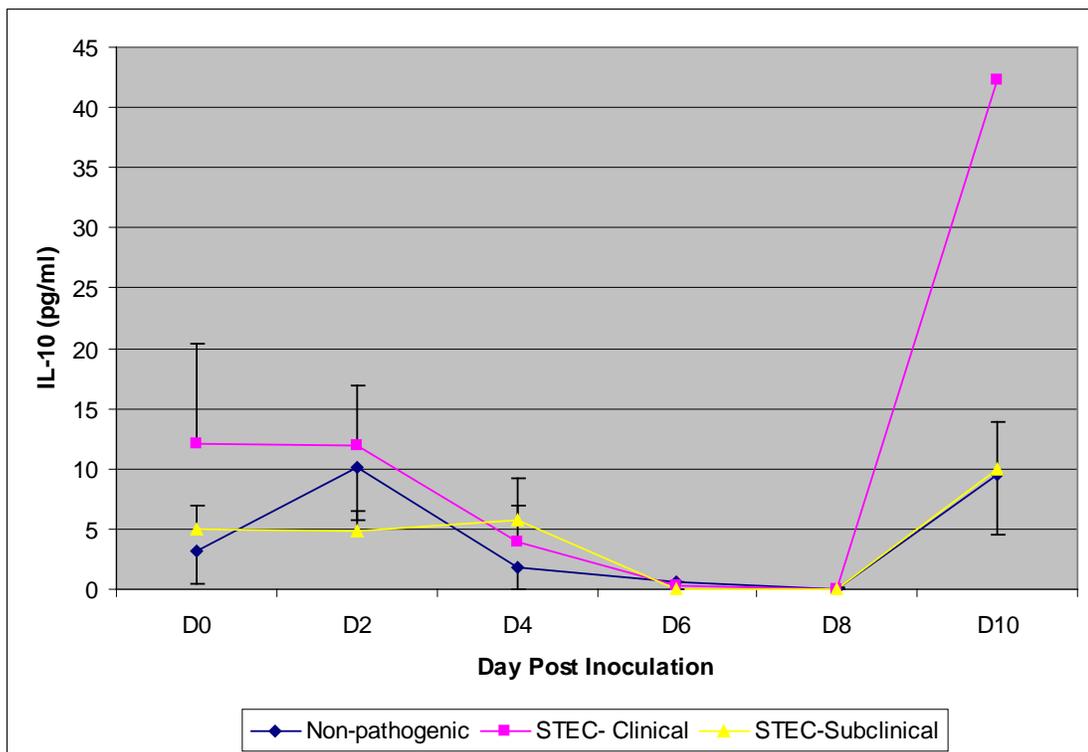
Figure 3. Cytokine (TNF-α, Il-1β and IL-10) levels were measured using ELISA in tissue homogenates from ileum, brainstem, kidney, and liver following STEC infection. Clinical pigs within the Shiga toxin-producing *Escherichia coli* (STEC) group had significantly greater levels of TNF-α (A) and IL-1β (B) in liver than either subclinical pigs or controls (p<0.05). Clinical pigs also had higher levels of liver IL-10 (C) than controls (p<0.05). Subclinical pigs had greater levels of IL-1β in ileum than both clinical pigs and controls, and higher levels in brainstem than clinical pigs (B; p<0.05).

A.
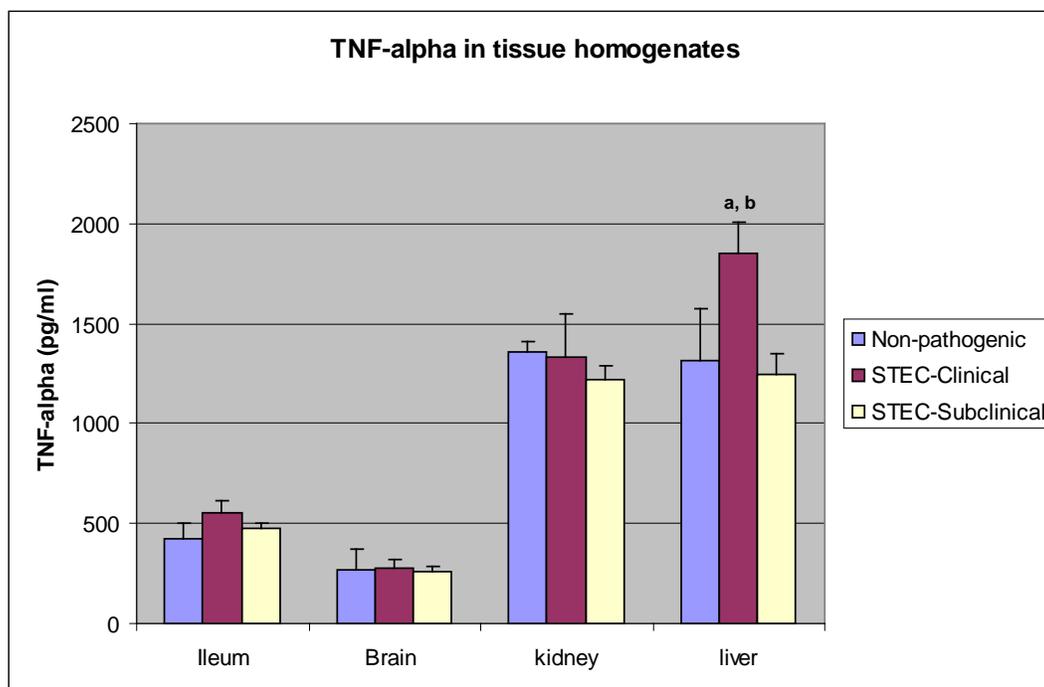
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B.
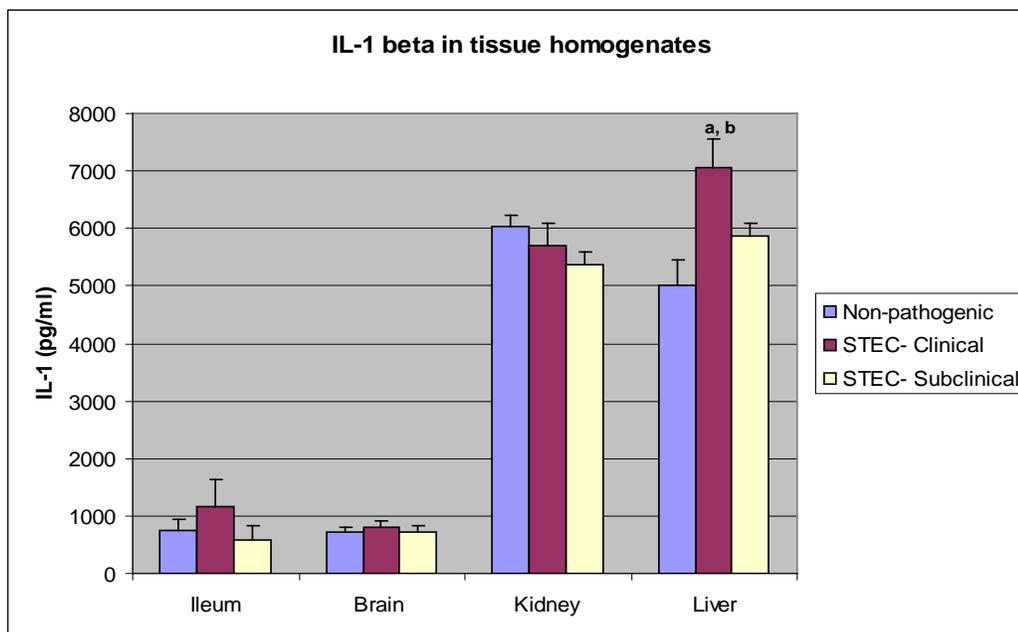
C.
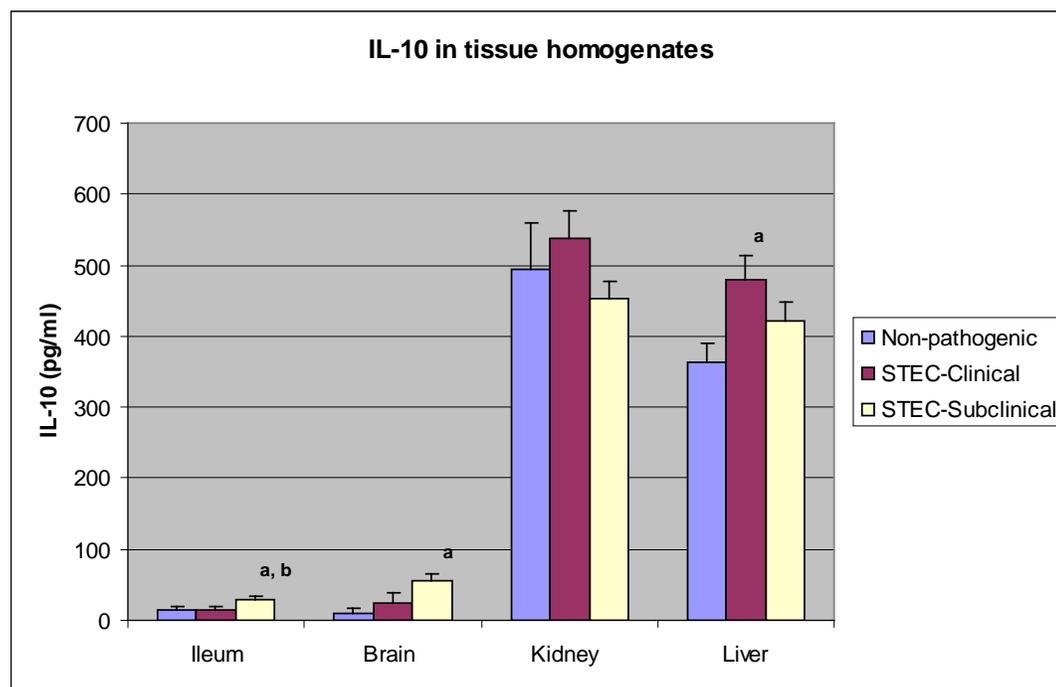
Figure 4. Relative mRNA expression for three cytokine targets (TNF-α, Il-1β and IL-10) in ileum, brainstem, kidney, and liver was determined using one-step quantitative real-time PCR. A). TNF-α mRNA expression in ileum, brainstem, kidney, and liver. Significant differences between groups in mRNA expression were not identified. B). No differences in IL-1β mRNA expression were noted between groups in the tissues examined. C). IL-10 mRNA expression in liver was significantly lower in clinical pigs within the STEC group compared with controls ($p < 0.05$).
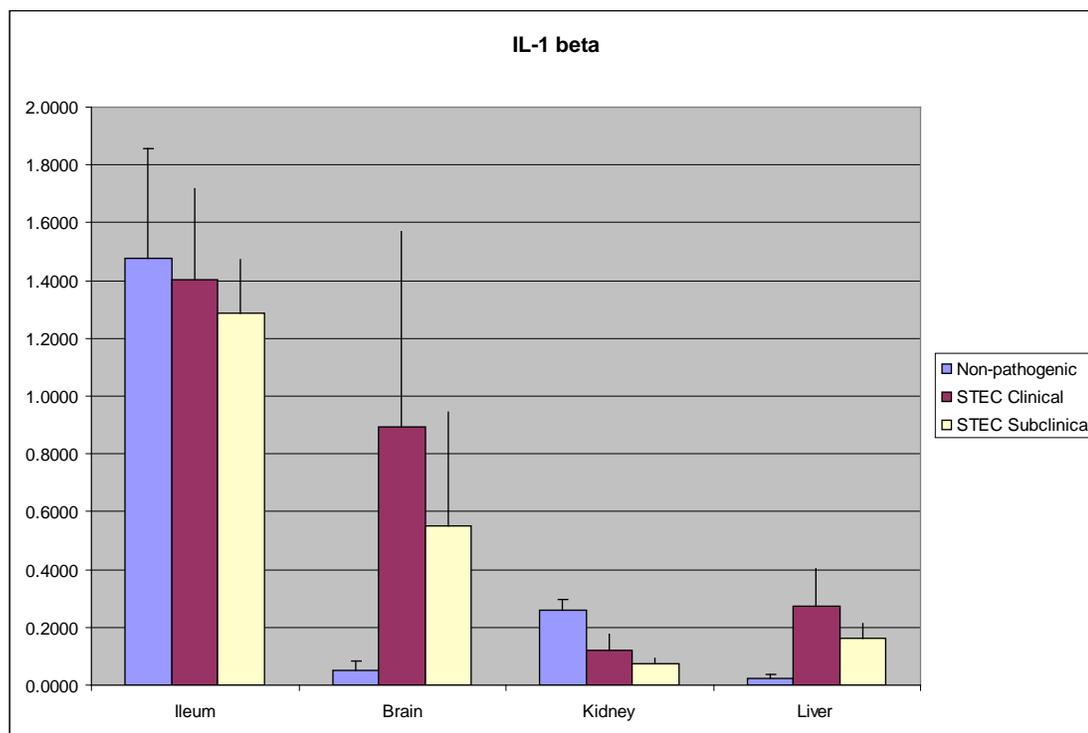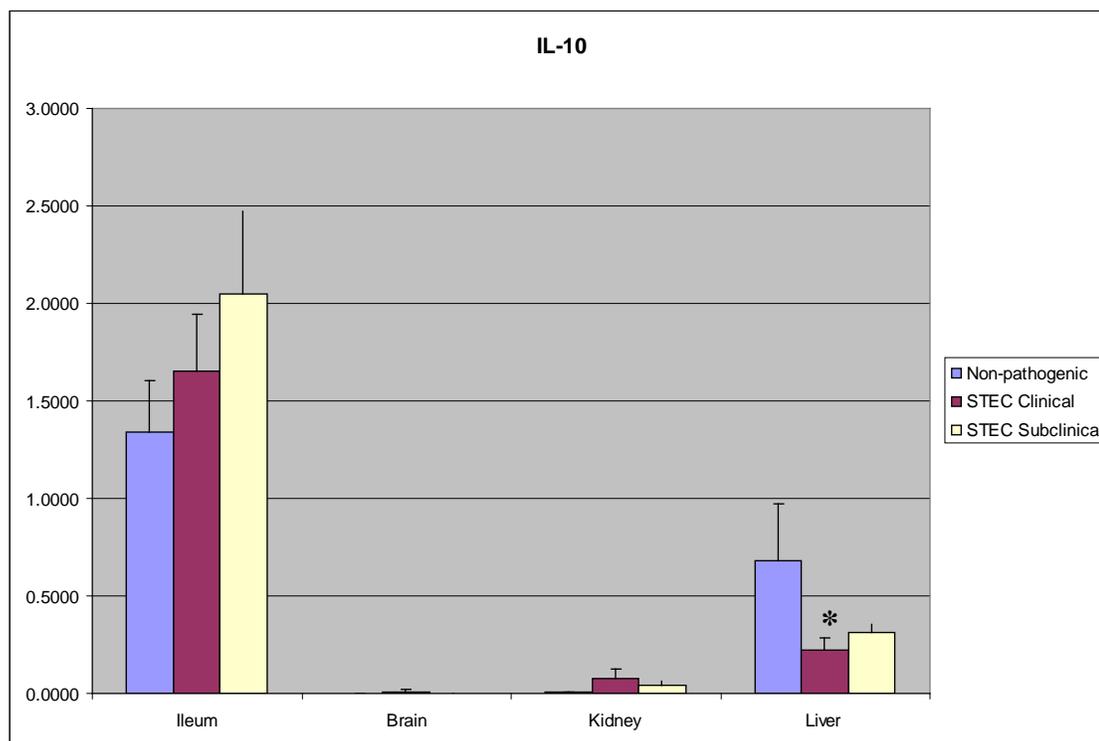
A.
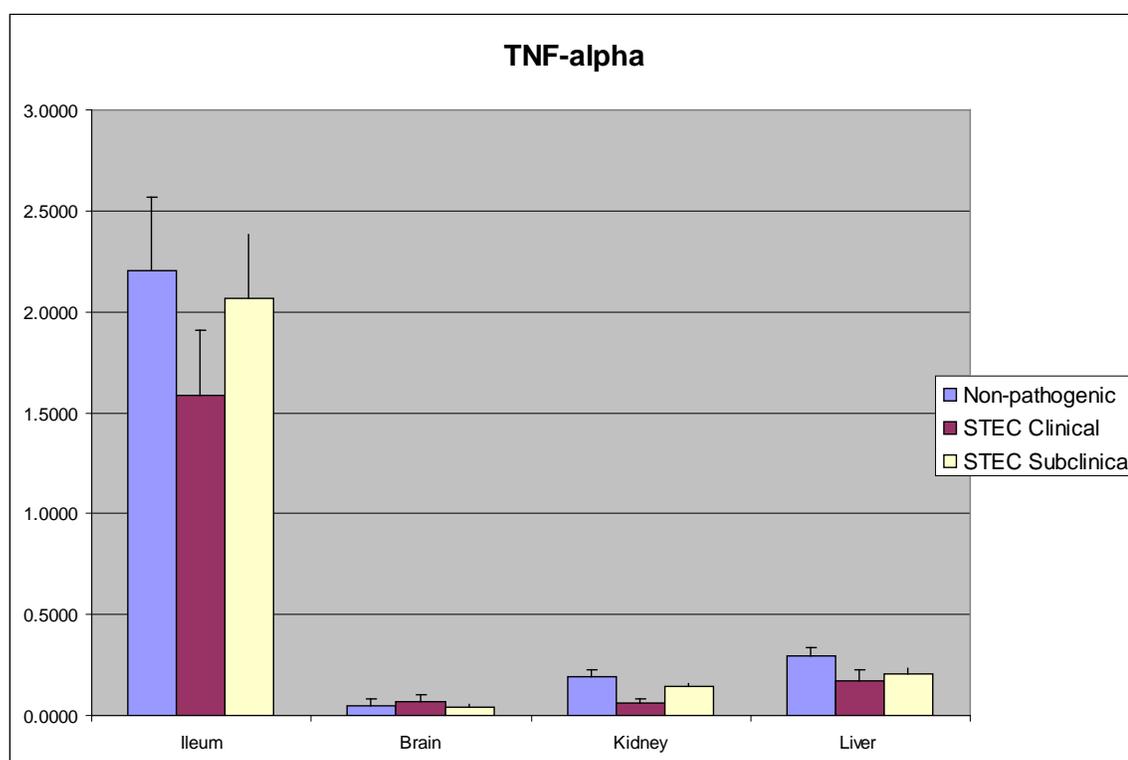
Figure 4 (continued)
B.

C.

Figure 5. TNF-α production by porcine macrophages was measured in cell culture supernatants via ELISA. Direct addition of purified Stx2e to porcine macrophages caused a mild increase in TNF-α ($p > 0.05$). In contrast, addition of LPS produced a marked increase in TNF-α (**, $p < 0.001$). In an attempt to more closely mimic in vivo conditions, porcine erythrocytes (RBC) were pre-incubated with either Hank's balanced salt solution (HBSS) or Stx2e (two doses) and added to cultured macrophages. Erythrocyte-bound Stx2e did not result in significant TNF-α production by porcine macrophages.
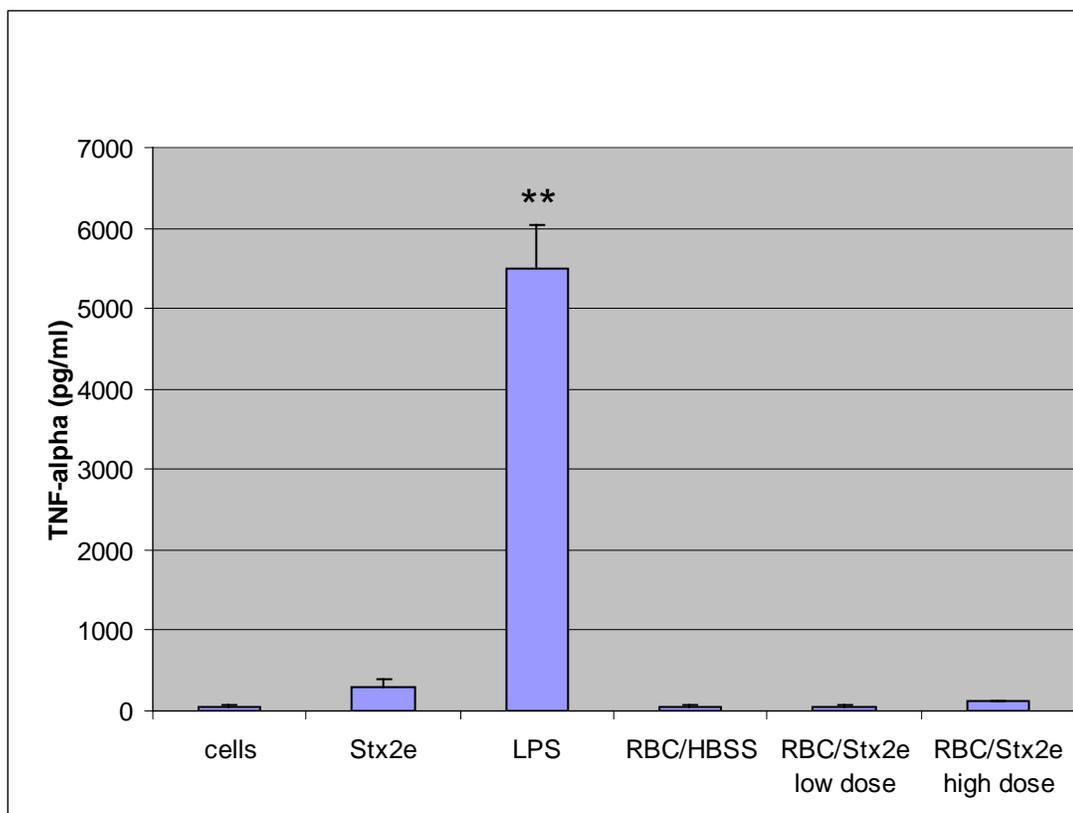
Chapter 4: Role of neutrophil-bound Shiga toxin in Shiga toxin-producing *Escherichia coli* infection.

A paper to be submitted to the journal *Infection and Immunity*

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Abstract

Hemolytic uremic syndrome (HUS), a systemic complication of Shiga toxin-producing *Escherichia coli* (STEC) infection, is the number one cause of renal failure in children in the United States. Currently, there is significant debate over how Shiga toxin (Stx) gets from the site of production, the intestinal lumen, to its site of action, vascular endothelium of the kidney, brain, and colon. The goal of this study was to further characterize the role of neutrophils as a potential means of Stx transport in STEC infection, and to assess the ability of neutrophil-bound Stx to influence the activity of other cell types. In this study we demonstrate specific binding of purified Stx2 to human neutrophils using flow cytometry. Neutrophil-binding of Stx2 was further confirmed using Vero cell assay. Neutrophil-bound Stx2 did not induce significant toxicity on cultured microvascular endothelial cells. Proinflammatory cytokine production secondary to neutrophil bound Stx was then assessed in the human monocytic cell line THP-1. Neutrophil-bound Stx2 caused significant IL-1β production by THP-1 cells. Neither purified Stx2 nor neutrophil-bound Stx2 induced significant TNF-α production by THP-1 cells, in contrast to LPS. The findings of this study support a direct role for

neutrophils in the binding of Stx2 and transfer to Vero cells. Neutrophil-bound Stx2 may also potentiate the proinflammatory cytokine response in STEC infection through the generation of IL-1β by macrophages.

Introduction

Hemolytic uremic syndrome (HUS), a systemic complication of STEC infection, is mediated by vascular damage induced by Shiga toxemia. HUS results in fatality in approximately 2-15% of individuals, and is a major cause of renal failure in those individuals who survive the acute infection (27). Shiga toxins (Stxs) from *Escherichia coli* are produced initially in the gut, enter the circulation via a yet undefined mechanism, and cause vascular necrosis in organs that express high levels of its preferred receptor, globotriaosylceramide (Gb_3), on vascular endothelium (kidney, brain). Stx is a classical AB_5 toxin, and interaction with its B binding subunits with Gb_3 mediates specific binding followed by toxin internalization. Once inside the cell, the inactive A subunit travels to the Golgi and endoplasmic reticulum via retrograde transport and is cleaved within the cytosol, forming an active *N*-glycosidase. The active enzymatic subunit then cleaves an adenine from the 28S rRNA and thus inhibits protein synthesis within the cell (19). Stx may also have an additional role in the pathogenesis of HUS. Stx are capable of inducing proinflammatory cytokine production in a variety of cell types, including mouse and human macrophages and the human monocytic cell line THP-1, and cytokines, in turn, greatly enhance the sensitivity of endothelial cells to the cytotoxic effects of Stx (31). Two major types of Stx have been described, Stx1 and Stx2. Although the two types

share the same mechanism of action, they are antigenically distinct and only share approximately 55% amino acid homology (26). Several variants of Stx2 have been described, and strains that produce Stx2 are more commonly associated with HUS than those that produce Stx1 or both toxin types (1).

One key aspect of HUS pathogenesis that remains to be elucidated is how Stx travels from the site of production in the gut to sites of vascular damage, e.g. kidney and brain. Stx binding to numerous peripheral blood elements has been reported, including platelets, erythrocytes and monocytes; however, most of the focus has been on Stx binding to neutrophils. The first study that identified specific binding of Stx1 to human neutrophils used both radiolabeled Stx1 with Scatchard analysis and FITC-labeled Stx1 with flow cytometry. Additionally, the investigators were also able to demonstrate transfer of the Stx1 from the non-Gb_3 receptor on neutrophils to Gb_3 on glomerular vascular endothelial cells (28). Further studies from the same group identified neutrophil-bound Stx in the peripheral blood of children with HUS, as well as healthy family members within the same household. The sensitivity of the neutrophil-bound Stx detection assay far surpassed that of traditional methods (serology, fecal culture) at identifying individuals with STEC infection (29, 30). These findings were corroborated by another study that identified Stx bound to human neutrophils in the blood of patients with HUS, even when additional evidence of STEC infection was not present (2). However, later studies by a different group of investigators failed to find a direct interaction between neutrophils and Stx1 and Stx2 (7). Moreover, the reliability of the original studies was brought into question recently, when Te Loo et.al. reported that their initial findings were an artifact, and that no true interaction between Stx and human

neutrophils exists (8). To further confound the matter, two more recent studies report either weak interaction between Stx and human neutrophils or interactions that are dependent on maturation status of neutrophils (3, 10).

The goals of this study were to further characterize the ability of Stx2 to bind specifically to human neutrophils and assess the ability of neutrophils to transfer surface-bound Stx2 to both microvascular endothelial cells and macrophages, thereby inducing cell death and proinflammatory cytokine production, respectively. The findings of this study support our hypothesis that human neutrophils indeed bind Stx2. Furthermore, neutrophil-bound Stx2 is readily transferred to Vero cells, thereby inducing cell death. Finally, we show that exposure of the human monocytic cell line, THP-1, to neutrophil-bound Stx2 may enhance IL-1β generation, but does not induce significant TNF-α production. The results of this study show that neutrophils can contribute to Stx transport and identify a potential role for neutrophil-bound Stx in the promotion of inflammatory responses.

Methods

Shiga toxin and cell lines: Crude Stx2 was prepared from whole cell lysate of *E. coli* strain DH5α containing a plasmid encoding the Stx2 gene as described previously (16, 25). Stx2 was purified using an affinity column containing a carbohydrate analogue of the Stx receptor, Gb_3 (17). The purified toxin was then dialyzed against PBS, filter sterilized, and stored at -80 degrees C until use. Crude Stx2 lysate was used in some flow

cytometry experiments. The final Stx2 concentration of the purified preparation was approximately 65 ng/ml (Vero cell titer 1: 131,072). As an additional control in binding and transfer experiments, crude whole cell lysates from the same *E. coli* DH5α strain containing the plasmid but lacking *stx2* were prepared. Human microvascular endothelial cells derived from neonatal dermis (HMVECnd) were purchased from Invitrogen™ (Carlsbad, CA) and cultured in supplemented Medium 131 (Invitrogen™) according to the manufacturers guidelines. HMVECnd were grown in either 25 cm^2 flasks or 96 well plates pre-coated with Attachment Factor (Invitrogen™). THP-1 cells were cultured in 24 well plates in supplemented RPMI at a density of 1 x 10^6 cells per well. They were induced to differentiate into macrophage-like cells by the addition of phorbol mystirate acetate (PMA, Sigma Aldrich®, St. Louis, MO) at a concentration of 50 ng/ml to the media for 48hrs. Fresh media, lacking PMA, was added daily until cells were used in experiments on day 5.

Neutrophil isolation and toxin incubation: Whole blood was collected from three healthy adult volunteers in accordance with a protocol approved by Iowa State University's Institutional Review Board. Human neutrophils were isolated from whole blood collected in sodium heparin following a technique Bryan Heit from the University of Calgary (http://www.bio.net/bionet/mm/immuno/2001-August/016695.html). The majority of erythrocytes were removed via dextran sedimentation. Briefly, whole blood was mixed 1:2 with 6% dextran in 0.9% sodium chloride using aseptic technique and incubated for one hour at room temperature. The supernatant containing leukocytes was

then removed, and centrifuged at 500 x g for 10 minutes. The supernatant was then

removed, and remaining erythrocytes were removed via hypotonic lysis. Mononuclear

cells and platelets were then separated out via density centrifugation using Ficoll-Paque

PLUS (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions.

The mononuclear cell layer and Ficoll layers were discarded, and the pellet was

resuspended in Hank's balanced salt solution (HBSS) lacking calcium, magnesium, and

Phenol red (Invitrogen™). Neutrophils were counted using the ADVIA® 120 automated

hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, IL). Cell purity was

approximately 98% neutrophils following isolation. Neutrophils (approximately 1 x 10^6

cells) were incubated with either Stx2 (approximately 6.5 ng), LPS-rich crude whole cell

lysate, (100 μl) or HBSS (100 μl) for one hour at room temperature immediately

following isolation. Cells were washed twice in HBSS and used immediately in binding

and transfer experiments.

Flow cytometry: Buffy coat preparations of leukocytes were made from whole

blood collected in sodium heparin. Whole blood was centrifuged at 500 x g for 10

minutes. The buffy coat and plasma were removed and added to a new 15 ml conical

tube and centrifuged at 1000 x g for 10 minutes. The supernatant was discarded, and

contaminating erythrocytes (RBC) were removed via hypotonic lysis. Neutrophils were

isolated as described above. Neutrophils or buffy coat leukocyte preparations were

incubated with Stx2 (approximately 6.5 ng) or Stx2-free whole cell lysate (no toxin

negative control) for one hour at room temperature and subsequently washed in HBSS

plus 2% rabbit serum. Cells were then incubated with either 10 µl polyclonal bovine anti-Stx2 (0.20 µg), mouse monoclonal anti-Stx2 (11E10 ascites diluted 1:10, 10 µl) or non-specific IgG (isotype control, 0.10 µg) for 30 minutes and washed in HBSS plus 2% rabbit serum (20). FITC-labeled rabbit anti-bovine IgG F(ab)$_2$ (0.15 µg) or rabbit anti-mouse IgG F(ab)$_2$ (0.20 µg) was subsequently added to all cell suspensions for 30 minutes. Cells were washed and re-suspended in HBSS. FITC fluorescence was evaluated on a FACScan flow cytometer and was measured with excitation and emission at 488 and 530 nm, respectively. Leukocyte populations were gated separately, and neutrophils were selected by forward scatter and side scatter properties.

Vero cell assay for Shiga-like toxin: Stx concentrations were assessed using a Vero cell assay as described previously (5, 9). Briefly, samples were diluted two-fold in HBSS and added to monolayers of Vero cells in microtiter plates. Toxin titers were expressed as the log of the reciprocal of the greatest dilution that resulted in $\geq 50\%$ Vero cell death. Specificity of the titers for Stx2 were confirmed by neutralization using specific antibody as previously described (12).

Neutrophil-bound Shiga toxin addition to THP-1 and HMVEC cells: Neutrophils were incubated with Stx2 (approximately 6.5 ng) as described above. After washing twice in HBSS neutrophils were then added to established cultures of THP-1 cells differentiated with PMA. Neutrophils were co-incubated with THP-1 cells for 1 hour at 37 degrees C, 5% CO_2, and then removed. THP-1 cells were washed with HBSS

to remove any remaining neutrophils, and fresh complete media was added to each well. THP-1 cells were incubated for an additional 24 hrs post treatment. The assays were replicated a minimum of three times.

ELISA for human TNF-α and IL-1β: Commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN) were used to determine TNF-α and IL-1β production by THP-1 cells. Cell culture supernatants were collected from THP-1 cultures at 24 hours post treatment. Supernatants were stored at -80°C and centrifuged at 3000 x g for 5 minutes prior to analysis to remove any cellular debris.   Levels of TNF-α and IL-1β were measured according to the manufacturer's instructions.

Statistical analysis: JMP software (SAS) was used for statistical analysis. The distribution of all data sets were initially assessed, and data were normalized via log transformation when necessary.   Data were analyzed with ANOVA followed by Student's t test. Results were considered significant at a value of $p<0.05$.

Results

Identification of specific binding of Stx to human neutrophils: Flow cytometry identified specific binding of Stx2 to human neutrophils by a shift in mean fluorescent intensity (MFI) compared to negative controls (both isotype and no toxin

controls were used) (Figure 1). Specific binding to monocytes and lymphocytes was not identified. Some variability in the magnitude of the shift in MFI was noted depending on the primary antibody used. Likewise, neutrophil-bound Stx2 induced a significant increase in Vero cell toxicity compared to neutrophils only, indicating specific binding of Stx2 to neutrophils (p<0.001, Figure 2). The cytotoxicity of neutrophil-bound Stx was abated by use of specific polyclonal antibody to Stx2.

Transfer of neutrophil-bound Stx to cultured microvascular endothelial cells:
To establish the potential role of neutrophils in the delivery and transfer of Stx2 to microvascular endothelial cells in vivo, the ability of neutrophils to transfer cell-bound Stx2 and induce toxicity in cultured microvascular endothelial cells was assessed. Neutrophil-bound Stx2 did not result in significant HMVECnd cytotoxicity.

Effect of neutrophil-bound Stx on proinflammatory cytokine production by THP-1 cells: To better understand the contribution of neutrophil-bound Stx2 to proinflammatory cytokine production by macrophages, the effects of neutrophil-bound Stx2 on TNF-α and IL-1β production by THP-1 cells was compared to that of direct addition of LPS or Stx2 using ELISA. Direct addition of LPS (200 ng) resulted in a marked increase in TNF-α production by THP-1 cells at 24 hours (p<0.001, Figure 3A). In contrast, addition of purified Stx2 (6.5 ng) did not result in an increase in TNF-α production. The addition of LPS plus Stx2 did not enhance TNF-α production beyond

that induced by LPS alone. Likewise, neutrophil-bound Stx2 did not cause a significant increase in proinflammatory cytokine production (Figure 3A).

A significant increase in IL-1β production was noted secondary to neutrophil-bound Stx2, as well as LPS and Stx2 alone in comparison to neutrophils only (p<0.05, Figure 3B). Interestingly, the magnitude of this effect was similar amongst all treatment groups.

Discussion

In this study, we were able to confirm that Stx2 binds specifically to human neutrophils using flow cytometry. Previous studies found a non-specific interaction between activated neutrophils and anti-Stx antibody caused a shift in mean fluorescent intensity mimicking Stx binding to neutrophils (8). To avoid the same potential complication, we added an additional control (crude bacterial cell lysate lacking Stx2) to our flow cytometry studies to help verify the specificity of our antibody- Stx interaction. Additionally, we were able to verify Stx2 binding to human neutrophils using a different methodology, Vero cell assay. This provides further evidence of the specificity of the neutrophil-Stx interaction identified in this study. We did not identify a direct role for neutrophil-bound Stx2 in microvascular endothelial cell injury. Prior treatment of cultured endothelial cells with LPS or proinflammatory cytokines is known to enhance the cytotoxicity of Stx, presumably due to upregulation of the cellular Stx receptor (6, 13, 14, 22, 31-33, 35). Because we were interested in dissecting out the effects of LPS and

Stx on host cells in this study, we looked at the cytotoxicity of Stx2 alone. Pre-treatment of HMVEC with LPS and/or TNF-α may have resulted in a greater cytotoxic response.

In this study we found less of a role for Stx2 in TNF-α production by macrophage-like cells than previously reported. Based on our findings, we now speculate that other STEC antigens such as LPS or flagellin may play a more primary role in the enhanced TNF-α responses observed in STEC infection. Previous work with human monocytes and the monocytic cell line THP-1 identified that purified Stx can induce both TNF-α and IL-1β production in vitro (23, 34). Stx1 was found to stimulate TNF-α and IL-1β production in THP-1 cells in a dose and time-dependent manner. A mild to moderate, yet statistically significant, increase in both cytokines was seen with a Stx1 dose of 100 ng/ml. A more marked response was seen with higher doses of Stx1 (400 ng/ml) (23). Furthermore, Stx1 affected cytokine production by THP-1 cells by stabilizing mRNA transcripts, thus enhancing the response. Although Stx1 produced a more muted cytokine response in THP-1 cells compared with equivalent concentrations of LPS, the cytokine transcripts were elevated for a longer period following exposure to Stx1 compared with LPS (11, 24). Similar work in human monocytes identified significant TNF-α and GM-CSF production secondary to higher doses of both Stx1 and Stx2 (18). Although these in vitro studies indicate a potential role for Stx in the promotion of proinflammatory cytokine responses during STEC infection, it is difficult to dissect out the exact stimulus for cytokine production in vivo, where exposure to other potent STEC antigens such as LPS are known to occur. Therefore, we were most interested in the ability of lower, more physiologically relevant levels of Stx and neutrophil-bound Stx to potentiate cytokine responses in an effort to more closely mimic

what is presumed to occur in vivo. Prior clinical studies from STEC patients found the maximal intestinal concentration of Stx to be approximately 20 ng/ml (Vero cell titer 40,960), based on fecal Stx titers (4). Based on this data, it is unlikely that Stx concentrations outside of the intestinal lumen, where exposure of monocytes and macrophages to STEC antigens would likely occur, would reach concentrations as high as 100 ng/ml. The lower doses of Stx2 used here (6.5 ng/ml vs. 100-400 ng/ml) likely account for the disparity between our results and those previously published. Another potential explanation for the lack of TNF-α response in this study could be the toxin preparation used. Previous work in THP-1 cells was performed using purified Stx1, whereas we used Stx2. To our knowledge, this is the first report of the effect of purified Stx2 on TNF-α and IL-1β production by the human monocytic cell line THP-1.

Interestingly, both Stx2 and neutrophil-bound Stx2 enhanced IL-1β production by THP-1 cells. This indicates that Stx2 is able to stimulate IL-1β production in a dose-independent manner, since approximately 100-fold less Stx2 was present in neutrophil-bound Stx compared with direct addition of Stx2. Additionally, Stx2 was as potent an activator of IL-1β production as LPS. Since neutrophils themselves did not stimulate IL-1β production, it is less likely that an active neutrophil product is responsible for this effect. IL-1β, a potent proinflammatory cytokine, is upregulated in macrophages following signaling through pattern recognition receptors (PRRs) and is an important mediator of innate immunity. Recently, a caspase-1 activating complex termed the "inflammasome" has been identified in macrophages. Bacteria and bacterial products are potent activators of the inflammasome, thereby inducing IL-1β production. Several pathogen associated molecular patterns (PAMPs), including LPS and flagellin, are known

to activate the inflammasome (15, 21). Although no studies have been performed to date to implicate Stx specifically in activation of the inflammasome, it is possible that Stx signaling through a PRR or danger signal could mediate IL-1β production in this manner. Further studies that decipher the specific mechanism for IL-1β production secondary to Stx are warranted.

In conclusion, the findings of this study support a potential direct role for neutrophils in the transport of Stx2 from the intestine to distant organs. Additionally, neutrophil-bound Stx2 is readily transferred to sensitive Vero cells, thereby inducing cell death. Neutrophil-Shiga toxin interactions also may potentiate the proinflammatory cytokine responses observed during STEC infection by contributing to IL-1β generation by macrophages. Additional studies that further elucidate the mechanisms of the enhanced proinflammatory response associated with HUS may enable the development of more advanced treatment modalities.

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Figures

Figure 1. Identification of Stx2 binding to neutrophils using flow cytometry. To assess binding of Stx2 to human neutrophils, leukocytes were incubated with Stx2, followed by specific anti-Stx2 antibody and a FITC labeled secondary antibody. Granulocytes (approximately 95-98% neutrophils) were gated upon based on forward and side scatter characteristics, and FITC fluorescence was measured. The histogram depicts a shift in mean fluorescent intensity (MFI) representing specific Stx2 binding (shaded) over control (gray line). Significant binding of Stx2 to lymphocyte or monocyte populations was not identified. This histogram represents Stx2 binding to neutrophils as identified using mouse monoclonal anti-Stx2 antibody 11E10. Smaller shifts in MFI were identified using a bovine polyclonal antibody.
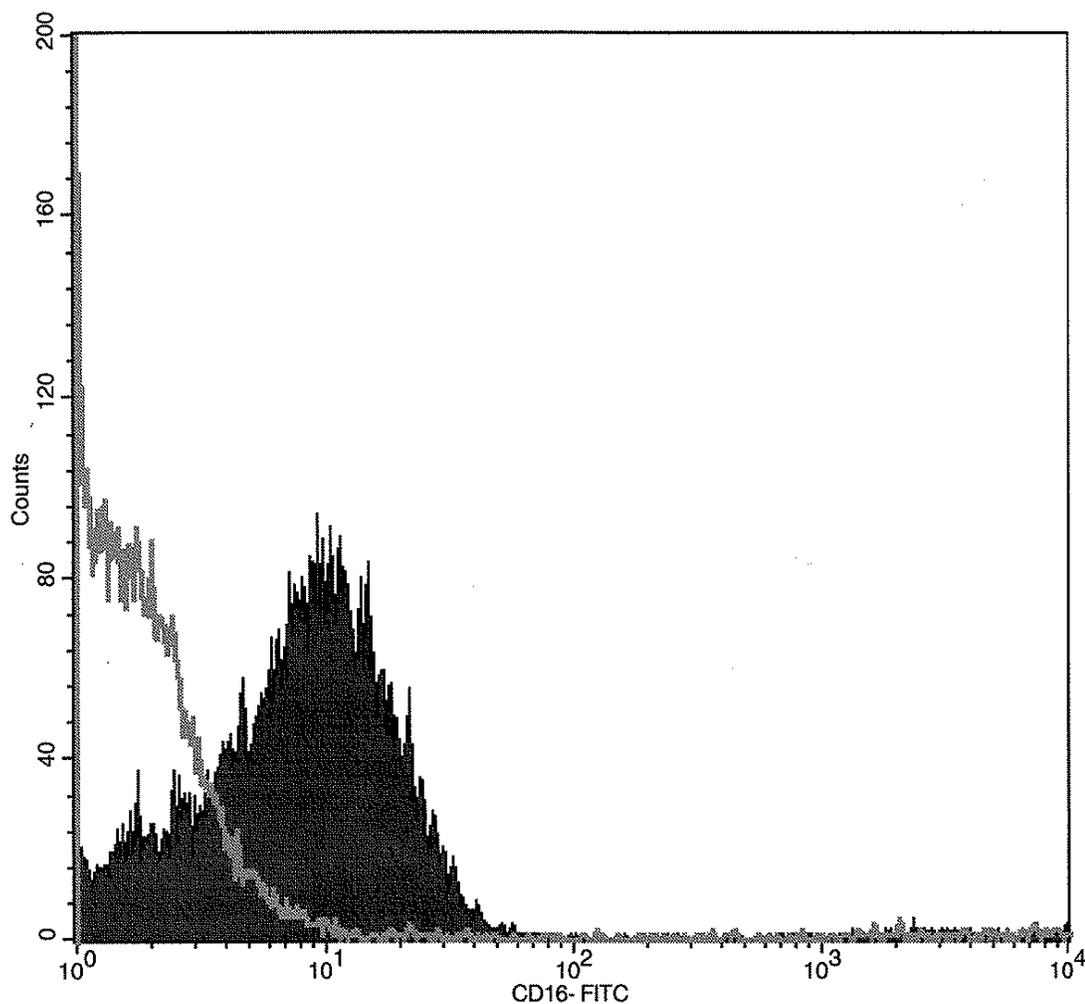
Figure 2. Binding of Stx2 to human neutrophils was assessed using Vero cell assay.
Neutrophils were incubated with either Hank's balanced salt solution (HBSS, negative
control) or purified Stx2 and added to Vero cells.   Neutrophil-bound Stx2 resulted in
significant Vero cell toxicity (*, p<0.001). Vero cell toxicity secondary to neutrophil-
bound Stx2 was neutralized with specific antibody (data not shown).
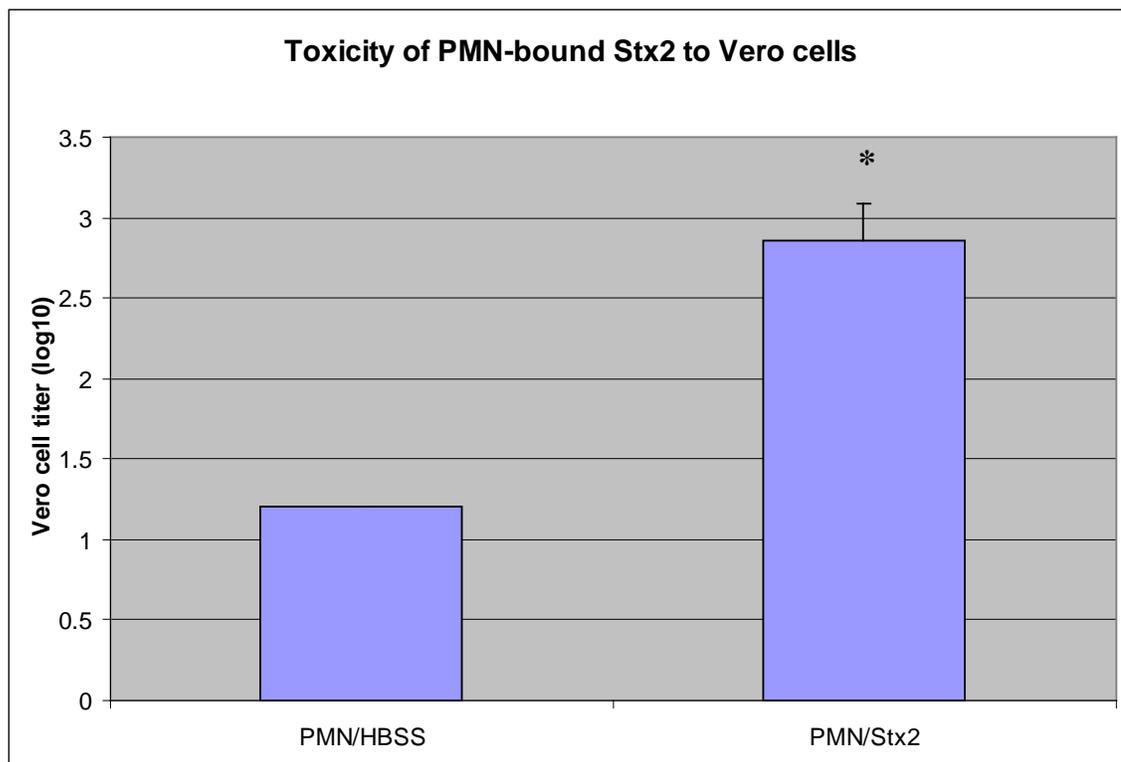

Toxicity of PMN-bound Stx2 to Vero cells

Figure 3. The ability of Stx2, LPS, co-addition of LPS and Stx2, and neutrophil-bound Stx2 to induce proinflammatory cytokine production in the human monocytic cell line THP-1 cells was assessed. A) LPS and Stx2 plus LPS induced significant TNF-α production in THP-1 cells (**; p<.001). In contrast, Stx2, either added directly or bound to neutrophils, did not result in considerable TNF-α production by THP-1 cells . B) THP-1 cells released significantly more IL-1β over baseline in all treatment groups assessed (*, p<0.05; **, p<0.001).
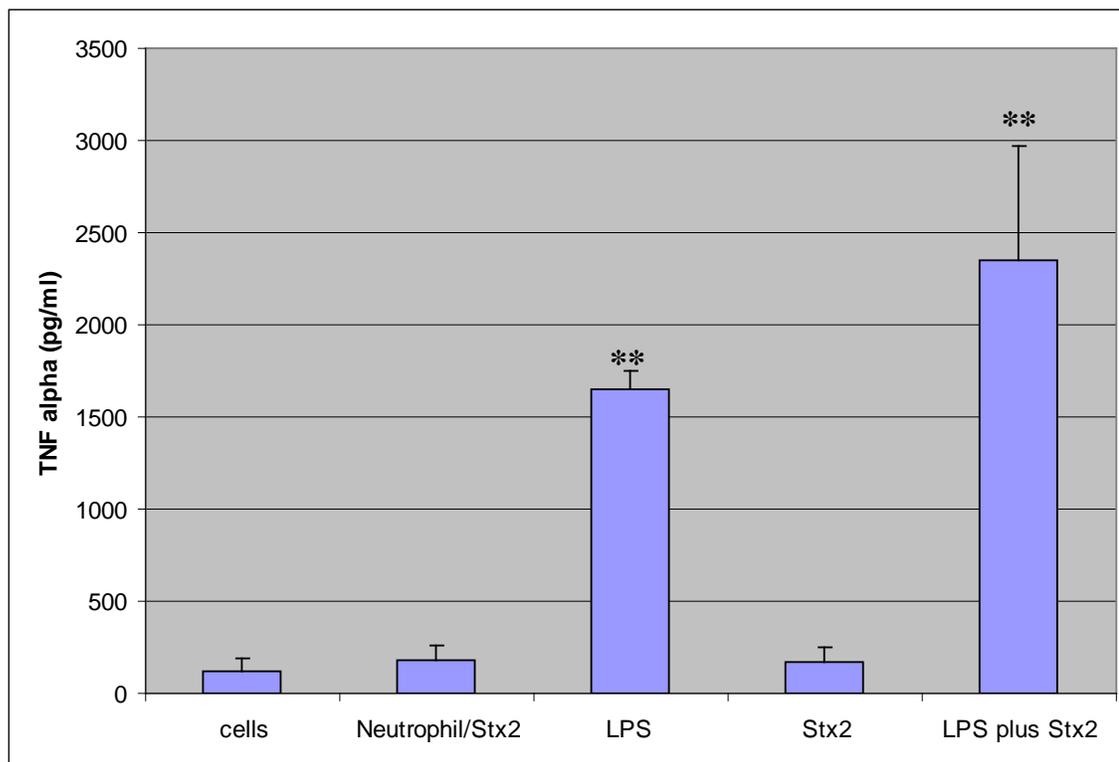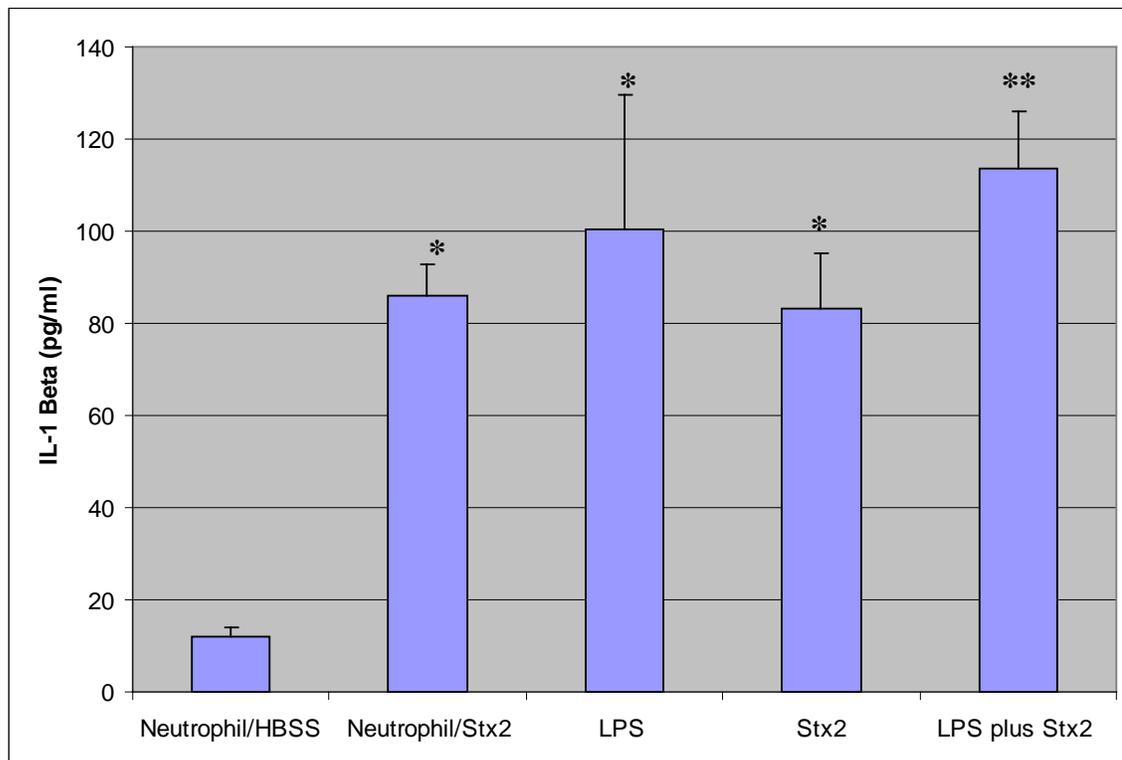
A.

Figure 3 (continued)
B.

Chapter 5: General conclusions

Shiga toxin-producing *Escherichia coli* (STEC) are a major cause of food borne illness in industrialized nations worldwide. Additionally, hemolytic uremic syndrome, a life threatening systemic complication of STEC infection in human beings, is the leading cause of acute renal failure in children in the United States. The goals of the experiments outlined in this thesis were to advance our understanding of HUS pathogenesis and to assess the efficacy of an experimental therapeutic using both in vitro studies and an animal model of HUS, edema disease of swine.

The first study tested the efficacy of an oral Shiga toxin 2e (Stx2e) binding agent, a receptor mimic probiotic, on the incidence of clinical edema disease. Our hypothesis was that treatment with the probiotic would bind and trap Stx2e within the intestinal lumen, thereby preventing systemic toxin absorption and subsequent clinical disease. We found that although the probiotic was effective at reducing intestinal Stx2e, it failed to prevent systemic absorption of Stx2e. Additionally, the probiotic did not reduce the incidence of clinical disease. The conclusion from this study was that an oral Stx2e binding agent is less effective than parenteral specific antibody at preventing disease due to systemic Shiga toxemia.

In the second study, proinflammatory cytokine responses following STEC challenge were assessed in pigs. We hypothesized that pigs with clinical disease would have a greater proinflammatory response than subclinical pigs. An additional goal of this study was to identify prodromal changes in systemic cytokine levels predictive of disease outcome. We hypothesized that clinical pigs would have a greater systemic

proinflammatory response prior to the development of clinical signs. Clinical pigs had greater levels of TNF-α and IL-1β in liver than subclinical pigs, and subclinical pigs had higher levels of the anti-inflammatory cytokine IL-10 in organs that develop vascular lesions. Interestingly, pigs that went on to develop clinical disease had higher plasma TNF-α on day 2 post-inoculation, prior to clinical disease onset. Finally, in vitro studies failed to identify a significant TNF-α response in porcine macrophages secondary to Stx2e exposure. The conclusions from this study were that clinical pigs had evidence of a greater proinflammatory cytokine response than subclinical pigs, and that plasma TNF-α may be a useful predictor of systemic disease development in STEC infected individuals.

In the last study, we sought to clarify the role of human neutrophils in the binding and transfer of Stx2. We hypothesized that neutrophils would bind Stx2, and that neutrophil-bound Stx2 would be readily transferred to other cell types. Specifically, we predicted that neutrophil-bound Stx2 would be transferred to endothelial cells and macrophages, thereby inducing vascular necrosis and proinflammatory cytokine production, respectively. The findings of this study show that neutrophils do bind specifically to Stx2, and that the toxin is then transferred to Vero cells. We did not, however, observe significant endothelial cell toxicity secondary to neutrophil-bound Stx2. Finally, neutrophil-bound Stx2 may serve to potentiate proinflammatory cytokine responses through macrophage-mediated IL-1β generation.

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