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The effect of inflammation on Shiga toxin absorption in vivo

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The effect of inflammation on Shiga toxin absorption *in vivo*

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General Introduction

Rationale and Hypothesis

Hemolytic Uremic Syndrome (HUS) is a potentially lethal complication of hemorrhagic colitis (HC), an enteric infection caused by Enterohemorrhagic *Escherichia coli* (EHEC) [1-4]. HUS is the leading cause of acute renal failure in children in the United States and other industrialized countries [5, 6], and is characterized by the triad of thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure [7]. The hemolysis in HUS is severe, often resulting in a packed cell volume that falls below 30%. Platelet counts can drop to 150,000 or less, and many patients require dialysis for the acute renal failure. HUS is caused by Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), which are produced by EHEC bacteria. Histologic lesions of HUS include endothelial cell necrosis and fibrin thrombi in the blood vessels of the renal cortex, the ileum and large intestine, and in some cases the brain of the affected individuals. Children, especially those under 5 years of age, and the elderly are more susceptible to HUS than adults [8, 9].

EHEC infections are common in the United States and throughout the developed and developing countries of the world. In the United States, it is estimated that 70,000 cases of EHEC infection occur annually. EHEC infections may be asymptomatic, or may present as moderate to severe bloody or non-bloody diarrhea. HUS develops in up to 10% of children infected with EHEC, and is fatal in up to 5 to 35% of cases [7-9]. The most common EHEC in the United States is *E. coli* O157:H7, accounting for approximately 80% of cases. This strain is prevalent in ruminants, and is thought by some to be adapted to cattle, where it

colonizes the ileum and large intestine, and has recently been found to persist at the ano-rectal junction. *E. coli* O157:H7, like other EHEC, forms closely adherent, intimate attachments to intestinal mucosal cells that are known as attaching and effacing (A/E) lesions. Up to 75% of cattle herds in the United States are thought to be infected with *E. coli* O157:H7, although estimates of individual animal infection within these herds range from 3 to 28%. Infections in cattle and other ruminants are more common in the spring and summer, which correlates with the highest incidence of hemorrhagic colitis and HUS in humans. Clinically, it is difficult to determine whether or not an individual with HC will develop HUS, and there are currently no effective treatments for HUS.

Although it is known that HUS is caused by Stx, the mechanism of movement of Stx from the intestinal lumen, where it is produced, to the systemic circulation, where it causes the potentially fatal lesions of HUS, is poorly understood. Stx, like endotoxin and many other bacterial toxins, is not readily absorbed by the intestinal mucosa under normal circumstances. Although in vitro studies have shown that Stx I translocates across polarized intestinal epithelial cells by a transcellular pathway, and that Stx II translocates across polarized epithelial cells by a paracellular pathway, the dynamics and movement of Stx in vivo is poorly understood [10]. It is possible that the A/E lesions formed by most EHEC bacteria allow passage of the toxin into the altered mucosal cells, or that movement of the toxin is transcellular in vivo. Alternatively, it is possible that the host inflammatory response induces changes in mucosal permeability that allows Stx to move from the intestinal lumen to the systemic circulation.

It is our hypothesis that the host inflammatory response to EHEC induces HC and allows or enhances movement of Stx from the intestinal lumen to the systemic circulation. If this hypothesis is correct, it may be possible to design effective therapeutic intervention strategies based on regulating the host inflammatory response to EHEC.

The objective of the first study in this dissertation was to develop a model of EHEC infection that would allow testing of the hypothesis that the host inflammatory response enhances movement of Stx from the intestinal lumen to the systemic circulation in EHEC infections. Although there are numerous animal models of EHEC infection, including the pig, most are used to study systemic effects of Stx or colonization factors associated with EHEC rather than mucosal permeability to Stx. Prior to work in this dissertation, previous pig models have used newborn (<24 hour old) pigs. However, newborn pigs acquire immunoglobulins and other macromolecules by passive transfer during the first 48 hours of life, making the newborn pig model inappropriate for studies of the mechanisms of Stx translocation. In order to circumvent this problem, a model of 3-day-old pig infection with EHEC O157:H7 was developed.

The objective of the second study was to induce an inflammatory response in the cecum and large intestine of the 3-day-old pig, sites of intestinal colonization, and to determine the effects of the inflammatory response on mucosal permeability to Stx in this model. In the first study, the 3-day-old pigs were colonized by EHEC O157:H7 and developed classic A/E lesions, but lacked or had only a very mild histologically detectable inflammatory response to infection.

The objective of the third study was to compare the clinical and systemic effects of EHEC infection and Stx absorption in 3-day-old pigs with and without colonic inflammation. This would allow determination of whether the host inflammatory response does or does not enhance Stx absorption.

Dissertation Organization

This dissertation consists of a general introduction and literature review (Chapter 1), followed by three manuscripts that have been prepared for submission to peer reviewed scientific journals (Chapters 2-4), and a general conclusion (Chapter 5). References are cited at the end of each chapter.

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Chapter 1: Literature Review

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) species, notably *E. coli* O157:H7, are pathogens that cause a wide range of clinical syndromes in people, ranging from asymptomatic carriers, to non-bloody diarrhea, to bloody diarrhea, with or without systemic complications such as Hemolytic Uremic Syndrome (HUS), Thrombotic Thrombocytopenic Purpura (TTP), and death [1-4]. EHECs gained notoriety following an outbreak of hemorrhagic colitis (HC) and HUS associated with a fast food restaurant in the Pacific Northwest. The main cause of concern with EHECs is their ability to induce systemic disease, namely HUS [3, 5]. This review presents and discusses findings of EHEC studies, with emphasis on the host inflammatory response to EHEC and that response's possible effects on intestinal mucosal permeability to Shiga toxins (Stx).

HUS

General information

HUS, and the related condition TTP, are disease syndromes known as thrombotic microangiopathies (TMA). The majority of HUS cases (90% in the U.S.) are secondary to EHEC infections [6, 7]. HUS is the leading cause of acute renal failure in children in the U.S. and the industrialized world [8, 9]. HUS is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure [10]. Approximately 10% of EHEC infections will proceed to HUS [11] and the majority of HUS cases occur in either the

very young or the very old [12]. HUS is caused by Shiga toxins (Stx) produced by EHEC. Stx 2 is primarily responsible for HUS [13]. Although Stx 1 is toxic to human glomerular endothelial cells (HGEC) *in vitro* it is not generally associated with HUS [14].

Shiga toxins

The Shiga toxins are a group of structurally similar but antigenically distinct toxins that are formed by EHEC, and are composed of a single A unit and five B subunits [15]. Stx 1 is differs by only a single amino acid from the toxin produce by *Shigella dysenteriae*, from which the name was derived [16]. Stx 2 shares 56% amino acid homology with Stx 1 [6]. The alpha unit of Stx 1 and II is the functional toxin and works by irreversibly cleaving a specific adenine residue on the ribosomal RNA that encodes the 28s subunit [17]. This halts protein production in the target cell and rapidly leads to cell death, or sublethal cell injury.

Stx binding

The B subunits of the toxin bind to receptors on the target cell, and confer target specificity [18]. Stx 1 and 2 bind to the globotriacylceramide (Gb3) receptors, while the edema disease toxin, Stx 2e binds to globotetracylceramide (Gb4) receptors [18]. Binding to target cells is dependent not only on receptor density, but also upon the fatty acid composition of the Gb3 receptor. Kiarash et al. reported that increased fatty acid chain length and unsaturated fatty acid chains improved Stx binding, and concluded that several overlapping moieties were recognized by the Stx B subunit, and that these moieties are differentially available based upon the fatty acid composition of the Gb3 receptor [19]. The Gb3/Gb4 receptors are differentially expressed in different tissues at a constitutive level [20], and can be up-regulated by TNF alpha, butyrate, IL-8, and other substances [20-22]. Gb3 receptor expression in human glomerular endothelial cells (HGEC) is 1000 x higher than in human

umbilical vein endothelium, which likely contributes to the exquisite sensitivity of HGEC to Stx, and makes the kidney a primary target of Stx 1n HUS [23].

Once bound to the target cell, Stx 1s taken into the cell by endocytosis where it moves in a retrograde direction through the Golgi [24]. As previously mentioned, the A subunit acts by enzymatically depurinating a residue of the 28s rRNA, shutting down protein synthesis and killing or sublethally injuring the target cell.

Symptoms of HUS

Symptoms of HUS are related to the killing or sublethal injury of the target cells and the cascade of events that this induces. There are several cell types that are preferentially targeted by Stx due to their Gb3 expression, including glomerular endothelial cells, renal tubular cells, intestinal epithelial cells, and central nervous system endothelial cells [20, 23, 25, 26]. When endothelial cells in the glomeruli and other locations are damaged, they respond by releasing vonWillebrands factor (vWF), a potent pro-coagulant factor that contributes to hemostasis. Large multimers of vWF released from lethally or sublethally injured endothelial cells attract and activate platelets, which subsequently degranulate and start to form microthrombi in affected vessels. If the endothelial damage is sufficiently wide-spread, this microthrombus formation leads to consumptive thrombocytopenia, which is one of the hallmarks of HUS [27, 28]. Microthrombi in small vessels cause turbulent blood flow, which has a shearing effect on red blood cells, and is believed to contribute to the hemolysis and red blood cell fragmentation seen in HUS [28]. Additionally, peroxidation of the lipid membranes of red blood cells can lead to increased fragility. Turi et al., reported increased lipid peroxidation by measuring malonyl dialdehyde levels in blood samples from HUS

patients, accompanied by increased oxidized glutathione levels and increased red blood cell fragility [29].

Uremia in HUS is caused primarily by decreased glomerular filtration, secondary to microthrombi within the renal arterioles and glomerular capillaries [30]. Toxin has been shown to be bound to renal tubular cells in human cases of HUS, and the direct toxic effect of Stx on tubular epithelial cells may also contribute to renal dysfunction seen in this disease [30]. 30-50% of HUS cases in humans also develop neurologic disease [31], which is believed to be secondary to Stx 1nduced endothelial damage with microthrombosis of arterioles within the brain, leading to small, multifocal areas of ischemic neuropathy [32-34].

Acute death occurs in 3-5% of HUS cases [7, 9]. Chronic renal sequelae can also occur, and include chronic proteinuria, and chronic renal insufficiency. There is progression to end-stage kidney disease in 3-5% of cases [7]. Most deaths that occur in HUS are due to brain injury rather than renal injury [35].

EHEC-induced HUS generally develops around 6 days after the onset of EHEC symptoms (bloody or non-bloody diarrhea, cramping, etc.)[6]. There are several known risk factors for developing HUS, including increased urinary IL-6 levels, increased serum TNF and IL-8 levels, high peripheral white blood cell count, and, as previously noted, extremes in age [9, 30, 36-38]. The increased urinary IL-6 levels, increased serum TNF and IL-8 levels, and high peripheral WBC counts may be due to a heavier bacterial load or more severe infection,

or they may reveal a more robust host response to a bacterial infection than one that is only average in severity.

Treatments

Currently, there is no effective treatment for HUS beyond supportive care. Several studies suggest that antibiotics may be an additional risk factor for developing HUS, which may be due to the fact that Stx 1s not released from the periplasmic space of the bacteria until bacteriolysis. However, other studies have shown that there is no effect, either positive or negative of antibiotic use in HC, and at least one study showed that antibiotic treatment with Fosfomycin had a beneficial effect of decreasing the likelihood of HUS [27]. Additionally, anti-diarrheal medications have been shown to be a risk factor, presumably due to increased retention of feces that contains Stx [39, 40]. Interestingly, Cornick et al., recently showed that there is discordance between fecal Stx and HUS development in children. That is, high fecal Stx levels are not always associated with the development of HUS in children [41].

Although no effective treatments besides supportive care have been identified for HUS, there are several treatment strategies that have been tested, are currently being tested, or that have been proposed for the treatment of HUS. These treatments include toxin binding agents that may decrease or prevent Stx absorption [42], and hyper-immune globulin [27]. Binding agents are typically highly branched, non-absorbed molecules that bind Stx and keep it in the intestinal lumen until it is eliminated from the body. A somewhat different spin on the binding agents is the use of recombinant bacterium as a probiotic that expresses an Stx receptor mimic, and in one study protected mice from an otherwise 100% lethal challenge dose of STEC [43, 44]. Unfortunately, clinical trials, to date, have been somewhat

disappointing. Hyper-immune globulin is given in an attempt to bind or deactivate Stx that has already been absorbed into the body and prevent it from reaching sensitive cell populations. In pigs, Dean-Nystrom et al., have shown that vaccination of sows with intimin produces antibodies that are protective to suckling piglets [45]. Similarly, Mukherjee et al., showed that human monoclonal antibodies to Stx 2 protected mice and gnotobiotic piglets from disease following experimental challenge with EHEC strain 86-24 [46].

In studies preceding the ones presented in this thesis, colostrum deprived dairy calves infected with *E. coli* O157:H7 and treated with an agent that inhibits neutrophil recruitment by blocking selectins, TBC 1269, had less severe clinical signs of *E. coli* infection. Although it was later shown that cattle lack vascular receptors for Stx [47], if the hypothesis that the host inflammatory response enhances Stx translocation is correct, anti-inflammatory agents could play a role in treatment of HC and HUS.

EHEC

Introduction

E. coli is an ubiquitous organism that colonizes the large intestinal tract of animals that range from invertebrates to humans. Most *E. coli* are non-pathogenic, however, there are numerous strains of *E. coli* that produce a relatively wide variety of diseases. *E. coli* are classified by antigenic differences in the LPS (O antigen), flagellar (H antigen), or capsular (K antigen), if present [48]. By this system, different serotypes of *E. coli* are recognized. For example, *E. coli* O157:H7 is a strain of *E. coli* that reacts the O (LPS) antigen number 157, and H

(flagellar) antigen number 7. *E. coli* are also classified based upon their pathogenic characteristics, or virulence factors (virotype). By this system, pathogenic *E. coli* are classified as Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), and Shiga toxigenic *E. coli* (STEC) [48]. The classifications can be overlapping, for example, some STEC are also EHEC.

Virulence factors

The EHEC are a group of *E. coli*, including *E. coli* O157:H7, that contain the virulence factors Stx 1, Stx 2, or both. These bacteria have been recognized as a cause of HUS since the early 1980's [27]. The EHEC's that are most commonly responsible for HUS have a number of virulence factors, including a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE) that contains the genes required for the attaching and effacing (A/E) phenotype, and a lysogenic bacteriophage that contains the gene sequences and operon control sequences for producing Stx's [49].

LEE

The LEE is located on the bacterial chromosome and contains the genes for the so-called "*E. coli* Secreted Proteins", or Esp's, intimin, and Tir, which are responsible for the intimate binding seen in A/E bacterial lesions, and a type III secretion apparatus encoded by the sep genes [50-52].

A/E Lesion formation

Initial, loose, attachment of EHEC is incompletely understood, but is thought to be due to non-specific binding interactions between the bacteria and enterocytes. EPEC's, which are similar to EHEC's in that they form A/E lesions, form a pilus structure called a "bundle

forming pilus" that is thought to participate in early binding activity, however, this pilus is not formed by EHEC [53]. The initial loose attachment of EHEC is followed by A/E lesion formation, which is dependent on eae and other genes encoded by the LEE [54-58], and is controlled by quorum sensing and other mechanisms [59]. Quorum sensing is a form of bacterial communication in which a reporter molecule, usually an acyl homoserine lactone-signaling molecule, is produced by bacteria and released into the environment [60]. When the reporter molecule in the environment reaches a certain concentration, it induces a metabolic change or protein production in the bacteria. Quorum sensing in *E. coli* uses the luxS gene [61]. When the population density of *E. coli* O157:H7 reaches a certain point, as reported by quorum sensing, the LEE is activated [59]. Presumably the first genes expressed are the sep and esp genes that encode the type III secretion system. Type III secretion systems are characterized by a filamentous structure that extends through the inner and outer bacterial membranes. This filamentous structure, along with the secreted Esp proteins, forms a large syringe-like structure that is used to inject proteins notably Tir [56], into the host/target cell [51]. Once Tir is in place within the host cell membrane, it binds to intimin, the product of the eae gene. The host cell responds by rearranging actin filaments surrounding the bacteria to form small pedestal-like structures that are characteristic of A/E attachment [62]. This rearrangement of actin fibers is thought to be at least partially controlled by EspB [51]. Thus, the bacteria actually inserts it's own receptor into the host cell in order to accommodate it's attachment to enterocytes [56].

Stx production

Control of Stx production, and the genes required for Stx production are located on a lambda bacteriophage, unlike the Stx gene in *Shigella dysenteriae*, which is located on the bacterial

chromosome [63]. Stx production is regulated *in vivo* by the iron concentration in bacterial environment. This is done by an upstream functional promotor that is regulated by the transcriptional repressor, Fur, which is iron-dependent [64]. Experimentally, Stx production can also be increased by phage regulators, but this is probably not significant *in vivo* [64].

Stx translocation

In vitro studies have shown that Stx 1 and Stx 2 translocate across intestinal epithelial cell monolayers by different mechanisms [65]. Stx 1 translocation is saturable, requires energy, and can be blocked by colchicine, which inhibits cytoskeleton microtubule formation that is required for many endocytic pathways [65-67]. These findings suggest that Stx 1 translocates in a transcellular manner. Stx 2 translocation, on the other hand, does not require energy, and is not blocked by colchicine. This suggests that Stx 2 translocates by a paracellular pathway [65]. However, these studies were done *in vitro* in polarized tissue cultures, and may or may not represent the *in vivo* translocation of Stx.

Stx trafficking

The Stx's, like several other toxins including ricin, a plant toxin from castor beans, and cholera toxin, exert their toxic activity in the cytosol of the affected cell [24]. Stx works by enzymatically cleaving a specific purine base on the 28s rRNA, which blocks protein chain elongation by preventing the peptide elongation factors EF-1 and EF-2 from interacting with the rRNA [17, 68, 69]. This rapidly shuts down protein synthesis and results in lethal or sublethal cell injury. The cell death that occurs in Stx 1nduced lethal cell injury is thought to be apoptotic [70].

Trafficking of Stx within the target cell is retrograde through the Golgi apparatus to the ER [24, 71], and is dependent on Rab11 and Rab6a', which are GTP binding proteins [24]. Once the toxin reaches the ER, it translocates to the cytosol [24]. In order to become active, the A subunit of Stx 1s cleaved by an enzyme called Furin [72], which is also responsible for activating several other protein toxins [73]. Furin is a serine protease that is present in most cell types, and is located primarily within the trans-Golgi network, where it cleaves precursors of some secretory membrane-bound proteins [74].

EHEC carriers

E. coli O157:H7 is commonly carried by cattle, which, as adults do not exhibit clinical signs of infection and are a large reservoir of infection [75]. Although *E. coli* O157:H7 is not pathogenic for adult cattle, it colonizes the ileum and large intestine in young cattle (< 3 weeks), causing A/E lesions and bloody diarrhea [76]. Pruimboom et al. showed that cattle lack vascular receptors for Stx, which likely explains the lack of systemic disease seen in infected adult cattle [47]. Recent studies have shown that the bacteria are carried in adult cattle on the skin of and immediately surrounding the anus [77]. There is also evidence that the bacteria are carried in the mouth of cattle. A recent report shows that ropes tied to fences at locations where cattle can easily access and chew become contaminated by *E. coli* O157:H7 in carrier herds [78]. These ropes can be useful for determining the *E. coli* O157:H7 infection status of a herd. Additionally, watering troughs of herds carrying *E. coli* O157:H7 also become contaminated [79]. Shedding of *E. coli* O157:H7 is variable throughout year, with increased shedding occurring during the warmer months, which correlates with increased human infections at this time [80].

In addition to cattle, EHEC's are found in sheep, goats, and pigs. Some strains of STEC have been isolated from avian species, although Vero cell cytotoxicity, a test that detects the presence of Stx, in these strains is rare, and none were positive for eae [81]. In the United States, the main STEC's in pigs are O139, O138, O141, and O147, which produce Stx 2e and causes edema disease [82], although *E. coli* O157:H7 has been isolated from pig herds. In other countries, like Japan, the incidence of *E. coli* O157:H7 in pigs is actually higher than the incidence in cattle. Among non-domestic animals, *E. coli* O157:H7 has been found in the feces of deer that co-graze with cattle at a prevalence of 2.4%, suggesting that deer may also be a carrier [83].

EHEC transmission

EHEC's, including *E. coli* O157:H7 are usually transmitted to humans through the food supply, although there have been outbreaks that were associated with farm visits, and petting zoo visits, as well as person to person transmission (fecal-oral) within families, at daycare centers, in nursing homes, and at swimming pools. Within the food supply, the most common source of human infection, and the most well known is under-cooked hamburger meat. Hamburger is especially dangerous because of the large number of animals that may go into a single batch of meat, and the fact that the grinding process mixes the bacteria throughout the meat, where it is difficult to detect and is protected from surface heat that kills bacteria on other cuts of meat, such as steak. The economic impact of *E. coli* O157:H7 in the food supply can be tremendous. Since the U.S. government has adopted a "zero tolerance" attitude toward *E. coli* O157:H7, numerous large recalls of meat have occurred throughout the U.S. In 2000, 2,631,180 pounds of hamburger were recalled in the United States and

Canada after being found to be contaminated with *E. coli* O157:H7. The average recall was for 138,483 pounds of meat [84]

In addition to hamburger, EHEC's have been isolated from unpasteurized milk and cheese products, unpasteurized juice products, fruit, contaminated local water supplies, and fresh vegetables. In Japan, 526 school children and 35 adults were infected by eating at a contaminated salad bar [14].

Person to person transmission within families is common, and many times family members that carry *E. coli* O157:H7 are asymptomatic. Detection of infected family members by fecal examination showed an approximately 30% infection rate among parents of infected children and a 45% infection rate among siblings of infected children, however, another study found Stx to be bound to polymorphonuclear white blood cells (PMNs) in 82% of household members, vs. 21% who were positive by stool and serum testing [85].

EHEC control measures

Current control measures to detect *E. coli* O157:H7 in the food supply and prevent spread include random cultures, PCR, and ELISA tests of the meat supply, washing beef carcasses at slaughter, and adequate chlorination of municipal water supplies and swimming facilities. Perhaps the most important control measure is public education. The majority of cases of *E. coli* O157:H7 infection in the U.S. could be prevented by adequately cooking hamburger meat to an internal temperature of 158° F.

Intestinal barrier function

Introduction

The gastrointestinal (GI) tract faces the complex, amazing, and contradictory task of absorbing the fluids and nutrients required for the maintenance of an organism, while preventing absorption of bacteria and bacterial toxins and by-products. This occurs in the most bacterially contaminated environment of the body, an environment that contains approximately 10^{12} organisms per ml of contents in the large intestine, and typically has over 30 genera and over 500 species of identified bacteria [86]. In order to perform it's functions, the GI tract has evolved a complex set of mechanisms that allow for nutrient and fluid absorption and prevent, for the most part, absorption of harmful substances and colonization by harmful organisms.

The mucosal barrier consists of a collection of simple and more complex mechanisms to prevent colonization and bacterial by-product absorption. From the mouth to the anus, the alimentary canal secretes fluids and mucous that help to flush would be colonizers away from mucosal surfaces. In addition, there is a rapid turnover of cells in the GI tract, so that cells that do get colonized rapidly slough and are replaced by new cells. The stomach in monogastrics, and the abomasum in ruminates maintain an acid environment that kills most of the flora in incoming foods and from the mouth. In the small intestine, enzymes are added to the ingesta to aid in digestion, but many of these enzymes also have bacteriocidal and/or bacteriostatic activity. In addition, bile salts, goblet cell secretions, and secretory IgA are added to the ingesta, and these also have bacteriocidal and/or bacteriostatic activity.

Intestinal cell types

Crypt cells

There are multiple types of cells that line the alimentary tract, and each of these cell types contributes to barrier function. Crypt cells, so named because they line the small pits or crypts within the intestine, are the pluripotent mitotically active cell population within the intestine. They give rise to two populations of cells, one of which produces absorptive enterocytes, and the other of which produces the secretory cells of the intestine, including goblet cells, enteroendocrine cells, and Paneth cells [87]. The basal rate of crypt cell mitosis allows for complete turn over of the absorptive enterocytes in the intestine approximately every 3 – 5 days under normal conditions [88]. Intestinal injury stimulates crypt cells to proliferate more rapidly in order to replenish the damaged absorptive cells.

Absorptive enterocytes

The most numerous cell type in the small intestine is the absorptive enterocyte. These are the major absorptive cells in the small intestine, and, as mentioned above, are derived from the crypt cells. These cells have numerous (~600) microvilli along the luminal border and are anchored to the basement membrane of the intestinal villi [89]. The microvilli increase the surface area of the apical membrane of the cell by a factor of 20 [89]. Combined with intestinal fold, villi, and microvilli, the surface area of the small intestine is increased about 600 fold, so that the surface area of the average human small intestine is equal to the surface area of a tennis court [89]. Numerous receptors for amino acids and monosaccharides are located along the microvilli of intestinal epithelial cells, which allow for transcellular absorption of digested food materials [89]. In addition, there are numerous Na/K pumps that allow the cell to move electrolytes, and thus fluid, in and out of the cell.

Paneth cells

Paneth cells are intestinal epithelial cells that are located in the crypts and contain eosinophilic secretory granules. These granules contain alpha defensins, lysozyme, and secretory phospholipase A_2, as well as antimicrobial peptides [90]. The granules are released from the apical surface of the Paneth cells by regulated exocytosis [91, 92]. Secretion of the granules can be stimulated by cholinergic agonists [92]. Paneth cells mature as they migrate down into the crypts, and have a lifespan of approximately 20 days [93].

Enteroendocrine cells

Enteroendocrine cells are formed from the same progenitor as Paneth cells [87], and are scattered throughout the intestinal mucosal cells. They function by producing and releasing certain hormones, such as glucagon-like peptides [94], serotonin, secretin, and substance P [95]. These cells have been shown, in mice, to have at least 15 regionally distributed subsets, based upon their primary secreted product [95].

Goblet cells

The mucous layer within the GI tract is produced by goblet cells. These cells originate from crypt cells as do the absorptive epithelial cells. Goblet cells are located along the villi throughout the GI tract. Histologically they are cup or goblet shaped, as their name suggests, and are filled with mucinous material that stains light blue to grey in H&E stained sections. Mucous within the GI tract acts as a lubricant to ease the passage of ingesta, and also has a protective role. In the stomach, the mucous layer helps prevent damage to the gastric mucosa in the acidic environment. In the small intestine, the mucous layer helps prevent autodigestion by the enzymes released from the exocrine pancreas. In addition, the glycoprotein structure of the mucous layer acts as a trap for bacteria, providing myriad

binding sites for flagella and other bacterial components. This allows the bacteria, in many cases, to be swept away with the mucous layer during normal peristalsis. However, in some instances, the mucous layer can actually act as the initial foothold that allows the bacteria to colonize the intestinal mucosa [86]. An example of this is the oligomassosides of mucin the binds a type I pili produced by certain strains of E. coli O157:H7 [96].

Barrier function and protection

Tight junctions

Intestinal epithelial cells are joined laterally by "tight junctions", which are electron-dense areas at the lateral margins of the cells that are tightly interdigitated. Tight junctions are formed by at least two types of proteins that are membrane spanning, occludin and claudin. There are at least 20 different members of the claudin family of proteins, all of which are involved in tight junction formation. The occludin and claudin proteins are anchored within the cytoplasm to the ZO-1 and ZO-2 proteins, which in turn are connected to actin filaments. The extra-cellular portions of occludin and claudin form loop structures that interdigitate and presumably act like a zipper (reviewed in [97, 98]). Since these molecules are anchored to the ZO proteins, which in turn are connected to actin molecules, tight junction permeability can be modulated by traction on the associated actin molecules [99, 100].

The tight junctions are the major site of fluid secretion and absorption in the intestine. The fluids move back and forth following relative electrolyte concentrations. Absorption through tight junctions is called "para-cellular" absorption. Tight junctions are also the sites through which inflammatory cells transmigrate through the mucosa into the intestinal lumen [101].

Although tight junctions are permeable to fluids, under normal circumstances, bacterial toxins are not allowed through [99].

Innate Immunity & Antimicrobial peptides

The intestinal mucosa protects itself from pathogens with a variety of innate immune factors, including pH, mucus, commensal microflora, and peristalsis as well as secreted products such as bile and pancreatic enzymes. More recently, endogenous peptides with antimicrobial properties have been discovered [86]. These peptides are divided into the alpha defensins, the beta defensins, and cathelicidins. They work by inserting themselves into the membranes of their target cells and forming pores that lead to rapid lysis of the cell [102]. The defensins currently has the greatest number of known family members. Defensins are peptides that contain 29-35 amino acid residues, are cationic, arginine-rich, and contain 3 disulfide bridges formed by 6 cysteines [103]. The defensins are divided into alpha and beta defensins, based upon the pattern of the disulfide bonding [86]. Alpha defensins include Human Defensin 5 (HD-5) and Human Defensin-6 (HD-6), and expression appears to be limited to the Paneth cells [86]. The beta defensins including LAP, on the other hand, are expressed more widely throughout the GI tract. In humans, hBD-1 and hBD-2 are expressed in the GI tract, as well as in the lung and other epithelial tissues. Some defensins, such as HD-5 and hBD-1, are constitutively expressed, while expression of others, such as HD-6 and hBD-2, are inducible [86]. The activity of defensins is tightly regulated by the salt concentration in the microenvironment, and is thus active at the borders of the intestinal epithelial cells where it is secreted, but not in the lumen of the gut, where salt concentrations are lower. In this way, beta defensins act as border guards that attack only those bacteria that closely approach the intestinal mucosa.

Toll-like receptors

Intestinal epithelial cells have Toll-like receptors (TLRs) that recognize certain highly conserved bacterial components known as pathogen associated molecular patterns (PAMPSs), and are involved in identifying bacterial invaders and inducing responses in the intestinal epithelial cells [104]. TLR's were discovered in Drosophila, and appear to be conserved throughout evolution. TLR's are transmembrane proteins. The extra-cellular portion contains numerous leucine-rich motif repeats, which are common to other pattern recognition proteins. The cytoplasmic portion of the protein is capable of triggering intracellular signals via homology to the IL-1 receptor [105]. TLR activation has been shown to result in induction of IL-1, IL-6, IL-8, IL-10, IL-12, and TNF alpha [105]. Through the activation of IL-12 and subsequent Th response, TLR's may form a bridge between the innate and the adaptive immune system [105].

Adaptive immune system

The adaptive immune system also plays a role in mucosal barrier function and mucosal protection. The adaptive immune system consists of lymphocytes, plasma cells, and antigen presenting cells. The intestinal adaptive immune system, like the intestinal tract itself, has a somewhat contradictory function. It must respond to non-self antigens in order to protect the system, but at the same time, must modulate its' responses so that it does not over react to the molecules and flora that are normal residents of the intestinal tract. This is a fine balancing act that can have devastating consequences if it tips too far in either direction. Over reaction to normal flora and GI contents is thought to be the underlying cause of Crohns disease and ulcerative colitis in humans, as well as inflammatory bowel disease (IBD) syndrome in cats and dogs. Under reaction of the intestinal immune system can lead to chronic diarrhea, mal-

absorption, sepsis, and even death of the individual. The mechanisms of control of the adaptive immune system in the GI tract is beyond the scope of this literature review, but the contribution of immune system to mucosal barrier function will be briefly addressed. The adaptive immune system ultimately produces effector cells that kill targeted organisms or produce proteins, i.e. immunoglobulins that bind to targeted organisms or molecules. Immunoglobulins activate other attack mechanisms, such as the complement cascade, and opsonize the molecule or organism for phagocytosis by the innate immune system cells. Within the GI tract, immunoglobulin A (IgA) is secreted as a dimer that is linked at the Fc portion of the immunoglobulin. IgA functions to specifically target pathogenic bacteria and products for degradation.

Intestinal repair

It is inevitable that the intestinal mucosa will be damaged while performing its absorptive and mucosal surveillance functions. The same characteristics that allow the GI tract to absorb nutrients and fluids (increased surface area, single cell layer mucosa, etc.) make it prone to mechanical, chemical, and biological damage. In the contaminated environment of intestinal tract, it imperative that any damage to the mucosa be repaired as quickly as possible in order to prevent or limit translocation of unwanted substances into the body.

There are several mechanisms that the intestinal tract uses to rapidly repair damage. When ulceration of the intestinal mucosa occurs, the villi rapidly contract, reducing the villous surface area. At the same time, undamaged epithelial cells flatten out and migrate along the exposed basement membrane to cover ulcerated areas in a process called restitution. Following injury, crypt cell mitosis increases and production of intestinal epithelial cells

speeds up, providing cells to replace the damaged cells. The villous will eventually relax and return to its' normal size after the defect is repaired. Damaged intestinal epithelium releases a variety of cytokines, including IL-8, and TNFa, and CXC chemokines, as well as prostaglandins. Prostaglandins produced by the injured tissues contribute to the contraction of villi and the epithelial cell restitution mentioned above [106]. Argenzio et al., showed that prostaglandins also stimulate barrier function recovery by causing tight junctions to close. The prostaglandins apparently accomplish this by increasing the secretion of chloride (Cl^-) and inhibiting reabsorption of sodium (Na^+). This induces an osmotic gradient that is associated with decreased tight junction permeability [107, 108]. In fact, it is the prostaglandin mediated closure of tight junctions that restores resistance to injured intestine in Ussing chambers rather than restitution of the intestinal epithelium [109]. In addition to their functions in restoring mucosal barrier function, the cytokines and chemokines induce an inflammatory response, up regulate the innate and adaptive immune systems, and are chemotactic for a variety of inflammatory cells, including neutrophils and macrophages. These cells defend the border against possible invading organisms and substances while the mucosal barrier function is reduced. In some instances, though, the inflammatory cells can cause collateral damage.

Host Inflammatory Response

General Inflammation Information

Inflammation is a response of living tissues to physical, chemical, or biologically induced damage. The classic inflammatory response, first described by Cornelius Celsus as the

cardinal signs of inflammation in the first century CE, includes increase in temperature (calor), redness (rubor), swelling (tumor), and pain (dolor). The fifth cardinal sign, loss of or decrease in function, is attributed to Galen in the second century CE, but many people believe it originated with Rudolf Virchow in the nineteenth century CE [110]. The inflammatory response has been called a surface oriented response because the process, from sensing damage, to initiating cellular responses, to inflammatory cell migration, takes place at the cell surface or through receptors and ligands expressed on the surface of cells. Inflammation is primarily a vascular response. The swelling, redness, increase in temperature, and to some extent the pain and loss of function associated with inflammation can be traced to changes in blood flow and vascular permeability that are induced by inflammatory mediators. In response to substance P, histamine, and kinins, small blood vessels dilate to increase the blood flow, while the vascular endothelial cells contract to allow plasma to leak out into surrounding tissues. Likewise, the recruitment of inflammatory cells such as neutrophils and macrophages occur at the vascular level. Vascular endothelial cells, in response to inflammatory mediators such as IL-8 and TNF express E- and P- selectins, which cause circulating inflammatory cells to marginate and roll along the vascular endothelium, where they express adhesion molecules such as β_2 integrins that encounter and bind to intercellular adhesion molecules (ICAMs) that allow the marginated cells to bind more strongly and then migrate through the vessel into the surrounding tissue.

Innate and adaptive immunity

Inflammatory leukocytes can be divided into two classes in regards to their contribution to immunity: the innate immune cells (neutrophils, macrophages, eosinophils, and basophils) and the adaptive immune cells (B and T lymphocytes, and plasma cells). The innate

immunity inflammatory cells are further classified as mononuclear cells (monocytes/macrophages) and polymorphonuclear cells, or PMNs (neutrophil, eosinophil, basophil). Some mammals, such as rabbits, and all avian species have heterophils instead of neutrophils, based upon the staining characteristics and enzyme inventory of the granules within the predominate PMN population.

Neutrophils

Introduction

The earliest responding inflammatory cell in most mammals, as well as the most numerous, is the neutrophil. Neutrophils are formed in the bone marrow from a pluripotent line of stem cells. Neutrophils have a short functional life, surviving only 48 to 72 hours after entering the circulating pool. If not recruited to an inflammatory site, neutrophils will generally enter the intestine, the lung, or the urogenital tract to die, not unlike the mythical elephant burial grounds in novels by Edgar Rice Burroughs or J. Ryder Haggard.

Neutrophil granules

Neutrophils have several kinds of intracytoplasmic granules that contain different enzymes and proteins. The primary granules are the first to appear, and can be found in the promyelocyte stage of myelogenesis. These granules contain myeloperoxidase (MPO), defensins, and proteinase 3 (PR3), and stain azurophilic. At the metamyelocyte stage of development, the secondary or "specific" granules appear. These granules contain collagenase, lactoferrin, gelatinase, and other proteins, including bactericidal/permeability-increasing protein (BPI), which is cytotoxic to gram-negative bacteria at nano molar concentrations. Another set of granules known as tertiary or "gelatinase" granules also forms. These granules are much like specific granules, but, as their name implies, they

contain large concentrations of gelatinase. When the neutrophils become mature, they form rapidly mobilizable vesicles called secretory granules that have adhesion molecules on their membranes and contain PR3 in addition to plasma proteins that are presumably acquired by endocytosis. In addition, secretory granules contain Cytochrome b_{558}, which is essential for activation of the NADPH, an enzyme that produces reactive oxygen species. When neutrophils are stimulated, the granules are secreted in a defined order. Secretory granules are secreted first, followed by tertiary (gelatinase) granules, secondary (specific) granules, and primary (azurophilic) granules. Granules can also fuse with phagocytic vacuoles to kill bacteria that have been phagocytosed by the neutrophil (reviewed in [111]).

In addition to enzymes, neutrophils contain anti-microbial peptides including defensins and cathelecidins that function by forming pores in the cell membranes of many pathogenic bacteria [111]. These peptides work synergistically with BPI. Antimicrobial peptides were discussed in the mucosal barrier section of this review.

Neutrophil migration

As briefly mentioned above, neutrophils are recruited from the circulating pool by activated vascular endothelial cells. When activated, endothelial cells express E-Selectin, and release stored P-selectin and the selectin ligand Sialyl Lewis X (SLX) that is stored in Weibel-Palade bodies on the surface of endothelial cells [112]. Neutrophils constitutively express L-selectin [113]. L-Selectin from the neutrophils binds with the SLX receptor on endothelial cells, while P and E-selectin binds with SLX on the neutrophils. This interaction causes a loose binding that "tethers" cells, and allows them to roll along the endothelial surface of the blood vessel. While undergoing this slow rolling, the neutrophil can bind more tightly to ICAM

molecules. Tight ICAM bonding is mediated through members of the B_2 integrin family, which are composed of a variable alpha subunit (CD11a, -b, or –c) and a common beta subunit (CD18). CD11a/CD18, also known as LFA-1, binds to ICAM1 and ICAM2. CD11b/CD18, also known as MAC-1, binds to ICAM1, ICAM2, fibrinogen, heparin, and factor X. In a mouse model, mice deficient in CD11b/CD18 were able to move neutrophils out of blood vessels, while mice deficient in CD11a/CD18 were not, suggesting that vascular transmigration is more dependent on CD11a/CD18 than CD11b/CD18. In addition to the ICAMs, Platelet/Endothelial Cell Adhesion Molecules (PECAMs) are important in vascular transmigration. PECAMs bind to $alpha_V beta_3$ integrins. In most instances, neutrophils migrate between endothelial cells, disrupting adherent junctions in the process [114, 115]. Endothelial adherent junctions are formed by cadherins, which are transmembrane proteins that homophilically bind to each other and are anchored by the intra-cellular proteins alpha catenin, beta catenin, and plakoglobin. After binding of the neutrophil to the endothelial cell, beta catenin and plakoglobin disappear from the cadherin/catenin complex . Del Maschio raised questions as to whether the junction disruption was caused by proteases secreted by neutrophils, and suggested that the junction disruption was actually due to intra-cellular signaling induced by neutrophil binding to endothelial cells [116]. However, subsequent studies by Moll et al., suggest that this process is mediated by a neutrophil protease rather than a cell signaling event between neutrophils and endothelial cells [117]. Another recent study suggests that rather than being disrupted, the vascular endothelial cell cadherin adherent junctions were relocated or pushed away from the area of transmigration, although the PECAM junctions were opened during neutrophil transmigration [118].

Whatever the case, neutrophils migrate between, and sometimes through [119] endothelial cells into the surrounding tissue.

Once outside the blood vessels, the neutrophils migrate along the surface of cells or extracellular matrix by extending pseudopodia expressing β_1 integrins and moving in an amoeboid fashion. Neutrophils are chemotactic to small gradients of inflammatory mediators, such as IL-8, as well as to bacteria and bacterial products. The chemotaxis and recognition of bacteria and their products, such as LPS, occurs via Toll-like receptors. These evolutionarily conserved receptors recognize conserved sequences of key bacterial components or metabolites, and are crucial to target recognition by neutrophils and other cell types, including enterocytes, as described previously.

In order to move through tissues and extra-cellular matrices, neutrophils utilize several enzymes including proteases, metalloproteinases, collagenases, and elastases. These enzymes allow the neutrophil to move through inter-cellular junctions, including tight junctions and adherent junctions as previously mentioned. Intestinal epithelial cells release proinflammatory cytokines in response to many stimuli including EHEC infection [120-123], although one recent paper claims that STEC suppresses pro-inflammatory cytokine production via NF-kB suppression that was dependent on EspB [124]. IL-8, which as previously mentioned is released by damaged intestinal epithelial cells and induced and up regulated by EHEC, is strongly chemotactic for neutrophils. However, IL-8 concentration does not form a gradient within intestinal epithelial cells, i.e. the concentration of IL-8 is the same at the basal side of the intestinal epithelial cell as it is at the apical side. This means

that, while IL-8 is important in recruiting neutrophils to the site of intestinal epithelial infection, it does not induce the recruited neutrophils to cross the intestinal epithelial barrier. Neutrophil movement through the mucosal barrier is induced by a gradient of bacterial products [123], as well as by a chemokine called pathogen-elicited epithelial chemoattractant (PEEC) that is secreted apically by intestinal epithelial cells when they encounter pathogenic bacteria [125].

Neutrophil induced damage

While migration of neutrophils and other inflammatory cells is necessary so that they can reach the site of inflammation and defend the host, it increases the vulnerability of the host to certain methods of attack. *In vitro* studies have shown that transmigration of neutrophils across epithelial surfaces, including intestinal epithelial surfaces, increases the permeability of those surfaces to surrounding molecules [101, 126-129]. This disruption of barrier function lasts approximately 12-20 hours and is dependent upon the number of neutrophils that transmigrate [129]. The disruption of barrier function and increase in permeability is related to the disruption of tight junctions by the migrating neutrophils. During this period of increased permeability, *in vitro* studies have shown that Stx 1s one of the molecules that moves more rapidly and easily across the intestinal epithelial barrier, which is not surprising since Stx 2 apparently transmigrates through the mucosal barrier by the paracellular route anyway [65, 130].

When neutrophils reach the site of inflammation, they react to the inflammatory stimuli by engulfing and breaking down the stimulus or by degranulating and releasing their battery of defensive weapons into the affected area. While some of the defensive mechanisms

employed by the neutrophil, such as the defensins, are target specific, somewhat like smart bombs, many other mechanisms, such as lysozyme, the superoxide ions induced by the oxidative burst, etc., are indiscriminate, more like hand-grenades. The non-specific defense mechanisms frequently injure surrounding host cells and tissues, a phenomenon known as "innocent bystander phenomenon", or "collateral damage", to continue the military parallel. This self-inflicted damage, like the damage induced by transmigration, can further degrade the barrier function when the inflammation is occurring on or in close proximity to the mucosal barrier, which is the case in EHEC [131-135].

Animal Models

Several animal models have been used to study EHEC and HUS, including neonatal and older calves, adult cattle, mice, rabbits, ferrets, primates, gnotobiotic pigs, newborn conventional pigs, and grower pigs [46, 55, 57, 58, 62, 75, 76, 136-148]. These models have been reviewed extensively [46, 55, 57, 58, 62, 75, 76, 136-148], and none of the animal models is a perfect correlation with human disease. Each has its' advantages and disadvantages, especially concerning the role of the inflammatory response.

Conclusions

Based upon the observations that 1) EHEC colonizes and is closely adherent to the mucosal epithelium in cases of hemorrhagic colitis [54], 2) hemorrhagic colitis is characterized by a robust host inflammatory response including neutrophilia in most species effected [11, 76, 141-143, 148], 3) host inflammation and specifically neutrophil infiltration and activation increase mucosal permeability [101, 126-132, 134], 4) there is a discordance between fecal Stx titers and risk of HUS [41], but, 5) there is a correlation between inflammatory mediator

concentrations, neutrophil infiltration, and risk of HUS in humans [37, 38, 149], we asked the question "does colonic inflammation, and specifically neutrophil infiltration, affect Stx absorption in EHEC?" In addition, current animal models do not appear appropriate to critically test this question.

Interestingly, it has been shown that EHEC and human neutrophils can modulate the activity of other neutrophils in ways that enhance host disease and, if our hypothesis is correct, could increase the risk of HUS. For example, Stx 1nduces IL-8 and other pro-inflammatory chemokine secretion by intestinal epithelial cells, and induces neutrophilia and neutrophil activation in the host [121, 122, 148, 150]. This is especially important in light of evidence that human neutrophils bind Stx and can transfer Stx to glomerular endothelial cells [151, 152]. Human neutrophils and the products that they secrete, specifically H_2O_2, are known to stimulate Stx production by prophage induction in EHECs [153]. Additionally, Stx has been shown to prolong neutrophil life [154], and induce superoxide production, while impairing phagocytosis [155]. All of these host-pathogen interactions increase inflammation or Stx production. If inflammation increases Stx absorption, then this is a potent combination of events that could increase the likelihood or extent of Stx translocation from the intestinal lumen to the systemic circulation and the subsequent development of systemic disease (i.e. HUS).

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Chapter 2: *E. coli* O157:H7 colonization of 3-day-old pigs does not induce inflammation and lacks detectable Stx absorption

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

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Abstract

Escherichia coli O157:H7, an enterohemorrhagic *E. coli* (EHEC), are bacteria that cause hemorrhagic colitis and the complicating condition, hemolytic uremic syndrome (HUS) in the United States and throughout the world. In EHEC infections, the bacteria colonize the mucosal epithelium and remain within the intestinal lumen where they release Shiga toxins (Stx), which enter the systemic circulation, and can cause the systemic lesions of HUS. Although it is known that HUS is caused by the Shigatoxins Stx 1 and Stx 2, the mechanism of Stx movement from the intestinal lumen to the systemic circulation *in vivo* is poorly understood. It is our hypothesis that the host inflammatory response to EHEC enhances translocation of Stx from the intestinal lumen to the systemic circulation. Newborn pigs (less than 24-hrs-old at time of inoculation) infected with *E. coli* O157:H7 develop vascular lesions similar to those seen in humans with HUS. However, the newborn pig is capable of absorbing macromolecules such as immunoglobulins and Stx until the second or third day of life, which limits its usefulness for studying the mechanism of Stx absorption. In this experiment we infected 2 and 3-day-old suckling pigs with *E. coli* O157:H7 strain 86-24 or

non-pathogenic *E. coli* 123. Our 3-day-old suckling pigs developed typical attaching and effacing (A/E) lesions with *E. coli* O157:H7, but lacked a histologically detectable inflammatory response and systemic signs of Stx absorption. We therefore concluded that the 3-day-old pig can be colonized by *E. coli* O157:H7 without the development of clinical signs suggestive of toxin absorption.

Introduction:

Diarrheal diseases caused by enteropathogenic Gram-negative bacteria are responsible for morbidity and mortality in people who live in both underdeveloped and developed countries including the United States. In the U. S., two common causes of severe diarrheal disease are *Salmonella sp.* and, more recently, enterohemorrhagic *E. coli* (EHEC). These infections are contracted through ingestion of contaminated food products, or by fecal oral inoculation from other infected individuals (11). During the past decade, EHEC's in general and *E. coli* O157:H7 in particular have become recognized as a cause of hemorrhagic colitis and the associated complicating condition, hemolytic uremic syndrome (HUS) (24). Up to 10% of young children affected by *E. coli* O157:H7 may go on to develop HUS, which is the leading cause of acute renal failure in children in the U.S. (7, 24). Shiga toxins 1 and 2 (Stx 1 and Stx 2), produced by *E. coli* O157:H7 are responsible for HUS (8, 16, 18, 23, 29). These structurally similar but antigenically distinct toxins both bind to the Gb_3 receptor via B subunit mediated receptor specificity (16). Studies have shown that the Gb_3 receptor is highly expressed in human glomerular endothelial cells, which likely accounts for their exquisite sensitivity to the Stx (18). Stx functions by enzymatically degrading the ribosome,

thereby halting protein synthesis and leading to rapid cell death (23).

Although it is known that Stx is responsible for HUS, it is not known how the toxin crosses from the intestinal lumen into the systemic circulation to reach the kidneys, brain, and other systemic sites of action *in vivo*. *In vitro* studies have shown the Stx 1 crosses polarized intestinal epithelial cells by a transcellular pathway and that Stx 2 crosses polarized intestinal epithelial cells by a paracellular pathway (1, 14), however, it is not known if these paradigms hold *in vivo*. Evidence that the *in vivo* situation is not this straight forward includes the finding that fecal Stx titers do not correlate with the risk of developing HUS (3), which they should if Stx translocation *in vivo* was simply concentration dependant transcellular and paracellular translocation.

EHEC form intimate, tightly adherent attachments, known as attaching and effacing (A/E) lesions, to the intestinal epithelial cells of affected individuals (21). The bacteria remain in the intestinal tract and release Stx that somehow crosses the intestinal mucosal barrier and enters the systemic circulation, where it causes systemic disease and systemic vascular lesions. Newborn pigs less than 24-hrs of age are susceptible to infection by EHEC and develop intestinal A/E lesions and systemic vascular lesions similar to those seen in humans by 24 to 36 hours after infection (2, 6, 19, 25). This makes neonatal pigs an appropriate animal model for human EHEC infections. However, the intestinal tract of newborn pigs is capable of absorbing certain large molecules, such as immunoglobulins, intact for the first 48 to 72 hours of life (26-28). This process, called passive transfer of maternal antibodies,

provides newborn pigs with passive immunity for the first 2 to 3 months of life (26). Previous studies have used pigs less than 24 hours of age to study the systemic effects of Stx (6, 13). Although this approach has worked well to define the systemic effects of Stx, passive transfer limits the usefulness of the newborn (<24-hr-old) pig in studies of the mechanism of absorption of Stx, since Stx appears to be passively absorbed along with immunoglobulin and other colostral proteins (6).

In order to study the mechanism of absorption of Stx 2 from the intestinal lumen to the systemic circulation in pigs, we determined the effect of EHEC infection in 3-day-old pigs. The advantage of the 3-day-old pig versus the newborn pig model is that by three days, passive transfer of maternal antibodies and other intestinal macromolecules has ceased, and thus is not a complicating factor (26, 27). To the best of our knowledge, EHEC infections in pigs inoculated at three days of age have not been reported. In this study, we demonstrated that 3-day-old pigs were readily colonized by EHEC and developed typical A/E lesions in the cecum, spiral colon, and to a lesser extent, the rectum.

Materials and Methods:

Animals

All procedures and treatments were approved by the Iowa State University Animal Care and Use Committee. Bred York/Duroc cross sows were obtained from Atlantic pig farms in Atlantic, Iowa. The sows were placed in a biolevel 2 holding facility at Iowa State University in Ames, Iowa at least a week before the scheduled farrowing date to allow the

animals to acclimate. At 3 days before the scheduled farrowing date, the pigs were placed in standard farrowing crates. The sows were monitored 5 times per day and allowed to farrow naturally. Farrowing date was designated as either the day the sows were seen farrowing, or the morning that the piglets were discovered with sows that farrowed overnight (last observation time, 9 PM). The average litter size was 10 piglets (range 9-13). One sow delivered 13 piglets. Of these, the three smallest piglets were removed from the study to maintain consistent piglet size.

Piglets were allowed to suckle for the entire length of the experiment. At 2 days (Groups C and D, n=20 pigs) or 3 days (Groups A and B, n=20 pigs) of age the piglets were numbered with a paint stick and inoculated with 10^8 CFU of EHEC *E. coli* O157:H7 strain 86-24, an Stx 2+ and eae+ strain that has been used in studies of EHEC infection in pigs (6). Control litters (n=16 pigs) were inoculated with 10^8 CFU of the non-pathogenic *E. coli* strain 123.

Observations

Throughout the experiment, diarrhea, activity level, and clinical signs of central nervous system lesions (incoordination, head pressing, seizures, and paddling) were recorded daily.

Blood Samples

Blood samples were collected in EDTA and serum tubes by anterior venous sinus venipuncture immediately prior to inoculation and immediately prior to euthanasia at 4 days post-inoculation (day 6 or 7 post-partum respectively). These samples were analyzed for Stx

activity using the Vero cell assay as previously described (9).

Tissue collection

The pigs were euthanized by pentobarbital overdose (Beuthanasia, Schering Plough, Union) on day 4 post-inoculation (day 6 or 7 post-partum respectively). Necropsies were performed on all pigs and samples of jejunum, ileum, cecum, spiral colon, rectum, kidney, and brain were collected in 10% neutral buffered formalin for histologic examination.

Histologic exam

Histologic samples were fixed in 10% neutral buffered formalin for 24 hours and then embedded in paraffin. Paraffin embedded blocks were cut in 5 μm sections, adhered to a glass slide and stained with hematoxylin and eosin (H&E). H&E stained histologic sections were examined for colonization by A/E bacteria, and inflammation (*Figure 1*). Inflammation was defined as edema, congestion, and/or neutrophil infiltration in the lamina propria, intestinal epithelial cells, or intestinal lumen. Loss of apical cytoplasm was judged present when the lumenal cytoplasmic border of enterocytes with adherent bacteria was appreciably recessed as compared to the lumenal cytoplasmic border of adjacent enterocytes without A/E lesions. Colonization was judged to be present in a pig if adherent bacteria that stained for *E. coli* O157:H7 antigen by immunohistochemistry (see below) were present in one or more of the following: ileum, cecum, spiral colon, or rectum.

Immunohistochemistry

E. coli O157:H7 bacteria were detected and identified by immunohistochemical staining using an Optimax Plus stainer (Biogenics, San Ramon, CA) using a modified protocol developed by Dean-Nystrom et al. (4). Briefly, paraffin embedded sections were cut to 5 μm and adhered to glass slides. 5 μm sections were de-paraffinized in xylene and re-hydrated through a series of ethanol/water baths. De-paraffinized sections were incubated with 3% hydrogen peroxide for 30 minutes to block peroxidase activity. The sections were then rinsed in Tris-buffered saline containing 0.05% Tween (TBS-T20) (Sigma, St. Louis, MO). Using a wax pen (Pap-pen, Biogenics, San Ramon, CA), two lines were drawn on the glass slide, one above and one below the section, to prevent excessive spreading of the reagents and tissue drying. The slides were loaded into the slide processor and a previously optimized staining protocol was run (4). The slides were incubated with 1:20000 dilution of affinity purified goat anti-*E. coli* O157:H7 polyclonal antibody (Kirkgaard-Perry, Gathersburg, MD) 2 times for 45 minutes, and rinsed in TBS-T20. Biotinylated rabbit-anti goat antibody was used as a secondary antibody (Kirkgaard-Perry). Signals were developed using the Nova Red substrate system (Vector Laboratories, Burlingame, CA). The slides were counter-stained with hematoxylin. Negative controls were processed using the same technique, but primary antibody in the incubation step was replaced by incubation with TBS-T20. In addition, the normal bacterial flora within the intestinal lumen provided an internal control, since only adherent bacteria and occasional individual bacteria in overlying mattes stained positive.

Immunohistochemically stained sections (3 sections/pig/region of intestine) of ileum, cecum, spiral colon, and rectum were scored for attaching and effacing lesions to determine colonization (*Figure 2*) by the following: 0= no A/E lesions; 1= sections with A/E lesions present and covering 10% or less of the mucosal surface; 2= sections with A/E lesions affecting between 10% and 30% of the mucosal surface; 3= sections with A/E lesions affecting between 30% and 50% of the mucosal surface; and 4= sections with A/E lesions affecting >50% of the mucosal surface.

Results:

Colonization

Four groups of pigs (A, B, D, and E, n = 10, 10, 11, and 9 respectively) were inoculated with *E. coli* O157:H7 strain 86-24. Of these, 26 (65%) had colonization at the time of necropsy (*Figure 3*).The amount of colonization ranged from less than 1% of the section affected to approximately 50% of the section affected. Site of colonization varied by age of pig at the time of inoculation. Pigs inoculated at two days of age were colonized in the cecum and spiral colon, but not the rectum. Pigs inoculated at three days of age were colonized in the cecum, spiral colon, and rectum (*Figure 4*). Pigs inoculated at 2 days of age had greater average colonization than pigs inoculated at three days of age (*Figure 5*). Colonization was not seen in the jejunum or ileum of treatment pigs. Loss of apical cytoplasm in association with colonization was seen in 18/26 (69%) of the colonized pigs. No colonization was seen in control pigs inoculated with *E. coli* strain 123.

Inflammation

Histologically, inflammation was detected in 4/40 infected pigs (10%), but when present was not locally associated with areas of *E. coli* strain 86-24 colonization. The inflammation was characterized by small clusters of neutrophils located within scattered, dilated crypts (crypt abscesses). Inflammation in control pigs was similar in severity and distribution to that seen in the infected pigs.

Clinical signs of systemic lesions

No clinical signs or systemic lesions (gross or microscopic) of Shiga toxicosis were seen in any of the pigs infected with *E. coli* strain 86-24 or controls. Clinical signs in the treatment piglets and controls were limited to a mild, transient diarrhea that started on day 2 or 3 post-inoculation and cleared spontaneously by day 4 post-inoculation. The Vero cell assay did not detect Stx 1n the blood of any pig, whether infected with *E. coli* O157:H7 strain 86-24 or *E. coli* strain 123.

Discussion:

Both 3-day-old suckling pigs, and 2-day-old suckling pigs were readily colonized by *E. coli* O157:H7 strain 86-24 and, by 4 days post-infection, developed attaching and effacing lesions in the cecum, spiral colon, and, depending on the age of the pig at the time of inoculation, in the rectum. In spite of the fact that 2 and 3-day-old pigs were colonized, they did not develop a histologically detectable host inflammatory response, they lacked Stx 1n the blood

at levels detectable by the Vero cell assay, and they did not develop the signs of systemic Stx toxicosis that were seen in pigs inoculated before 24 hours of age (6).

The lack of host inflammatory response in the face of colonization by EHEC in our pigs is puzzling, but is consistent with the results seen in <24 hour piglets (6). The results of this study are consistent with the hypothesis that Stx 1s absorbed along with colostrum in <24 hour old pigs. Dean-Nystrom et al. have shown that suckling newborn pigs given *E. coli* O157:H7 strain 86-24 at less than 24 hours of age develop CNS signs of Stx toxicosis and die rapidly (by 36 hours post-inoculation), sometimes before colonization was detectable (6). By contrast, Caesarian-derived/colostrum-deprived (CDCD or gnotobiotic) pigs inoculated at less than 24 hours of age did not develop systemic signs of Stx toxicosis until up to 72 hours post-inoculation (4-6, 25), suggesting that Stx is being co-absorbed with colostral proteins in the newborn pig.

A possible explanation for the apparent lack of Stx absorption seen in our pigs, if one hypothesizes receptor mediated transfer as an *in vivo* Stx translocation pathway, is a change in receptor expression in intestinal epithelial cells between birth and three days of age, although this does not explain the difference in suckling vs. CDCD pigs seen by Dean-Nystrom et al. (6).

Alternatively, it is possible that other factors such as the host inflammatory response play a role in changes in mucosal barrier function that allow Stx to be absorbed into the systemic

circulation. The intestinal inflammatory response involves the movement of neutrophils and other inflammatory cells into the intestinal lumen. During migration, these cells disrupt the tight junctions between intestinal epithelial cells resulting in increased intestinal permeability to ions, markers such as [^{51}Cr]EDTA, and certain sugars (20, 22). In addition, antimicrobial and proteolytic enzymes released by activated neutrophils can cause "innocent bystander" damage to intestinal epithelial cells, further degrading mucosal barrier function (12). Some studies in human medicine have described a correlation between circulating cytokine inflammatory mediator concentration and the severity of renal lesions in HUS (17), and others have found peripheral neutrophilia to be a risk factor for HUS development in cases of hemorrhagic colitis (10). Hurley et. al have shown that neutrophil migration across polarized intestinal epithelial cells increases Stx translocation *in vitro* (15), and it is possible that neutrophil migration could have a similar effect *in vivo*. Since neonatal pigs have relatively low numbers of neutrophils, and our pigs apparently lacked a host inflammatory response, they may have been resistant to Stx translocation. This explanation is consistent with Hurleys findings that Stx movement across polarized intestinal epithelial cells *in vitro* is enhanced by the movement of neutrophils across the cells (15). Hurley and Acheson speculated that paracellular movement of Stx 1s increased by neutrophil mediated temporary disruptions *in vitro* (15). We hypothesize that a similar mechanism may occur *in vivo*.

The apparent lack of a host inflammatory response makes the 3-day-old pig a good model to study the effect of induced inflammation on Stx translocation. A method of reliably inducing an inflammatory response in the cecum and colon of young piglets would allow *in vivo*

comparison of Stx absorption across inflamed vs. non-inflamed intestinal mucosa.

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List of Figures

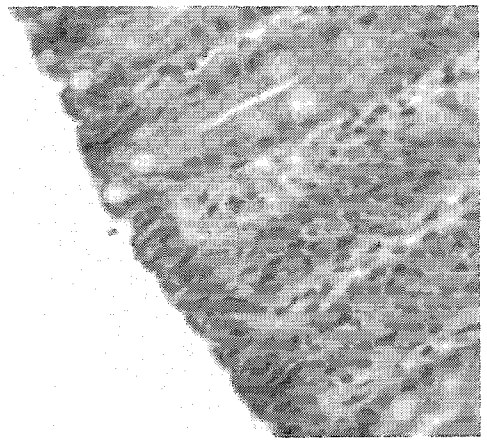

Figure 1. Cecum of a pig infected with *E. coli* O157:H7 attaching and effacing (A/E) lesions 4 days after inoculation. Note the lack of inflammation associated with A/E colonization. H&E stain.

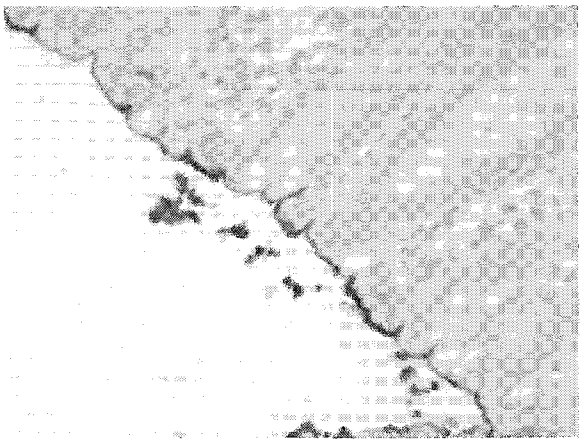

Figure 2. Section of cecum from a pig infected with *E. coli* O157:H74 days after inoculation. The dark brown staining along the margin is antigen of *E. coli* O157:H7 in sites of attaching and effacing (A/E) lesions. Hematoxylin counterstain.

Group	Treatment	A&E	Inflammation	Cytoplasm Loss	Clinical Signs of Systemic Lesions	Blood Stx
A	8624	9/10	4/10	6/10	0/10	0/10
B	8624	3/10	0/10	1/10	0/10	0/10
C	123	0/10	2/10	NA	0/10	0/10
D	8624	9/11	0/11	7/11	0/11	0/10
E	8624	5/9	0/9	3/9	0/9	0/10
F	123	0/10	0/10	NA	0/10	0/10

Figure 3. Number of pigs in each treatment group (A-F)(see Materials and Methods) that have attaching and effacing (A/E) lesions, inflammation detectable by light microscopy, intestinal epithelial cytoplasm loss secondary to A/E lesions, and detectable blood shiga toxin (Stx) via Vero cell assay.

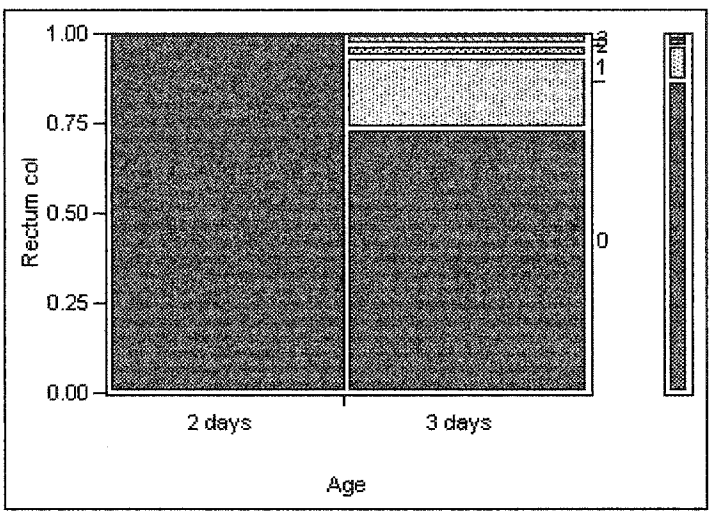

Figure 4. *E. coli* O157:H7 attaching and effacing (A/E) colonization score in the rectum of pigs based upon age at time of innoculation. Pigs inoculated at 2 days of age did not have A/E lesions in the rectum, while pigs inoculated at 3 days of age had A/E lesions in the rectum. The difference is significant (p=0.026). The colors represent the proportion of animals with each colonization score (0-5)(see Materials and Methods).

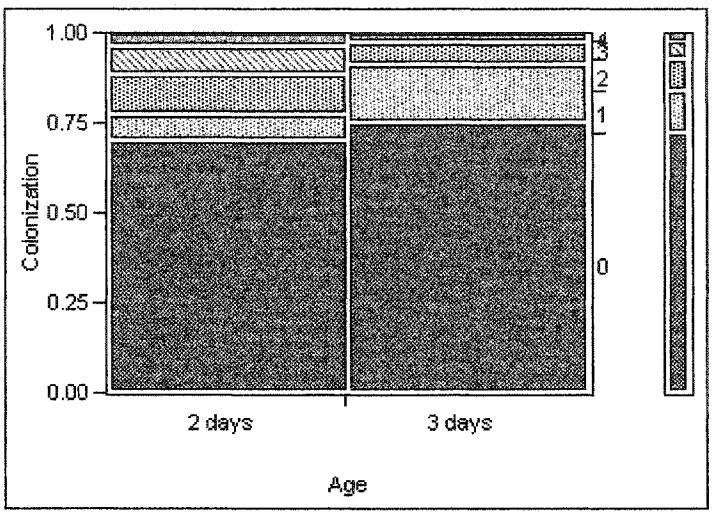

Figure 5. Colonization scores of *E. coli* O157:H7 of pigs based upon age (2 & 3 days) at time of innoculation. The colors represent the different colonization scores (0 - 4)(see Materials and Methods). Two day old pigs had significantly greater colonization scores (p=0.06) versus three day old pigs.

Chapter 3: DSS induces suppurative and ulcerative inflammation in the large intestine of colostrum-fed neonatal pigs.

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Abstract

Previously, we have developed a 3-day-old pig model of *E. coli* O157:H7 infection in which pigs are colonized by *E. coli* O157:H7 and develop classic attaching and effacing type lesions, but develop neither a microscopically detectable host inflammatory response nor systemic signs or lesions of Shiga toxin (Stx) absorption. A method of reliably inducing a host inflammatory response in the large intestine of neonatal pigs is needed to test the hypothesis that the host inflammatory response may influence absorption of Stx. Dextran Sulfate Sodium (DSS) induces suppurative inflammation and ulceration in mice, rats, and guinea pigs. In this study, we showed that DSS at concentrations of 2.5% in 20 mls of milk replacer consistently induced fibrinosuppurative inflammation and ulceration in the ileum, cecum, and large intestine of neonatal pigs in a time and dose-dependent manner by 3 days post ingestion. DSS consistently induced hemorrhagic colitis by 4 days post-ingestion (8/8 pigs), which became more severe by 6 days, at which time the pigs had severe suppurative inflammation and ulceration in the cecum, spiral colon, and rectum. Similar changes were seen in the ileum but were limited to the mucosal epithelium that covered the Peyer's patches. This work demonstrates that DSS consistently induces acute inflammatory lesions in the

large intestines of pigs.

Introduction:

Diarrheal diseases are an important cause of morbidity and mortality in both developed and developing countries throughout the world. It is estimated that every year, 2.1 million children under the age of 5 die of diarrheal diseases worldwide. In the United States and other developed countries, many people suffer from non-infectious diarrheal diseases, such as Crohn's disease and other chronic inflammatory bowel diseases. A better understanding of the mechanism of bowel inflammation and it's contribution to diarrheal diseases is essential to developing treatments and cures for both infectious and non-infectious diarrhea. Animal models of acute and chronic diarrheal disease currently include the mouse [9], the rat [3], and other animals [4]. In several animal models, Dextran Sulfate Sodium (DSS) has been used to induce bowel inflammation and ulcerative colitis. Studies have shown that DSS given in the drinking water induces suppurative and ulcerative colitis in mice [2,9], rats [3], and guinea pigs[6]. In addition, several studies suggest that the ulcerative colitis induced by DSS in rats and mice can be attenuated by suppressing neutrophil function and/or infiltration [8,18]. Since our previously developed 3-day-old pig model lacks both a histologically detectable host inflammatory response and systemic signs of Stx absorption [12], a method of reliably inducing hemorrhagic colitis in neonatal pigs would be ideal to study the role of the host inflammatory response to Stx absorption in *E. coli* O157:H7 infection and could be useful as a model of other inflammatory bowel diseases. In this study, we tested the ability of various doses of DSS to induce inflammation and ulceration in the large intestine of neonatal pigs. The purpose of this study was to: 1) determine if DSS caused intestinal inflammation in pigs, 2)

characterize these changes, and 3) determine the extent to which DSS-induced inflammation alters mucosal permeability.

Materials and Methods:

All procedures were approved by the Iowa State University Animal Care and Use Committee. Neonatal Yorkshire/Durok cross pigs (<24 hrs) were obtained from a local producer (Laboratory Animal Resources, ISU). The pigs were farrowed in crates and allowed to suckle prior to removal within 12 hours from the sow in order to obtain colostral antibodies through passive transfer. Pigs were placed in stainless steel cages in a climate-controlled room and cages were maintained at 94° F. The pigs were fed Esbilac (Pet Ag) puppy milk replacer.

Initial range finding studies of 16 pigs showed that DSS dosages of 1% in 20 ml milk 2 times daily did not consistently induce inflammation by 6 days of dosing, and that DSS dosages of 7.5% twice daily consistently induced 100% ulceration with severe inflammation in pigs by 6 days of dosing (data not shown). Additionally, time trial studies showed that histologic inflammation started appearing between days 2 and 4 in pigs dosed with greater than 1% DSS 2 times daily (data not shown).

Treatment pigs were randomly divided into low dose and high dose DSS groups. Low dose groups received 2.5% DSS in 20 ml milk replacer 2 times daily (n=2) or 5% DSS in 20 ml milk replacer once daily (n=2) starting at day 2 of age. High dose pigs received 5% DSS in 20 ml milk replacer 2 times daily (n=2) or 7.5% DSS in 20 ml milk replacer once daily (n=2) starting at day to of age.

The pigs were monitored for food intake, activity level, diarrhea, grossly visible fecal blood, and hydration status. The average daily intake was normalized by considering the intake on the first day of dosing as 100% for each group. The pigs were dosed in the evening on the first day, so dosing did not affect intake for that day. Subsequent daily intake was compared to the intake from the first day of dosing.

Pigs were euthanized at 6 days after the start of DSS treatment. Complete necropsies were performed and samples of jejunum, ileum, cecum, spiral colon, and rectum were collected in 10% neutral buffered formalin for histologic examination.

At necropsy, the gastrointestinal (GI) tract was evaluated for signs of enteritis including spiral colon edema, mucosal hemorrhage, and intestinal discoloration. Sections of ileum, cecum, spiral colon, and rectum (3 sections of each tissue per pig) were evaluated histologically and were subjectively scored for degree of inflammation and ulceration. Inflammatory changes were scored as follows: 1=minimal infiltration of inflammatory cells, 2=mild infiltration of inflammatory cells, 3=moderate infiltration of inflammatory cells, 4=severe infiltration of inflammatory cells. Degree of ulceration was scored as follows: 0=ulceration of less than 1% of the mucosal surface, 1=ulceration of from 1-20% of the mucosal surface, 2=ulceration of 21-40% of the mucosal surface, 3=ulceration of 41-60% of the mucosal surface, 4=ulceration of 61-80% of the mucosal surface, 5=ulceration of greater than 80% of the mucosal surface.

Statistical analysis was performed using JMP 5.0.1a statistical analysis software. Parametric data was analyzed using analysis of varience (ANOVA). Non-parametric data was analyzed using non-parametric ANOVA.

Results:

DSS reliably induced grossly visible fecal blood by 4 days post inoculation at concentrations of 2.5% and greater in all pigs. The amount of fecal blood increased from day 4 on in all pigs (n=8). The appetite and activity level of the pigs decreased starting at day 3 post-DSS dosing and declined as long as pigs were dosed with DSS. By day 7, all treated pigs were dehydrated, weak, and lethargic with poor appetites (Figure 1).

At necropsy, the spiral colons of all treatment pigs were variably edematous with red to dark purple discoloration of the serosal surface (Figure 2). The serosal surface of the cecum and rectum were similarly discolored in treatment pigs, and there was consistent, but less severe edema of the mesorectum.

Histologically, an inflammatory infiltrate consisting mostly of neutrophils with fewer macrophages, lymphocytes, and plasma cells within the lamina propria, mucosa, and intestinal lumen of the ileum, cecum, spiral colon, and rectum of all pigs that received DSS. The degree of neutrophil infiltration varied by site and dosage of DSS in the ileum and spiral colon.

In low dose groups that received DSS 2.5% BID or 5.0% SID in 20 ml milk replacer, the

cecum and large intestinal sections were characterized by infiltration of neutrophils, macrophages, lymphocytes, and plasma cells into the lamina propria. There was villous atrophy throughout the sections with transmigration of neutrophils across flattened mucosal epithelium. In some areas, the mucosal surface was ulcerated and covered by a mixture of neutrophils, fibrin, and necrotic cell debris that also extended over non-ulcerated areas in some sections (Figure 3)(Figure 4). The villous atrophy and inflammation in the ileum was limited to the mucosa directly above the Peyers patches. No lesions were seen in the jejunum.

The high dose groups received DSS 5.0% BID or 7.5% SID in 20 ml milk replacer. In this group, the sections were characterized by near 100% ulceration and necrosis of the mucosal surface. The luminal surface of the intestine was composed of a thick layer of necrotic cell debris, fibrin, and inflammatory cells (fibrino-necrotic membrane) that replaced the entire lamina propria and extended almost to the tunica muscularis in some sections (Figure 5)(Figure 6). As was seen in the low dose group, the changes in the ileum were limited to the mucosa directly above the Peyers patches. The jejunum lacked lesions throughout the experiment.

The high dose DSS group had significantly greater ulceration ($p<0.0001$) (*Figure 7*) and significantly greater inflammation ($p<0.0001$) (Figure 8) than the low dose group.

Additional studies:

After determining an initial concentration of 2.5% DSS, based upon the results of this experiment (below) 48 additional pigs have been treated with 2.5% DSS in 20 ml of milk

with similar results.

Discussion:

Dextran Sulfate Sodium (DSS) reliably induces suppurative and ulcerative inflammation in the cecum, spiral colon, and rectum of neonatal pigs by 4 days of treatment, in a concentration and time dependent manner. The changes induced by DSS in neonatal pigs in this experiment were similar to those described in rats, mice, and guinea pigs, and included inflammatory cell infiltrates, mucosal cell death, ulceration, and crypt dilation. DSS 2.5% in 20 ml milk replacer twice daily reliably induced hemorrhagic colitis in all neonatal pigs by 3 days post-inoculation. The DSS induced colitis was characterized by severe suppurative inflammation, ulceration, and hemorrhage of the cecum, spiral colon, and rectum. Changes in the small intestine were less severe than those in the large intestine, and were limited to the areas directly above the Peyer's patches in the ileum, but were otherwise similar to the large intestinal changes. The degree of inflammation and ulceration between the high dose and the low dose DSS groups was significant ($p<0.0001$ for both inflammation and ulceration).

In the previously described hosts, DSS was added to the drinking water. Due to the expense of DSS, it was not feasible to add the material to all of the fluid (milk) that was consumed by the pigs. However, we found that DSS worked well when mixed in a relatively small volume of milk (20 mls) and administered twice daily.

The dose of 2.5% DSS twice daily induced a considerable amount of inflammation and ulceration without destroying the entire mucosal surface, as happened in the higher doses.

The effective concentration of DSS used in our studies (2.5%) was similar to the concentrations used in studies on guinea pigs (3%), but somewhat lower than the concentrations used in rats (4%), and mice (5%) [6,9,17].

In previous studies, we have shown that 3 day old piglets infected with *E. coli* O157:H7 develop classic attaching and effacing lesions, but lack both a host inflammatory reaction to the infection and systemic signs and lesions of Shiga toxin absorption [12]. By using DSS to induce hemorrhagic colitis in the neonatal pig, we plan to study the contribution of the host inflammatory response to Stx absorption in piglets.

When neutrophils migrate across mucosal surfaces in the intestine, the tight junctions between the mucosal cells, which provide much of the barrier function of the intestinal mucosa, are temporarily disrupted either by proteases within the neutrophil or by intracellular mechanisms within the mucosal cells that are induced by the signals from the neutrophil [1,11,13,14,16]. *In vitro* studies have shown that neutrophil migration across polarized mucosal cell cultures disrupts the tight junctions, leading to increased mucosal permeability [5,13]. In addition, areas of mucosal ulceration are known to allow increased movement of electrolytes and other materials between the underlying lamina propria and the intestinal lumen, although mucosal ulceration and villous contraction may also decrease bacterial attachment in EHEC infections. It seems reasonable to assume that ulceration and inflammation related disruption of mucosal barrier function may contribute to increased Stx translocation from the intestinal lumen to the systemic circulation in *E. coli* O157:H7 infections. Indeed, several studies have shown that increased peripheral neutrophilia, increased circulating pro-inflammatory

cytokines, and increased fecal excretion of neutrophils are associated with an increased risk of developing HUS in humans [7,10,15].

Acknowledgments:

I would like to thank the Iowa State University Laboratory Animal Medicine staff for their excellent animal husbandry.

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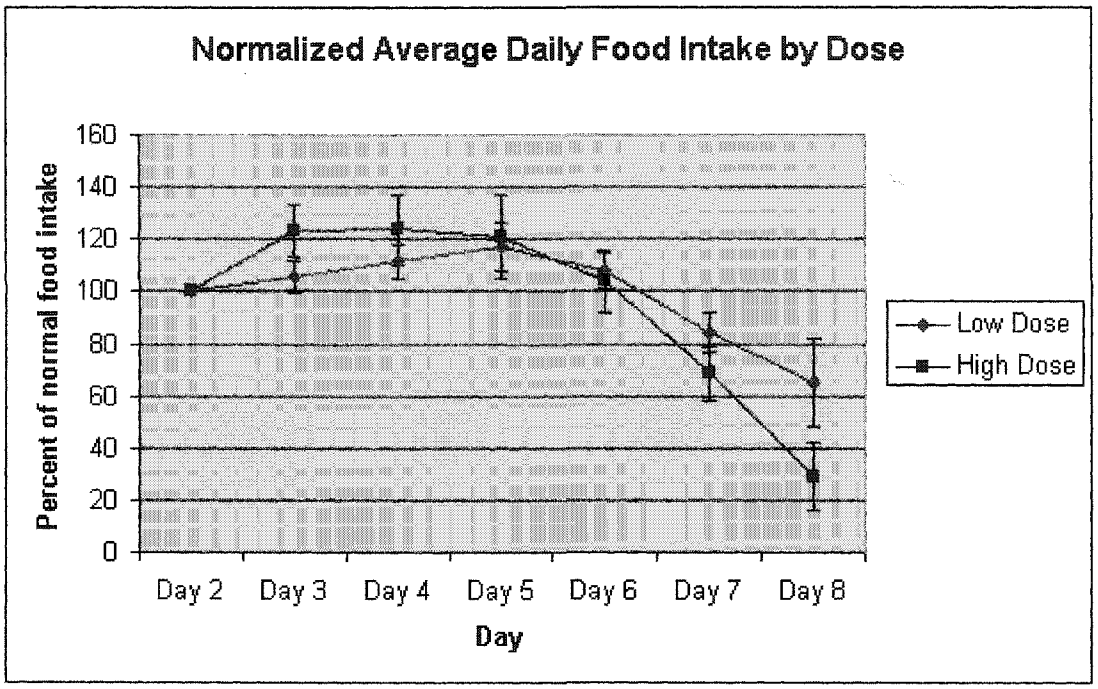

Figure 1. Daily food intake normalized to first day of treatment (see Materials and Methods). The high dose group recieved dextran sulfate sodium (DSS) 5.0% two times daily (n=2) or DSS 7.5% once daily (n=2). The low dose group received DSS 2.5% two times daily (n=2) or DSS 5.0% once daily (n=2). The error bars represent the standard error of the mean. There was a trend for high dose treatment pigs to consume less food by the end of the experiment, but the trend was not significant (p=0.13).

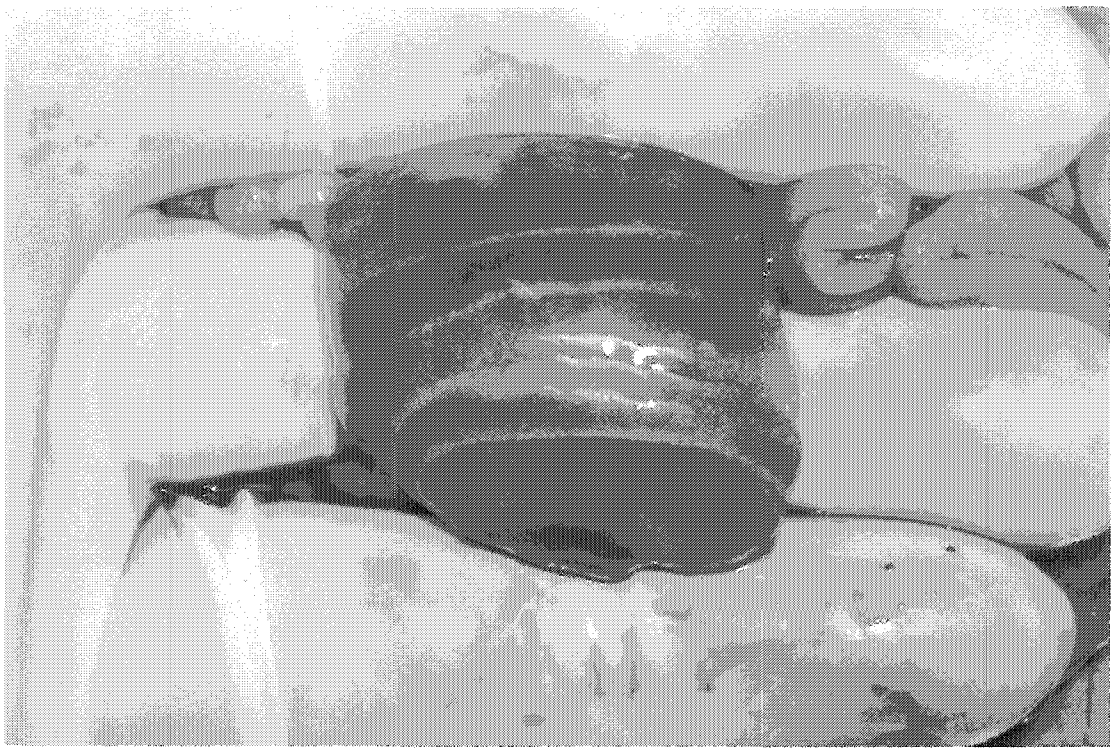

Figure 2.Gross photograph of the spiral colon from a pig treated with 2.5% dextran sulfate sodium (DSS) day 6 of DSS treatment. Note the dark purple discoloration of the spiral colon and gelatinous appearance of the mesocolon, due to edema.

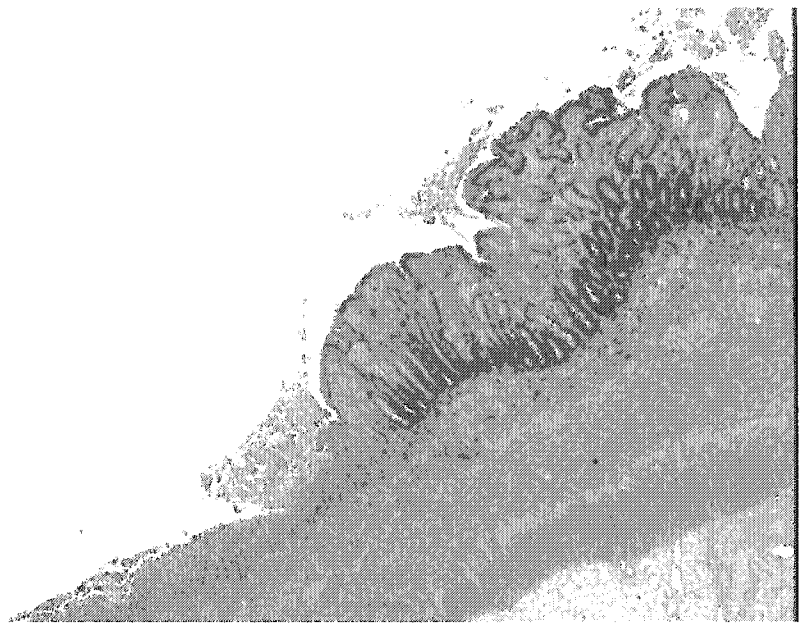

Figure 3. Photomicrograph (20 x) of spiral colon from a pig treated with low dose (2.5%) dextran sulfate sodium (DSS) at day 6 of DSS treatment. There are extensive areas of ulceration that border an area of intact mucosal epithelium. H&E stain.

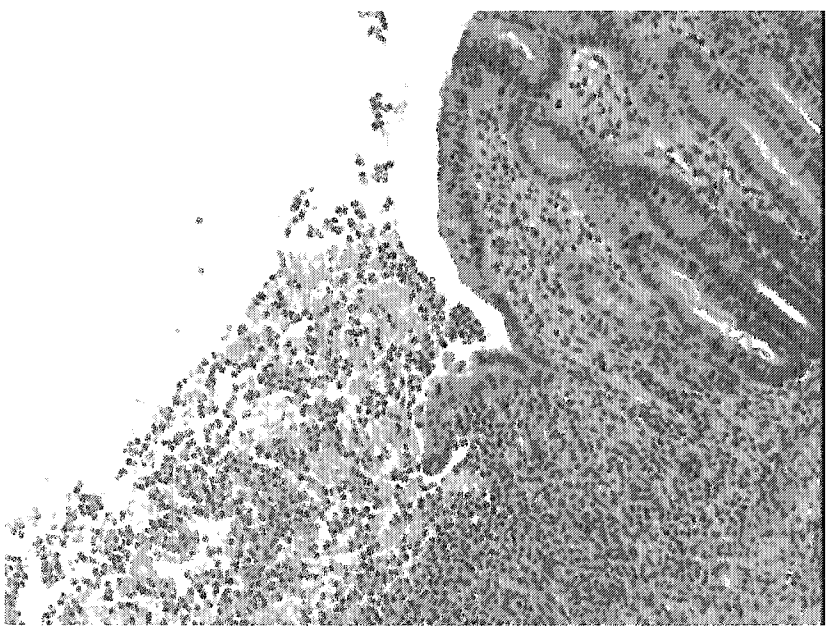

Figure 4. Photomicrograph (40 x) of the spiral colon of a pig treated with low dose (2.5%) dextran sulfate sodium (DSS) at day 6 of treatment. There is intense neutrophil infiltration in an ulcerated area, and in an area of intact mucosal epithelium. H&E stain.

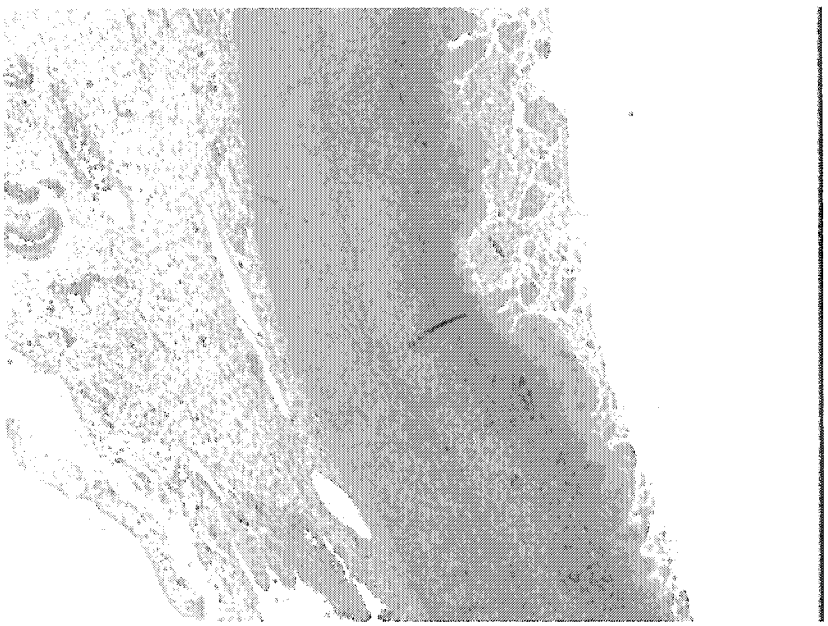

Figure 5. Photomicrograph (10x) of spiral colon from a pig treated with high dose (7.5%) dextran sulfate sodium (DSS) at day 6 of DSS treatment. The normal mucosal surface is replaced by a thick fibrino-necrotic membrane and there is complete loss of mucosal epithelium. H&E stain.

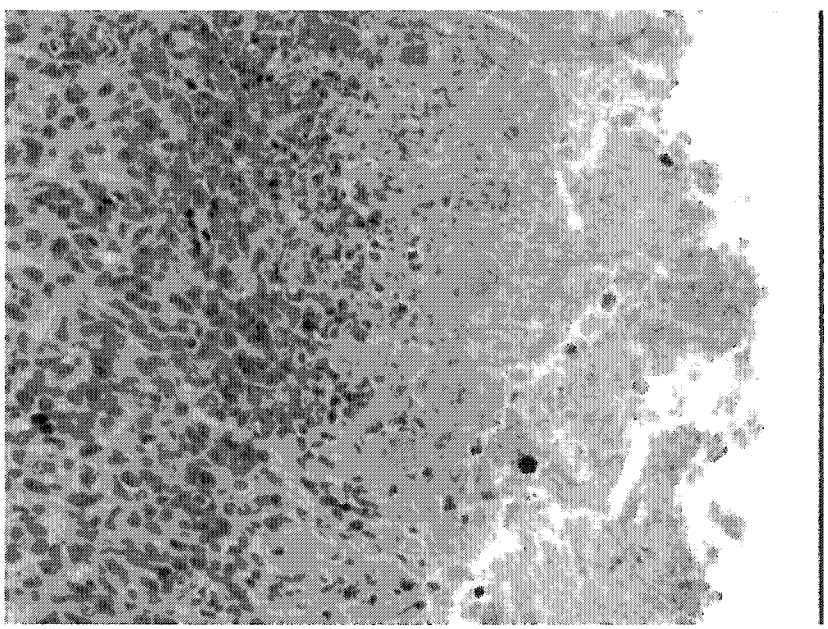

Figure 6. Photo micrograph (40 x) of the spiral colon of a pig treated with high dose (7.5%) dextran sulfate sodium (DSS) at day 6 of DSS treatment. A thick fibrino necrotic membrane has replaced the normal mucosal epithelium and there is total loss of mucosal epithelium. H&E stain.

Average Ulceration by Treatment

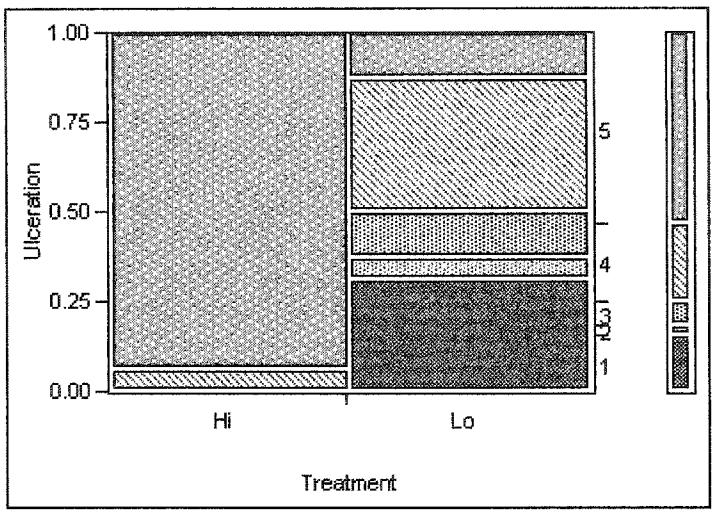

Figure 7. Mosaic plot of the average ulceration score for animals treated with high dose (5% 2x/day or 7.5% 1x/day) dextran sulfate sodium (DSS)(n=4) or low dose (2.5% 2x/day or 5% 1x/day) DSS (n=4). The high dose group had significantly greater ulceration ($p<0.0001$) than the low dose group. The colors represent the different ulceration scores (0-5)(see Materials and Methods).

Average Inflammation by Treatment

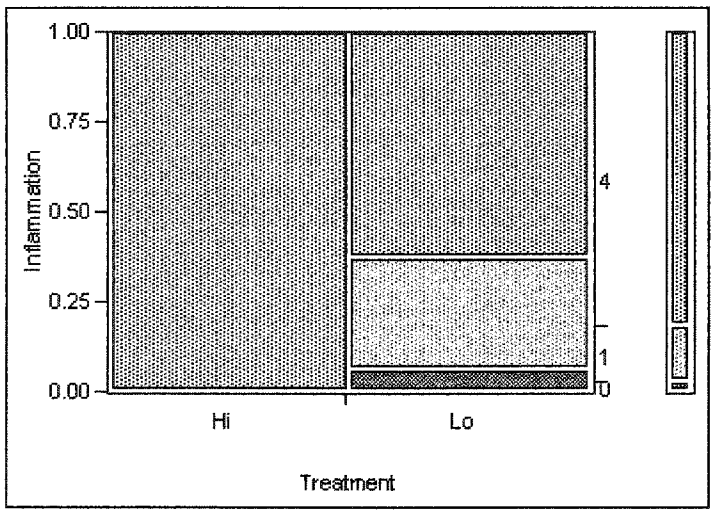

Figure 8. Mosaic plot of the average inflammation score for animals treated with high dose (5% 2x/day or 7.5% 1x/day) dextran sulfate sodium (DSS)(n=4) or low dose (2.5% 2x/day or 5% 1x/day) DSS (n=4). The high dose group had significantly greater inflammation ($p<0.0001$) than the low dose group. The colors represent the different inflammation scores (1-4)(see Materials and Methods).

Chapter 4: Effect of intestinal inflammation on Stx 2 absorption and Stx-induced extra-intestinal lesions *in vivo*.

A paper prepared for submission to the journal *Infection and Immunity*

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Abstract

Previous studies have shown that *E. coli* O157:H7 readily colonizes 3-day-old pigs, but does not induce histologically detectable inflammatory changes, and does not cause clinical signs of Shiga toxin (Stx) toxicosis. Dextran Sulfate Sodium (DSS) reliably induces suppurative and ulcerative inflammation in the cecum, spiral colon, and rectum of neonatal pigs by 4 days of treatment, in a concentration and time dependent manner. In this study, the effect of mucosal inflammation induced by DSS on systemic signs, absorption of marker sugars, and lesions of Stx toxicosis was determined in pigs either infected with *E. coli* O157:H7 or dosed with *E. coli* O157:H7 crude Stx toxin preparation. Colostrum-fed pigs with and without DSS treatment were inoculated with 10^9 CFU of *E. coli* O157:H7 at 3 days of age or were orally dosed with *E. coli* O157:H7 crude toxin preparation containing 10^7 CD 50/ml of Stx at 4 to 8 days of age. The strain of *E. coli* O157:H7 strain 86-24 that we used is a spontaneous Streptomycin resistant mutant, and in order to determine the effect of commensal microflora on *E. coli* O157:H7 strain 86-24 colonization, another group of pigs received Streptomycin prior to and during *E. coli* inoculations. All pigs inoculated with *E. coli* O157:H7 developed attaching and effacing (A/E) lesions and those receiving DSS developed inflammatory

lesions and had increased levels of absorption of marker sugars, indicating increased mucosal permeability. However, only pigs dosed with crude toxin preparation developed clinical signs of Shiga toxicosis. Treatment with DSS did not affect the number of periodic acid Schiff (PAS) stained droplets surrounding blood vessels in the medulla, or the rate of micro-hemorrhage development in the cerebellar folia, cerebellar pons, or medulla. Thus, the inflammatory response induced by DSS did not exacerbate signs and lesions of Shiga toxicosis. We conclude that severe enteric mucosal inflammation does not enhance Stx translocation *in vivo*.

Introduction:

Infections with enterohemorrhagic *E. coli* (EHEC) are a common cause of Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) in the United States and around the world (16). HUS caused by EHEC infections is the leading cause of acute renal failure in children in the U.S. and the developed world (11, 18, 34). EHEC bacteria, including *E. coli* O157:H7, form intimate, attaching and effacing (A/E) lesions with enterocytes (23), and produce Shiga toxins (Stx), which are responsible for the systemic signs of HUS (7, 20, 31, 44). *In vitro* studies have shown that neutrophil infiltration enhances mucosal permeability (26, 27) (8, 12, 29), and increases movement of Stx across polarized epithelial cells (14). In humans, and some animal species, EHEC infection is associated with an intense inflammatory response that includes neutrophil infiltration, ulceration, and hemorrhage (9, 17, 35, 45). Although pigs are infected with EHEC, the infection is typically not associated with an intense inflammatory response. In previous studies we found that pigs older than 3 days of age that are infected with *E. coli* O157:H7 were colonized, but lacked a histologically

detectable inflammatory response compared to age matched controls, and did not develop systemic or neurologic symptoms of Stx intoxication (25). By contrast, pigs infected before 3 days of age typically develop clinical and histiologic extra-intestinal signs of Stx toxicosis, although they also lack a histologically detectable host inflammatory response (3, 4, 13), which may be due to the fact that physiological absorption of macromolecules occurs until 3 days of age in the pig (42, 43).

Since young pigs are susceptible to systemic Stx toxicosis (3, 4, 13), but 3 day old pigs do not develop an inflammatory response to EHEC infection, and do not develop systemic signs of Stx toxicosis, 3 day old pigs are a reasonable model to test the role of inflammation in Stx absorption. Dextran Sulfate Sodium (DSS) has been used to cause inflammation in the intestine of guinea pigs, rats, and mice (6, 10, 15, 19). We previously showed that DSS reliably induces suppurative inflammation, ulceration, and hemorrhage in the cecum, spiral colon, and large intestine of neonatal pigs (24). In the current studies, DSS-induced inflammation was used to compare clinical signs and histopathologic lesions of systemic Stx as a measure of Stx translocation from the intestinal lumen to the systemic circulation in 3-day-old pigs with and without a host enteric inflammatory response.

Materials and Methods:

All procedures were approved by the Iowa State University Animal Care and Use Committee. Neonatal (<24 hr's old) Yorkshire/Durok cross piglets (N=35) were obtained from a local producer (Laboratory Animal Resources, ISU). The piglets were allowed to suckle for up to 12 hours prior to removal from the sow in order to obtain colostral antibodies

by passive transfer. Pigs were placed in cages that were maintained at 94° F in a climate-controlled room, and were fed Esbilac (Pet Ag) puppy milk replacer. All pigs were fed 1 mL of a solution of 5% lactulose, 1% L-rhamnose, and 1% mannitol as marker sugars to assess intestinal permeability (22, 40, 41).

Experiment 1, strain 86-24:

Pigs (N=11) were randomly divided into treatment (n= 8) and control (n= 3) groups and were allowed to acclimate until three days of age in order allow for gut closure and prevent passive transfer of maternal antibodies (42, 43). At three days of age, all pigs were infected with 10^9 CFU. of *E. coli* O157:H7 strain 86-24 per os in 20 ml of milk replacer. Starting 8 hr's after infection with *E. coli* strain 86-24, the treatment pigs were dosed with DSS 2.5% as previously described (24). Control pigs received a sham treatment of 20 ml milk twice daily. All pigs were evaluated as described in "Evaluation" below.

Experiment 2, strain 86-24 + streptomycin:

Pigs (N=8) were randomly divided in treatment (n=4) and control (n=4) groups and were allowed to acclimate until three days of age in order allow for gut closure and prevent passive transfer of maternal antibodies (42, 43). The pigs were dosed daily with a solution of non-metabolized marker sugars (mannitol, L-rhamnose, and lactulose). Five hours after dosing, urine was collected via cysto centesis and marker sugar concentration was detected using a previously described method (5) (see "Evaluation" below). At three days of age, all pigs were dosed with 2.1 x 10^{10} CFU's of *E. coli* O157:H7 strain 86-24 per os. In order to enhance *E. coli* O157:H7 colonization and suppress commensal microflora, all pigs were

given streptomycin 11mg/pig per os (21, 33). Half of the pigs (N=4) were dosed with 2.5% DSS as previously described (24) starting 8 hours after infection. Control pigs received a sham treatment of 20 ml milk twice daily. All pigs were evaluated as described in "Evaluation" below.

Experiment 3, high dose toxin (crude toxin preparation):

Pigs (N=8) were randomly divided into treatment (n= 4) and control (n=4) groups and were allowed to acclimate until three days of age in order allow for gut closure and prevent passive transfer of maternal antibodies (42, 43). The pigs were dosed daily with a solution of non-metabolized marker sugars (mannitol, L-rhamnose, and lactulose). Five hours after dosing, urine was collected via cysto centesis and marker sugar concentration was detected using a previously described method (5) (see "Evaluation" below). Half of the pigs received 2.5% DSS as described (24). Control pigs received a sham treatment of 20 ml milk twice daily. Starting at 7 days of age, pigs were orally dosed with 5 mL's of crude toxin preparation from *E. coli* strain 86-24 (1×10^7 CD^{50}/ml) two times daily. All pigs were evaluated as described in "Evaluation" below.

Experiment 4, low dose toxin (crude toxin preparation):

Pigs (N=8) were randomly divided into treatment (n= 4) and control (n=4) groups and were allowed to acclimate until three days of age in order allow for gut closure and prevent passive transfer of maternal antibodies (42, 43). The pigs were dosed daily with a solution of non-metabolized marker sugars (mannitol, L-rhamnose, and lactulose). Five hours after dosing, urine was collected via cysto centesis and marker sugar concentration was detected using a

previously described method (5) (see "Evaluation" below). The treatment group was further subdivided into short term (2 days of treatment, n=2) and long term (4 days of treatment, n=2). Starting at 4 days of age, the long term treatment group began receiving DSS 2.5% as previously described (24). Two days later (6 days of age) the short term treatment group began receiving DSS 2.5% in 20 as previously described (24). Control pigs received sham treatments of 20 ml of milk replacer twice daily. DSS administration was stopped on all treatment pigs on the evening of day 7 of age. On day 8 of age, all pigs received 1 ml of *E. coli* strain 86-24 crude toxin preparation (1×10^7 cd^{50}/ml) in 20 ml of milk. All pigs were evaluated as described in "Evaluation" below.

Evaluation:

Food intake, hydration status, activity level, bloody or non-bloody diarrhea, and neurologic signs of Stx toxicosis (head pressing, paddling, seizures (3, 4)) were recorded for all pigs throughout the experiments. Pigs were euthanized on day 11 of age, or when clinical signs dictated, for humane reasons, by barbiturate overdose (Beuthanasia) and complete necropsies were performed.

The non-metabolized sugars mannitol, L-rhamnose, and lactulose have been used in pediatric medicine as a non-invasive test of intestinal mucosal permeability (5, 22, 40, 41). A mixture of 5% lactulose, 1% L-rhamnose, and 1% mannitol in water is given by mouth, and urine is collected for 5 hours. The amount and ratio of marker sugars in the urine has been shown to give an accurate measurement of mucosal permeability in children (5, 22, 40, 41). A modified version of this test was used in pigs. One mL of 5% lactulose, 1% L-rhamnose, and

1% mannitol was given by mouth to the pigs, and urine was collected 4 hours later by cysto centesis, and frozen at –20 C until it could be evaluated for the presence of the marker sugars via gas chromatography (5).

Sections of ileum, cecum, spiral colon, rectum, and brain were collected in 10% neutral buffered formalin. Formalin fixed sections were processed and imbedded in paraffin, then cut into 5 μm sections and stained with hematoxylin and eosin (H&E), and periodic acid Schiff (PAS) stains. H&E stained sections of ileum, cecum, spiral colon, and rectum were evaluated histologically and were subjectively scored for degree of inflammation and ulceration.

Inflammatory changes were scored as follows: 1=minimal infiltration of inflammatory cells, 2=mild infiltration of inflammatory cells, 3=moderate infiltration of inflammatory cells, 4=severe infiltration of inflammatory cells. Degree of ulceration was scored as follows: 0=ulceration of less than 1% of the mucosal surface, 1=ulceration of from 1-20% of the mucosal surface, 2=ulceration of 21-40% of the mucosal surface, 3=ulceration of 41-60% of the mucosal surface, 4=ulceration of 61-80% of the mucosal surface, 5=ulceration of greater than 80% of the mucosal surface.

PAS stained sections of brain were evaluated for lesions of systemic shiga toxicosis as described by Dean-Nystrom and Pohlenz (30). PAS-stained mid-sagittal sections of the right half of the cerebellum and brain stem were examined starting at the dorsal midline of the brain stem directly below the cerebellar peduncles. 100 random arterioles were assessed for

the presence of PAS stained droplets in the space of Virchow surrounding the vessel in these areas for each pig.

H&E stained mid-sagittal sections of the right half of the cerebellum and brain stem were examined for the presence of micro-hemorrhages in the cerebellar folia, cerebellar pons, and medulla. Each area of the section was considered a significant lesion if there was at least one focus of red blood cell leakage into the neuropil surrounding a vessel. Leakage at the margins of the tissues or in areas that were obviously damaged during brain removal was not included.

Additional unstained 5 μm sections of ileum, cecum, spiral colon, and rectum were mounted to glass slides for immunohistochemical staining of *E. coli* O157:H7 bacteria using an OptiMax Plus stainer (Biogenics, San Ramon). Briefly, paraffin embedded sections were cut to 5 μm and adhered to glass slides. 5 μm sections were de-paraffinized in xylene and re-hydrated through a series of ethanol/water baths. De-paraffinized sections were incubated with 3% hydrogen peroxide for 30 minutes to block peroxidase activity. The sections were then rinsed in Tris-buffered saline containing 0.05% Tween (TBS-T20) (Sigma, St. Louis, MO). Using a wax pen (Pap-pen, Biogenics, San Ramon, CA), two lines were drawn on the glass slide, one above and one below the section, to prevent excessive spreading of the reagents and tissue drying. The slides were loaded into the slide processor and a previously optimized staining protocol was run (2). The slides were incubated with 1:20000 dilution of affinity purified goat anti-*E. coli* O157:H7 polyclonal antibody (Kirkgaard-Perry, Gathersburg, MD) 2 times for 45 minutes, and rinsed in TBS-T20. Biotinylated rabbit-anti

goat antibody was used as a secondary antibody (Kirkgaard-Perry). Signals were developed using the Nova Red substrate system (Vector Laboratories, Burlingame, CA). The slides were counter-stained with hematoxylin.

Colonization by A/E bacteria was scored on the immunohistochemically stained sections as follows: 0=no attached bacteria visible, 1=<5% of the mucosal surface area of the section colonized, 2=5-10% of the mucosal surface of the section colonized, 3=10-25% of the mucosal surface of the section colonized, 4=>25% of the mucosal surface of the section colonized.

Statistical significance of parametric data was determined by single factor analysis of variance (ANOVA) and statistical significance of non-parametric data was determined by the Wilcox test using the statistical software analysis package JMP 5.0.1a (SAS Institute).

Results:

Clinical Signs:

In all 4 studies, pigs that received DSS developed bloody diarrhea while pigs that did not receive DSS did not develop bloody diarrhea. Likewise, dehydration and lethargy were seen in DSS treated pigs, but not in those that did not receive DSS. This finding was consistent with our previous findings that DSS induces bloody diarrhea and dehydration in pigs (24).

Clinical signs of systemic Stx intoxication (head pressing, seizures, and paddling) were seen in the high dose Stx experiment (experiment 3), but were not seen in the 86-24 colonization

experiments (experiments 1 and 2) or the low dose Stx experiment (experiment 4). In the hi dose toxin experiment (experiment 3) clinical signs of Stx toxicosis were seen in both pigs that received DSS and those that did not receive DSS.

Marker sugars:

The concentration of marker sugars in the urine of pigs was below the level of quantification in the majority (49/52) of samples tested. However, trace amounts (detectable, but below the threshold level of quantification by GC (50 or 100 ppm depending on the sugar)) of one or more of the sugars were detected in 45 out of 52 samples. Using the presence of trace amounts of the sugars as a non-parametric measurement of marker sugar absorption, there was a significant difference in the absorption of mannitol ($p=0.05$) (Figure 1) and lactulose ($p=0.05$) (Figure 2), but not L-rhamnose in animals treated with DSS versus those not treated with DSS. Interestingly, mannitol was absorbed to a greater extent in animals that did not receive DSS versus those that did, while Lactulose was absorbed to a greater extent in animals that did receive DSS versus those that did not. Additionally, significantly greater absorption of mannitol ($p=0.03$), L-rhamnose ($p=0.04$), and lactulose ($p=0.04$) was seen in the high dose toxin group (DSS 18) than in the low dose toxin group (DSS 21) and the live bacteria group (DSS 17) (Figure 3) (Figure 4) (Figure 5).

Histopathology:

Examination by light microscopy showed that DSS caused a significant increase in both inflammation ($p<0.0001$) and ulceration ($p<0.0001$) in the cecum, spiral colon, and rectum of pigs that received DSS treatment vs. pigs that did not receive DSS treatment (Figure 6)

(Figure 7). Pigs treated with DSS had severe suppurative inflammation in the cecum, spiral colon, and rectum with numerous neutrophils in the intestinal lumen, within and between intestinal epithelial cells, and in the lamina propria, tunica submucosa, and in some cases within the tunica muscularis (Figure 8). In addition, DSS treated pigs had large areas of ulceration that covered anywhere from less than 5% to 100% of the surface area of the section (Figure 9). In general, ulceration was most severe in the spiral colon of DSS treated pigs, with the cecum and rectum usually being less severely affected. Non-DSS treated pigs had minimal to mild inflammatory infiltrates in the cecum, spiral colon, and rectum that were similar in severity to those seen in age matched controls from previous studies.

DSS caused a significant ($p=0.0055$) decrease in the extent of *E. coli* O157:H7 A/E lesions in the large intestine (Figure 10).

Extraintestinal lesions:

Stx is known to cause microvascular hemorrhages in the medulla, cerebellar pons, and cerebellar folia (Figure 11) and leakage of PAS positive material (Figure 12) from vessels (30, 39). PAS stained droplets were present in all pigs and were similar in DSS-treated and sham-treated pigs. Likewise, there was no significant difference in the presence of microvascular hemorrhages in either the cerebellar folia, the cerebellar pons, or the medulla between DSS-treated and sham-treated pigs in any of the experiments (Figure 13).

The oral toxin groups (experiments 3 & 4) had a significantly higher rate of microvascular hemorrhages ($p=0.0012$) compared to the groups that were inoculated with *E. coli* strain 86-24 (experiments 1 & 2) (Figure 14). However, within these groups, treatment with DSS made no significant difference on the rate of cerebellar micro-hemorrhages.

Discussion:

To the best of our knowledge, this is the first report of clinical signs and histologic lesions associated with oral administration of Stx 2 in pigs. The results of these studies were consistent with our earlier findings in that DSS induced significant increases in both inflammation and ulceration in the large intestine of pigs (24). However, this increase in inflammation and ulceration appeared to have mixed effects on the absorption of the marker sugars mannitol, lactulose, and L-rhamnose, and did not appear to increase the translocation of Stx from the intestinal lumen to the systemic circulation, based upon measurement of vascular leakage as determined by PAS staining in the medulla, and microvascular hemorrhages in the cerebellar folia, pons, and underlying medulla.

The finding that inflammation did not enhance Stx translocation is interesting because *in vitro* studies have shown that inflammatory changes, particularly neutrophil transmigration, increase the translocation of Stx across polarized intestinal epithelial cells in a manner that is dependent on the number of neutrophils that transmigrate across the epithelial cells(12, 14, 26, 27, 29). Additionally, Stx has been shown to bind to neutrophils in humans (36-38) and in pigs (personal communication, Shannon Jones Hostetter), and neutrophilia in humans is associated with a higher chance of developing HUS in EHEC infections.

It is possible that DSS, being a sulfonated carbohydrate chain, acts as a sink to bind Stx in the intestinal lumen like several other compounds are known to do (28, 32). Thus, it is possible that, although DSS was only given twice daily to treatment pigs, there was enough DSS in the lumen of the intestine to bind Stx, and therefore less Stx was available to cross from the intestinal lumen to the systemic circulation. However, in experiment 4, pigs were treated with DSS and then the DSS was withdrawn for 24 hours in order to allow it to clear the intestinal lumen prior to dosing with oral Stx. Even with this change, no difference between pigs with inflammation versus those without inflammation was seen, even when DSS was cleared from the intestinal tract.

In the bacterial colonization studies (experiments 1 and 2), it is possible that the decreased total enterocyte surface area associated with villous contraction and ulceration of the mucosal surface resulted in decreased bacterial growth/colonization and/or decreased Stx production, which could have obscured changes in Stx absorption. Additionally, DSS may have had direct inhibitory effects on the growth of *E. coli* O157:H7 strain 86-24, or may have suppressed Stx production by the bacteria. A possible complicating factor in the crude toxin preparation studies (experiments 3 and 4) is absorption of Stx in the small intestine before reaching the area affected by DSS. Additional studies to address these concerns are planned.

It is possible that inflammation and ulceration do affect the translocation of Stx from the intestinal lumen to the systemic circulation, but measurements of Stx translocation were not sensitive enough to identify the change. Stx translocation could not be detected via the Vero

cell assay, even in pigs that had extra-intestinal histopathologic lesions of Stx (cerebellar microhemorrhages). It could be that Stx is only very transiently present in the systemic circulation, making it difficult to detect, or that Stx is bound to a carrier substance in the systemic circulation, such as the neutrophil, and is thus not available to interact with Vero cells in the assay. Apoptosis is another measure of systemic Stx damage and Stx is known to cause apoptosis in endothelial cells in the kidney and brain. However, TUNEL staining procedures were unsuccessful in pig tissues in our lab and were not uniformly specific for apoptosis. Some of the caspase assays may be useful for detecting apoptosis in the pig, and may provide a more sensitive measure of systemic Stx damage.

Alternatively, it may be that our measurement of PAS and hemorrhagic lesions are adequately sensitive and that inflammation and ulceration does not influence Stx translocation in EHEC infections. The increased risk of HUS in individuals with elevated neutrophil counts and inflammatory mediators may simply indicate that the EHEC infection, rather than the host response, is more severe. It has been shown that small amounts of Stx II translocate across polarized intestinal epithelium. Since Stx is a very potent toxin, this small amount of physiologic translocation may be sufficient to induce HUS and other systemic effects of Stx. However, if this is the case, the discordance between disease outcome and fecal Stx remains puzzling (1).

Gas chromatography detection of marker sugars demonstrated that there are differences in marker sugar absorption between pigs that received DSS versus those that did not. However, a higher level of sensitivity of detection and quantification may more precisely differentiate

mucosal changes in permeability. The GC method is used humans, but pigs may have subtle differences in excretion. Also initial concentrations of marker sugars administered could be different, or the timing between dosing with the sugars and collecting urine could be different could be different in humans versus pigs. In humans, total urine output is typically collected, but this is not possible in neonatal pigs. More sensitive means of detection, such as high performance liquid chromatography, or alteration of the timing between dosing and urine collection and the initial dosing volume may prove beneficial to adapting the method of measurement in pigs.

The decreased absorption of mannitol in DSS treated groups versus sham treatment groups is surprising, since DSS increased lactulose absorption and DSS treatment clearly caused mucosal lesions, but may be due to interference or interaction of DSS with mannitol. Alternatively, since mannitol is used as a marker of transcellular permeability, the decrease in mucosal cells present may have been responsible for the decreased mannitol absorption in DSS treatment groups versus sham treatment groups.

Clearly, much work needs to be done in order to completely understand the mechanism of Stx translocation between the intestinal lumen and the systemic circulation.

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List of Figures

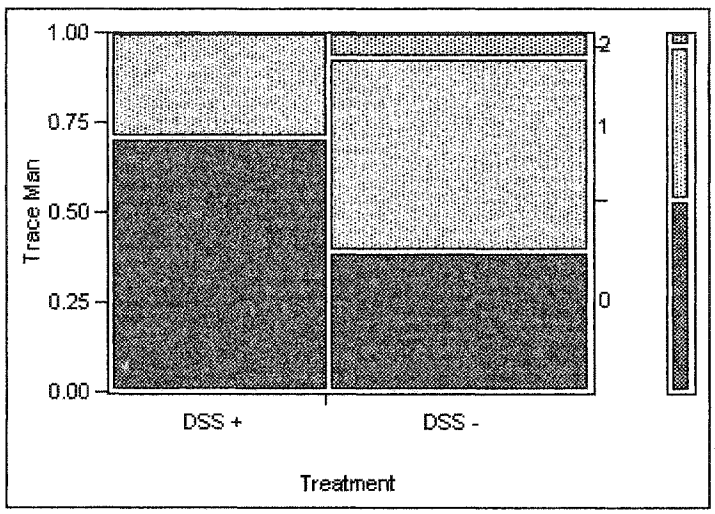

Figure 1. Mosaic plot of mannitol detection by gas chromatography in the urine of pigs treated with dextran sulfate sodium (DSS) versus pigs not treated with DSS. The colors red, green, and blue represent no detectable level of mannitol, trace levels of mannitol (<50 ppm) and measurable amounts of mannitol (>50 ppm) respectively. Mannitol detection in the urine is significantly greater (p=0.05) in pigs that were not treated with DSS (DSS-) versus pigs treated with DSS (DSS +).

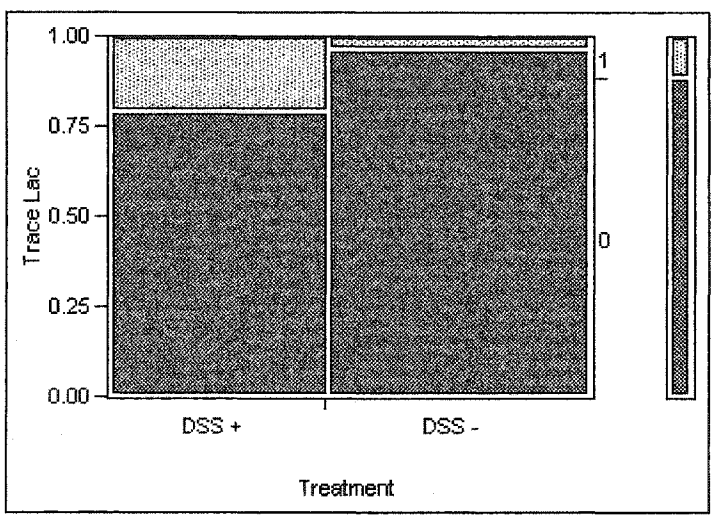

Figure 2. Mosaic plot of lactulose detection by gas chromatography in the urine of pigs treated with dextran sulfate sodium (DSS) versus pigs not treated with DSS. Lactulose detection in the urine is significantly greater (p=0.05) in pigs that were treated with DSS (DSS+) versus pigs that were not treated with DSS (DSS -). The colors red, green, and blue represent no detectable level of lactulose, trace levels of lactulose (<100 ppm) and measurable amounts of lactulose (>100 ppm) respectively.

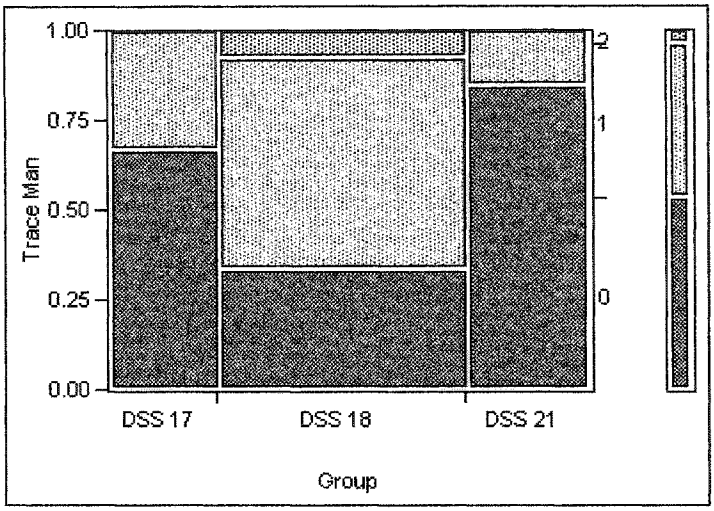

Figure 3. Mosaic plot of mannitol detection by gas chromatography in the urine of pigs. DSS 17 pigs (n=8) were dosed with *E. coli* O157:H7 strain 86-24 and streptomycin, DSS 18 pigs (n=8) were dosed with 5 mL of *E. coli* O157:H7 crude toxin preparation, 10^7 CD^{50}/mL daily (High dose Shiga toxin), and DSS 21 pigs were dose with 1 mL of *E. coli* O157:H7 crude toxin preparation, 10^7 CD^{50}/mL once (Low dose Shiga toxin). Mannitol dectection was significantly higher (p=0.02) in DSS 18 pigs versus DSS 17 and DSS 21. There was no significant difference in mannitol detection in DSS 17 versus DSS 21 pigs. The colors red, green, and blue represent no detectable level of mannitol, trace levels of mannitol (<50 ppm) and measurable amounts of mannitol (>50 ppm) respectively.

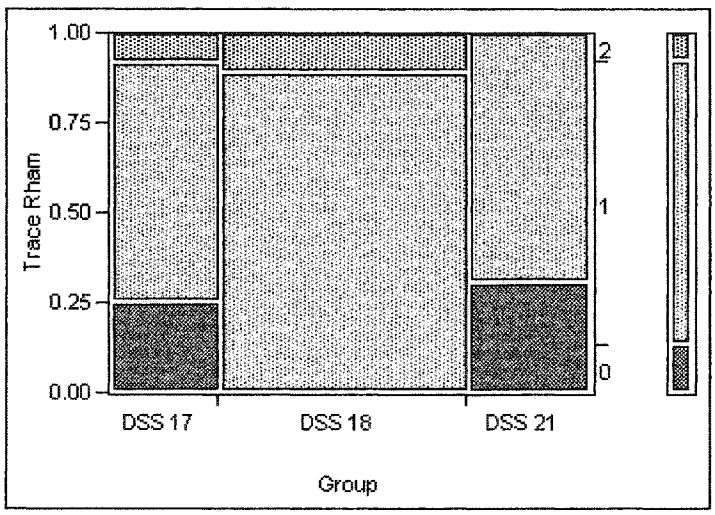

Figure 4. Mosaic plot of L-rhamnose detection by gas chromatography in the urine of pigs. DSS 17 pigs (n=8) were dosed with *E. coli* O157:H7 strain 86-24 and streptomycin, DSS 18 pigs (n=8) were dosed with 5 mL of *E. coli* O157:H7 crude toxin preparation, 10^7 CD^{50}/mL daily (High dose Shiga toxin), and DSS 21 pigs were dose with 1 mL of *E. coli* O157:H7 crude toxin preparation, 10^7 CD^{50}/mL once (Low dose Shiga toxin). L-rhamnose was significantly higher (p=0.04) in DSS 18 pigs versus DSS 17 and DSS 21. There was no significant difference in L-rhamnose detection in DSS 17 versus DSS 21 pigs. The colors red, green, and blue represent no detectable level of L-rhamnose, trace levels of L-rhamnose (<50 ppm) and measurable amounts of L-rhamnose (>50 ppm) respectively.

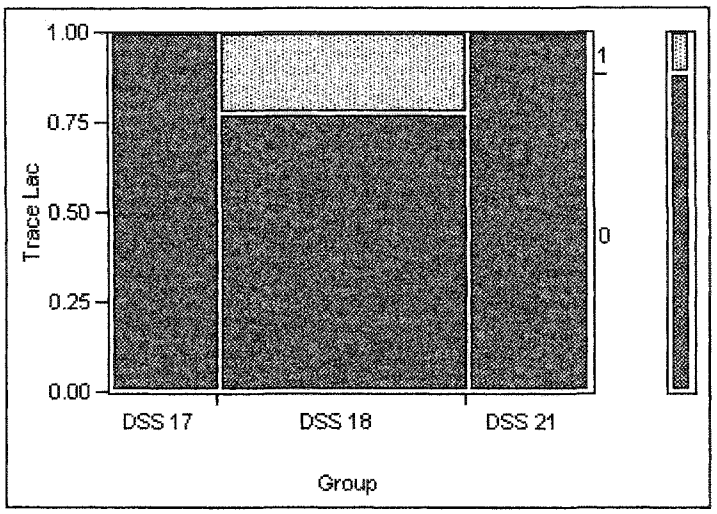

Figure 5. Mosaic plot of lactulose detection by gas chromatography in the urine of pigs. DSS 17 pigs (n=8) were dosed with *E. coli* O157:H7 strain 86-24 and streptomycin, DSS 18 pigs (n=8) were dosed with 5 mL of *E. coli* O157:H7 crude toxin preparation, 10^7 CD^{50}/mL daily (High dose Shiga toxin), and DSS 21 pigs were dose with 1 mL of *E. coli* O157:H7 crude toxin preparation, 10^7 CD^{50}/mL once (Low dose Shiga toxin). Lactulose was significantly higher (p=0.04) in DSS 18 pigs versus DSS 17 and DSS 21. There was no significant difference in L-rhamnose detection in DSS 17 versus DSS 21 pigs. The colors red, green, and blue represent no detectable level of lactulose, trace levels of lactulose (<100 ppm) and measurable amounts of lactulose (>100 ppm) respectively.

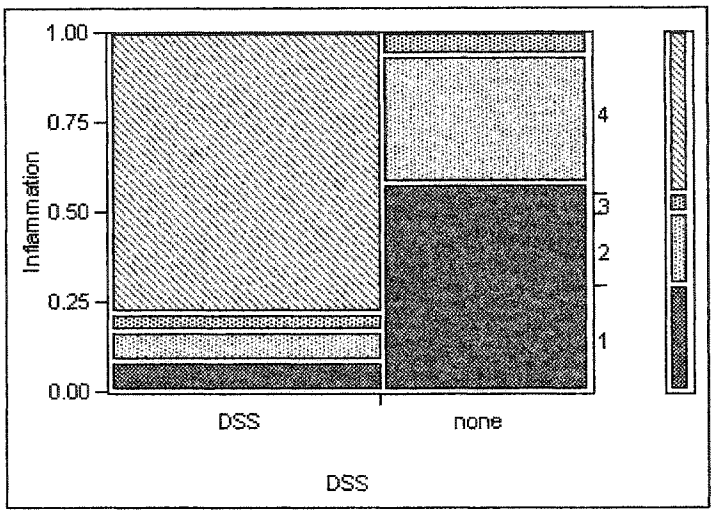

Figure 6. Mosaic plot of inflammation in pigs treated with dextran sulfate sodium (DSS) versus pigs not treated with DSS (none). The colors represent the different inflammation scores (1-4) assigned to cecal, spiral colon, and rectal histologic sections (combined) (see Materials and Methods). There is significantly greater inflammation ($p<0.0001$) in DSS treated pigs versus pigs not treated with DSS.

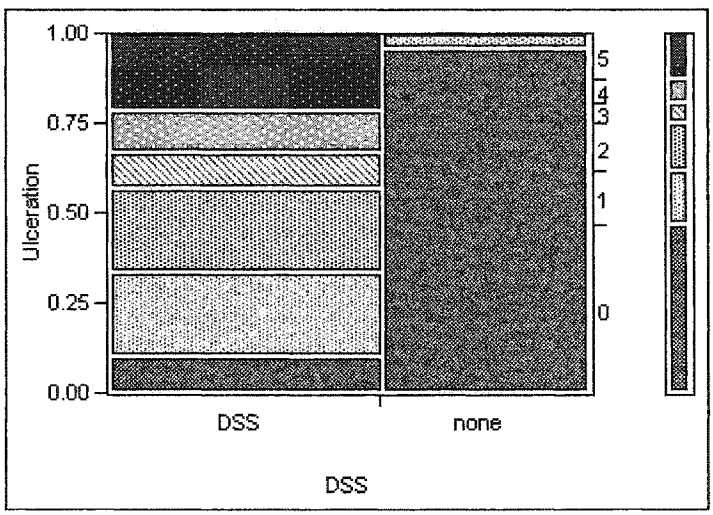

Figure 7. Mosaic plot of ulceration in pigs treated with dextran sulfate sodium (DSS) versus pigs not treated with DSS (none). The colors represent the different ulceration scores (0-5) assigned to cecal, spiral colon, and rectal histologic sections (combined) (see Materials and Methods). There is significantly greater ulceration ($p<0.0001$) in DSS treated pigs versus pigs not treated with DSS.

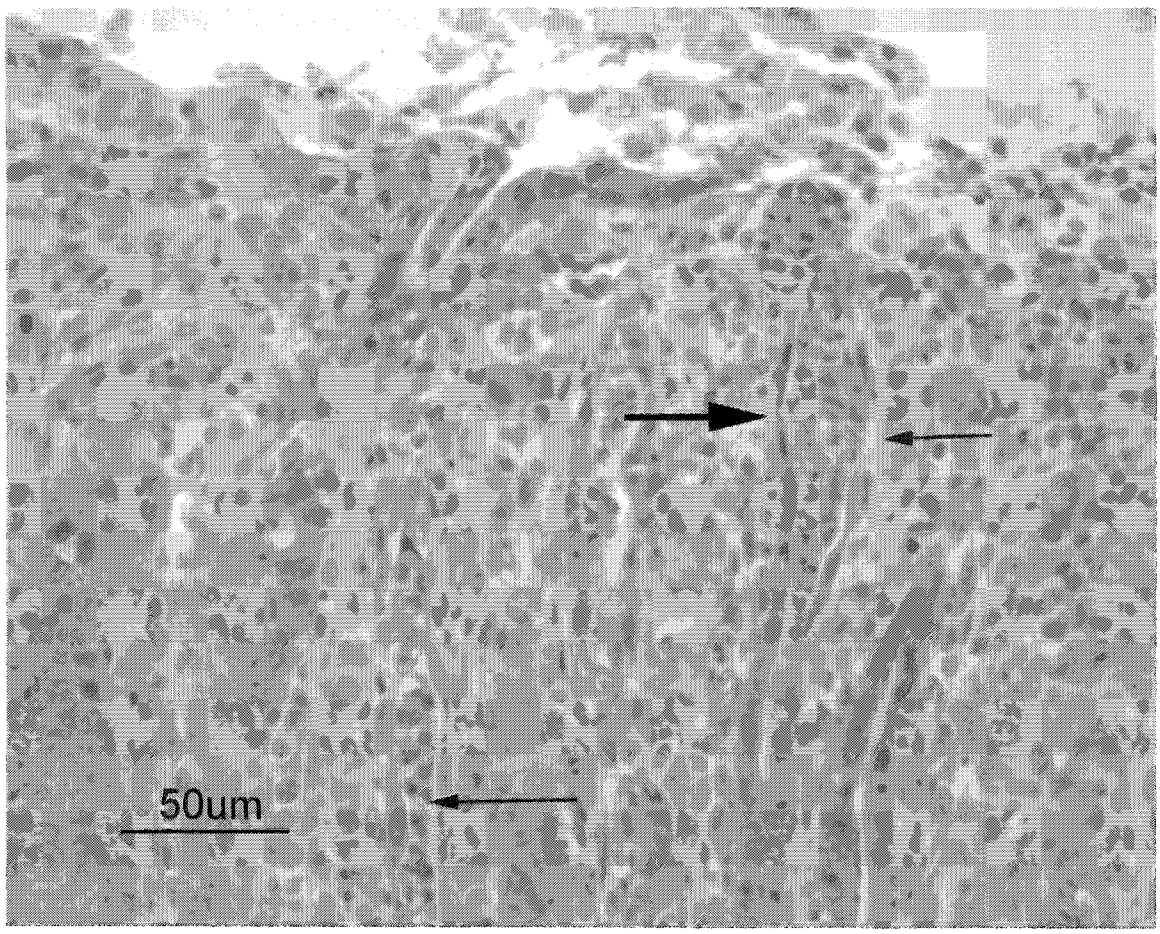

Figure 8. Photomicrograph (40 x) of the spiral colon demonstrating the typical inflammatory changes seen in the cecum, spiral colon, and rectum of pigs treated with dextran sulfate sodium (DSS). Note the crypt abscesses (small arrows) and areas of epithelial restitution (large arrow). Bar=50 micrometers.

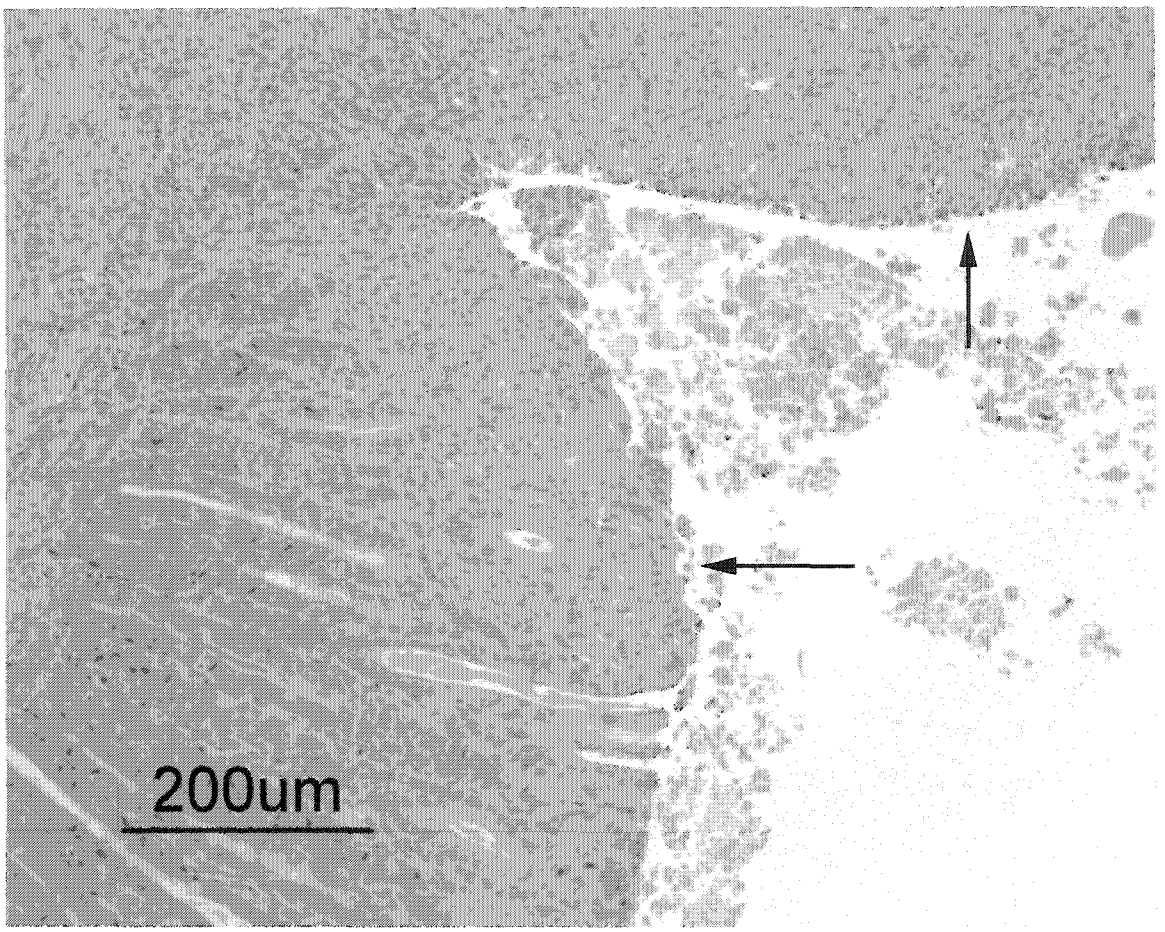

Figure 9. Photomicrograph (10 x) of the spiral colon demonstrating the typical ulcerative changes seen in the cecum, spiral colon, and rectum of pigs treated with dextran sulfate sodium (DSS). The arrows indicate a large area of ulceration, and smaller area of ulceration. Bar=200 micrometers.

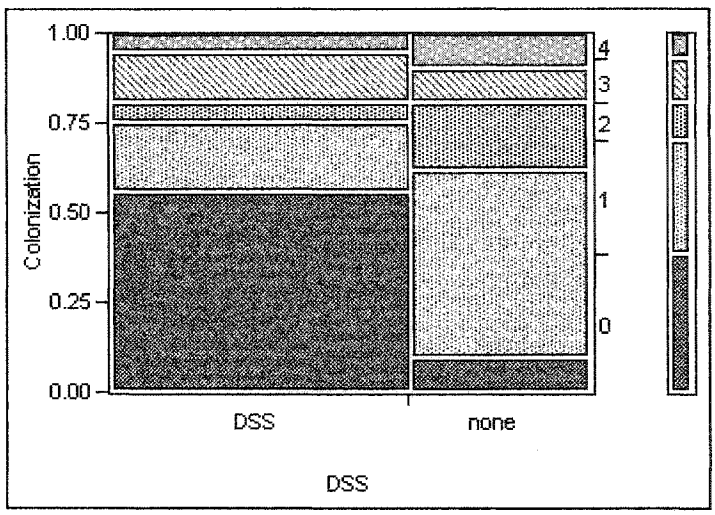

Figure 10. Mosaic plot of *E. coli* O157:H7 colonization scores in the large intestine (combined) of pigs treated with dextran sulfate sodium (DSS) versus those not treated with DSS (none). There was significantly less colonization (p=0.006), based upon the presence of attaching and effacing lesions, in pigs treated with DSS versus those not treated with DSS. The colors represent the different colonization scores (0-4) assigned to sections of cecum, spiral colon, and rectum (see Materials and Methods).

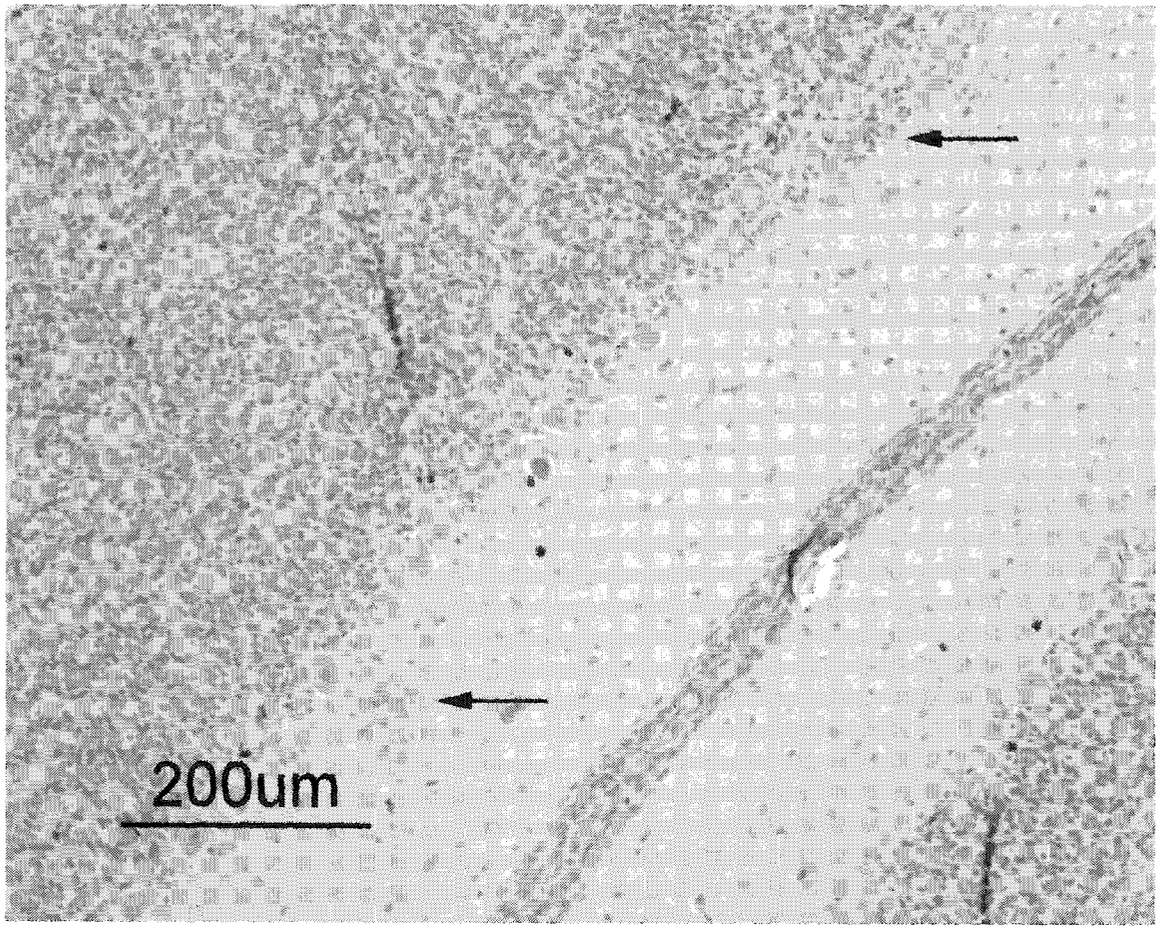

Figure 11. Microvascular hemorrhages in the cerebellar folium of a pig treated with Shiga toxin crude toxin preparation 5 days after onset of treatment. Arrows denote foci of microvascular hemorrhage. H&E stain. Bar=200 micrometers.

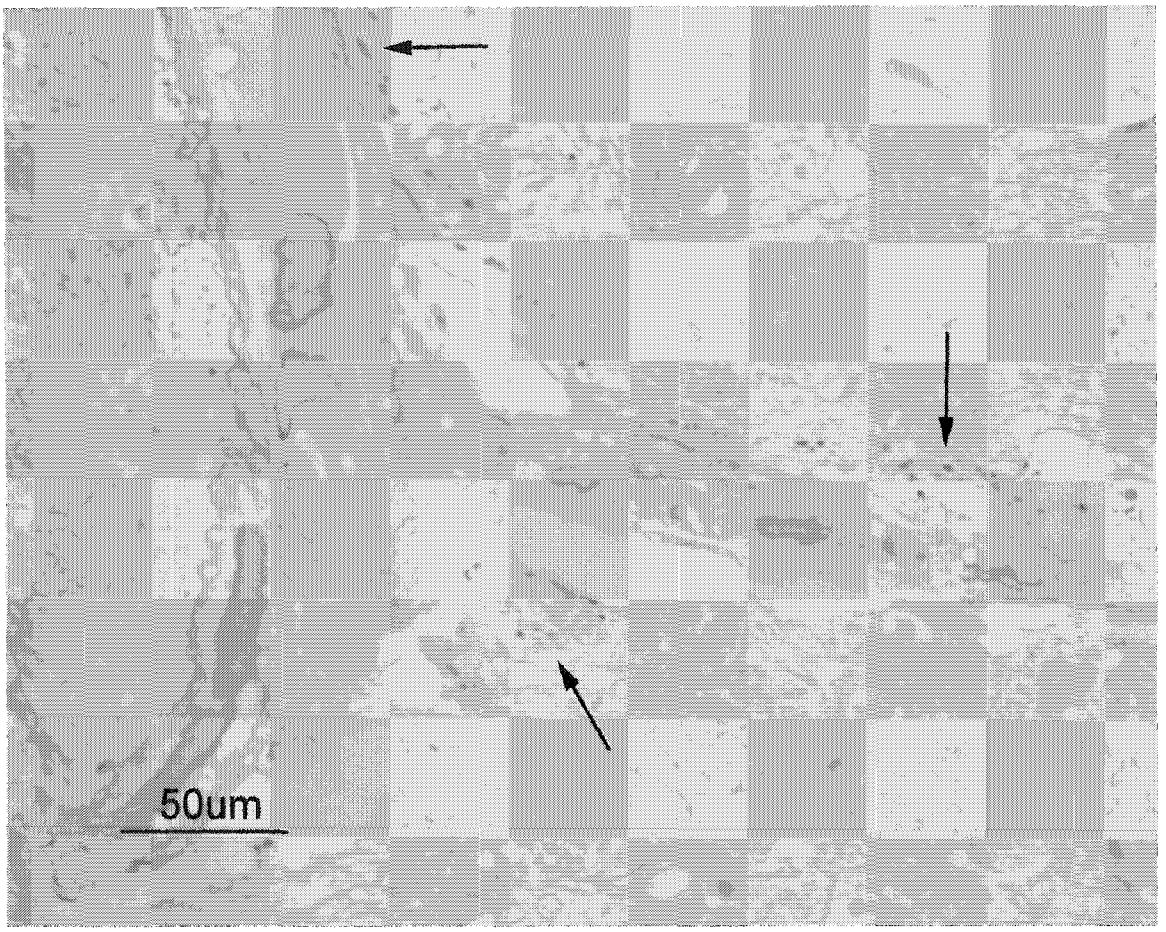

Figure 12. PAS stained droplets surrounding an arteriole in the medulla oblongata of a pig treated with Shiga toxin crude toxin preparation taken 5 days after onset of treatment. Arrows denote PAS positive droplets along the neuropil surrounding the vessel. These droplets are due to vascular leakage. PAS stain. Bar=50 micrometers.

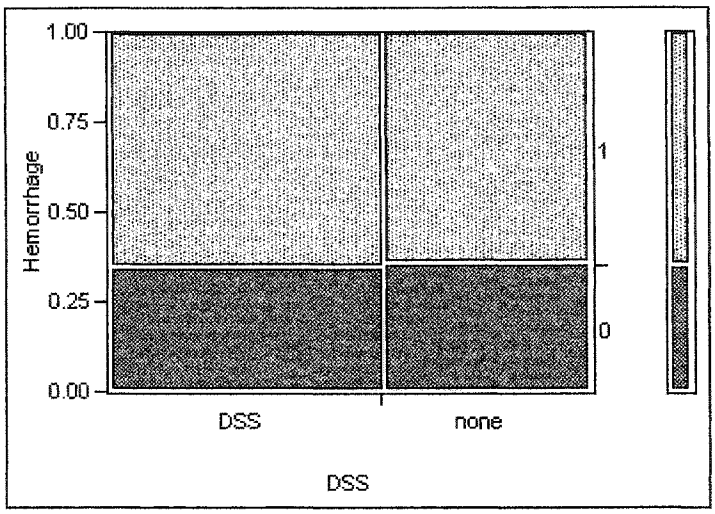

Figure 13. Mosaic plot showing the presence of hemorrhage in the cerebellar folia, pons, and medullar oblongata of pigs treated with dextran sulfate sodium (DSS) and those not treated with DSS (none). All pigs also received either *E. coli* O157:H7 bacterial inoculum or *E. coli* O157:H7 crude toxin preparation. There is no statistical difference between the groups, indicating the treatment with DSS did not have an effect on the presence of Shiga toxin induced microvascular hemorrhages in the pigs.

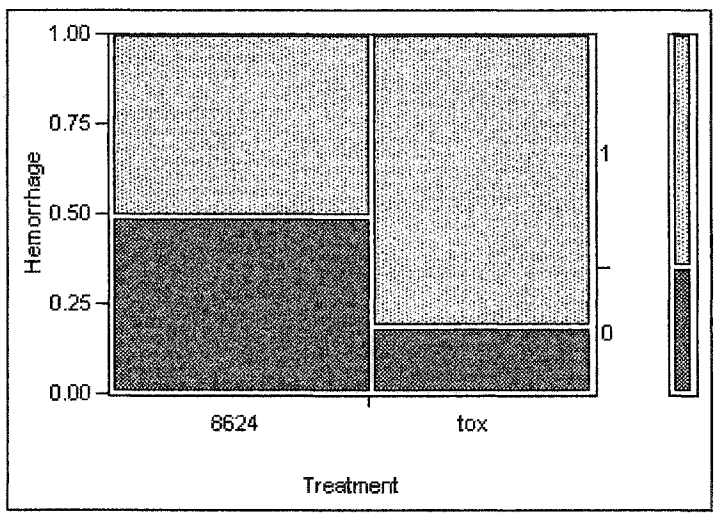

Figure 14. Mosaic plot of the presence of microvascular hemorrhages in the cerebellar folia, pons, and medulla oblongata in pigs that received either *E. coli* O157:H7 bacterial inoculum (86-24) or *E. coli* O157:H7 crude toxin preparation (tox). The pigs that received crude toxin preparation had a significantly higher (p=0.0012) presence of microvascular hemorrhages compared to pigs that received bacterial inoculum.

General Conclusions

E. coli O157:H7 is an enterohemorrhagic *E. coli* that is responsible for morbidity and mortality in children and adults throughout the industrialized world. Hemolytic uremic syndrome induced by *E. coli* O157:H7 is the leading cause of acute renal failure in children in the United States, and is a cause of chronic renal sequela in many of the children affected.

Much has been learned about the pathogenesis of EHEC colonization, bacteria-host interactions, and HUS development. One of the steps of the pathogenesis of EHEC infection that remains undefined is Shiga toxin (Stx) translocation from the intestinal lumen to the systemic circulation. Several *in vitro* studies have shown that Shiga toxin 2 (Stx 2) translocation is enhanced by neutrophil transmigration across polarized intestinal epithelial cells. Other studies have shown that neutrophils and their products induce Stx production, and bind Stx in humans. It seems likely that *in vivo* translocation of Stx would be enhanced by the host inflammatory response to EHEC.

The purpose of the papers presented in this thesis was to develop an *in vivo* model of EHEC infection where host inflammation could be controlled so that Stx translocation in the presence and in the absence of the host inflammatory response could be assessed.

The first experiment demonstrated that conventional pigs infected with *E. coli* O157:H7 at three days of age, after physiologic gut closure, were colonized by *E. coli* O157:H7 and developed classic attaching and effacing lesions of *E. coli* O157:H7, but lacked both a

histologically detectable host inflammatory response as compared to age matched controls, and clinical signs of systemic Stx toxicosis. This showed that the 3-day-old pig could be used as a model of EHEC infection.

The second experiment demonstrated that dextran sulfate sodium (DSS), which has been used in the guinea pig, mouse, and rat to induce colonic inflammation, reliably induced inflammation of the cecum, spiral colon, and rectum of the neonatal pig. The significance of the this experiment was that it provided a method of inducing inflammation and ulceration at the sites of EHEC colonization in conventional 3-day-old pigs.

The third experiment compared clinical outcome and histologic lesions of systemic Stx intoxication in pigs that had an intense inflammatory response in the large intestine versus pigs that had a minimal or no inflammatory response in the large intestine. Clinical and histologic measurements were used to determine whether Stx had translocated into the systemic circulation. We were unable to detect Stx in the blood of any pigs, even those that had systemic lesions of Shiga toxicosis using the Vero cell assay. Likewise, TUNEL staining did not work reliably in our lab on pig tissues. The lesions that we chose to examine have been described in the scientific literature in association with Stx intoxication in pigs [1-4], and since the most important measurement in real world disease is the outcome, systemic lesions of Stx are arguably the ultimate measure of success or failure of a treatment.

Based upon the model that we developed to test our hypothesis, the host inflammatory reaction to DSS does not enhance Stx absorption, as measured by the presence of systemic

lesions of Stx toxicosis, in the 3-day-old pig. However, it is possible that DSS directly or indirectly affected the growth/colonization and/or Stx production of *E. coli* O157:H7 strain 86-24 in these experiments. In addition, it is possible that less severe inflammation and/or ulceration may have a different effect on Stx translocation.

Additional *in vitro* tests to determine the effects of DSS on growth and toxin production of E. coli O157:H7 as well as additional *in vivo* tests involving less severe inflammatory changes will be helpful to further define the mechanism of Stx translocation from the intestinal lumen to the systemic circulation.

"It is a sad thing, indeed, to see a beautiful hypothesis crushed by cold, hard, observations." – original author unknown to me, but often repeated by my father.

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I see you Dad,
In the smile on Timmy's face
In Kaili's quick grin and giggle
I see you in the night sky
When the stars you loved so much
Are shining overhead

I hear you Dad,
In John's laughter
In the expressions that I use
I hear you in the songs of birds
And the creak of trees
As the wind touches their branches

I love you Dad,
As a father and a friend
As a mentor and a hero
With every fiber of my being

I miss you Dad

Tim