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14. ABSTRACT
Escherichia coli O157:H7 was first clearly documented as a human pathogen when it caused an outbreak of hamburger patty-associated bloody diarrhea, or hemorrhagic colitis (HC) in 1982. In the ensuing 28 years, E. coli O157:H7 has been responsible for multiple food- and water-borne outbreaks of diarrhea and/or HC worldwide. More importantly, a portion (4-15%) of such E. coli O157:H7-infected individuals, particularly young children, develop a life-threatening sequela of infection called hemolytic uremic syndrome or HUS. The major virulence factor that has been linked to both the presentation of HC and HUS is Shiga toxin (Stx), a potent cytotoxin that can cause inhibition of protein synthesis in sensitive target cells such as the vascular endothelial cells in the glomeruli of the kidneys.

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ABSTRACT

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"Impact of Anti-Shiga Toxin Type 2 (Stx2) Neutralizing Antibody on

Colonization and Pathogenesis of *Escherichia coli* O157:H7 in Mice"

Krystle L. Mohawk, Doctor of Philosophy, 2010

Thesis directed by:

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Escherichia coli O157:H7 was first clearly documented as a human pathogen

when it caused an outbreak of hamburger patty-associated bloody diarrhea, or

hemorrhagic colitis (HC) in 1982. In the ensuing 28 years, *E. coli* O157:H7 has been

responsible for multiple food- and water-borne outbreaks of diarrhea and/or HC

worldwide. More importantly, a portion (4-15%) of such *E. coli* O157:H7-infected

individuals, particularly young children, develop a life-threatening sequela of infection

called hemolytic uremic syndrome or HUS. The major virulence factor that has been

linked to both the presentation of HC and HUS is Shiga toxin (Stx), a potent cytotoxin

that can cause inhibition of protein synthesis in sensitive target cells such as the vascular

endothelial cells in the glomeruli of the kidneys. Currently, treatment of infection is

limited to supportive care with additional efforts focused towards disease prevention. However, several laboratories are also attempting to develop therapeutic strategies and vaccines for humans. The long-term goal of this research was to help elucidate the course of *E. coli* O157:H7 pathogenesis in an effort to facilitate treatment and prevention of disease. For that purpose, we first developed a conventional mouse model in which the *E. coli* O157:H7 introduced by pipette feeding or gavage had to compete with the resident microbiota to establish infection. This intact commensal flora (ICF) adult mouse model is one of the few such murine models described to date that permits the evaluation of both colonization by *E. coli* O157:H7 and the subsequent systemic disease associated with Stx production. Through the use of this ICF model, we next went on to demonstrate not only a role for Stx in *E. coli* O157:H7 colonization, but more importantly, a role for anti-Stx antibody in reducing colonization. Our results suggest that a vaccine to reduce *E. coli* O157:H7 disease should include a toxoid component not only because anti-toxin antibodies would prevent Stx toxicity but also because anti-toxin antibodies may reduce bacterial colonization. Thus, we propose that our animal model and findings on colonization by *E. coli* O157:H7 and the subsequent impact of Stx produced in the gut may further advance the development and use of toxoid-based vaccines and therapeutic strategies against *E. coli* O157:H7.

Impact of Anti-Shiga Toxin Type 2 (Stx2) Neutralizing Antibody on Colonization and Pathogenesis of *Escherichia coli* O157:H7 in Mice

By

Krystle Lee Mohawk

Dissertation submitted to the Faculty of the

Emerging Infectious Diseases Interdisciplinary Graduate Program of the

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F. Edward Hébert School of Medicine

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy 2010

With every achievement there is sacrifice…

May the sacrifices of all my furry pals herald the way for scientific progress.
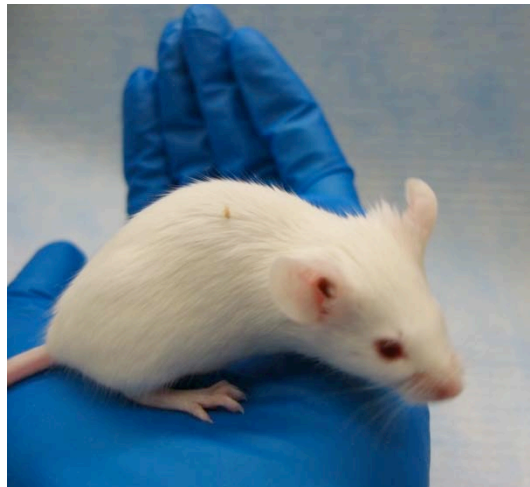
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CHAPTER ONE

INTRODUCTION

Overview

Escherichia coli are Gram-negative bacteria that primarily reside in the intestinal tract of humans and other mammals. Most strains of intestinal *E. coli* behave as commensals and only cause illness if they enter a sterile body site such as the bladder. Isolates of *E. coli* that cause diarrheal disease generally possess additional virulence characteristics when compared to normal-gut flora *E. coli*. Such *E. coli* strains fall into one of the following categories of *E. coli*: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), or enterohemorrhagic *E. coli* (EHEC). While all members of these groups are considered diarrheagenic *E. coli*, the intestinal site of colonization and mode of pathogenesis varies among these groups [reviewed in (144)].

E. coli O157:H7 is the prototypic organism among the enterohemorraghic *E. coli* or EHEC and is the subject of this dissertation. EHEC were so named because the organisms cause bloody diarrhea or hemorrhagic colitis (HC). EHEC produce Shiga toxins (Stxs), cause an attaching and effacing lesion, and contain a large plasmid (215).

E. coli O157:H7 is also a member of the larger category of Shiga toxin-producing *E. coli* (STEC). This group of *E. coli* is solely defined by its capacity to produce Shiga toxin type 1 (Stx1), Shiga toxin type 2 (Stx2), or both toxins (as well as variants of these). The capacity of the STEC in general and *E. coli* O157:H7 and other EHEC in particular to produce Stxs makes them of particular concern because Stx has been linked to the development of the hemolytic uremic syndrome (HUS) that can lead to kidney failure, particularly in children (150, 151). Worldwide, the most common STEC serotypes are: O157:H7, O26:H11, O103:H2, O145:H⁻, and O111:H⁻ (147).

History of *E. coli* O157:H7

Most papers cite 1982 as the year of the emergence of *E. coli* O157:H7 as a pathogen for humans. While the first recorded outbreak due to organisms of this serotype in the United States did occur in 1982, *E. coli* O157:H7 infection in both people and animals can be traced back as early as the 1970's; in fact, there are reports of a hemorrhagic colitis-type condition that occurred even earlier than that decade. The prevalence of this pathogen has grown since its first description and, despite our best control measures, *E. coli* O157:H7 remains a serious health concern (Figure 1).

A New Serogroup

The immune response to the surface of *E. coli* varies according to the polysaccharide component of the lipopolysaccharide or O antigen, flagella or H antigen, and, for some isolates, the capsule or K antigen. Specific antisera to these antigens are commonly used to type *E. coli* by serogroup (O antigen only) or serotype (O, H, and sometimes K antigen). *E. coli* O157 was first identified as a new serogroup of *E. coli* in 1972 (95). Subsequently, certain archival strains of *E. coli* were re-typed with the new anti-O157 reagent. These serogrouping studies revealed that an *E. coli* OX36 strain that had been isolated from pigs with colibacillosis (diarrhea due to *E. coli*) in Canada in 1970 was actually an *E. coli* O157 (110). Furthermore, some *E. coli* strains that were enteropathogenic in pigs and were previously categorized as serogroup O116 were re-typed as serogroup O157 (110). These re-classifications of the serogroups of certain existing porcine *E. coli* strains resulted in an increase in the estimated prevalence of O157 isolates that were considered responsible for enteric disease of swine. Furthermore,

Figure 1: Major outbreaks of *E. coli* O157:H7 infection in the United States in the context of key discoveries that linked Stx produced by the organism with HUS

McDonald's hamburger outbreak;
1st U.S. outbreak

O157:H7 gains notoriety following
Jack-in-the-Box outbreak

Cookie dough
outbreak

Epidemiological link made
between STEC, Stx, and HUS

Largest outbreak to date occurs in Japanese
schoolchildren and is associated with radish sprouts

Largest U.S. outbreak occurs in NY at
a fairground (contaminated well water)

Spinach outbreak

Odwalla apple juice outbreak

1982   1985   1993   1996   1999   2006   2009

YEAR

some of the original *E. coli* OX36 (O157) isolates with unidentified flagellar antigens may actually have been *E. coli* O157:H7 (125).

Prior to 1982

While the first reported *E. coli* O157:H7 isolation occurred in 1975, *E. coli* O157:H7 was not recognized as a human pathogen until 1982. However, there are reports in the literature to suggest that *E. coli* O157:H7 may in fact have been responsible for illness prior to 1982. These publications describe patients who developed a hemorrhagic colitis-like condition, a clinical presentation that is now considered characteristic of *E. coli* O157:H7 infection. For example, in 1963 a type of ischemic colitis in which elderly individuals suffered from transient to severe bloody diarrhea was first described (125). Then, in 1972, Clark and colleagues described four cases of transient HC in young adults (53). While *E. coli* O157:H7 was never confirmed as the cause of most of these cases or others like them, these reports of HC-like illnesses probably represent the earliest documented manifestations of *E. coli* O157:H7 as an emergent human pathogen.

Throughout the 70's and 80's *E. coli* of the serotype O157:H7 was rarely isolated as the cause of enteric illness. However, *E. coli* O157:H7 likely caused sporadic disease (bloody diarrhea) during this period based on a retrospective survey of *E. coli* isolates obtained from stools of patients. For example, *E. coli* O157:H7 was confirmed in this retrospective analysis as the cause of illness in a 50-year-old woman from California who presented with diarrheal disease that included evidence of gross blood in the stool (239, 310). In fact, this was the only *E. coli* O157:H7 isolate among the CDC Enteric

Reference Lab collection of more than 3,000 isolates taken from 1973 through 1982

(310). Similarly, *E. coli* O157:H7 was not recognized as a cause of human disease in

Canada until an O157 strain was isolated from a patient in 1978 (124). Furthermore,

between 1978 and 1981, the National Enteric Reference Centre at the Laboratory Centre

for Disease Control in Canada reported only 6 isolations of *E. coli* O157:H7 from people,

five of which were from patients who presented with HC (177). These infrequent

isolations of *E. coli* O157:H7 from ill persons in the U.S. and Canada are consistent with

the notion that through 1981 *E. coli* O157:H7 caused only sporadic enteric disease in

humans.

The Big Mac Attack

In 1982 the CDC released a case report that detailed the first two outbreaks of *E.

coli* O157:H7 disease in humans. These outbreaks occurred from February through

June1982 in two different states, Oregon and Michigan, and affected 47 people (239).

The disease was characterized as "an unusual gastrointestinal illness" that manifested as

HC, or bloody diarrhea (239, 302). Although two thirds of infected individuals were

hospitalized (302), none of the patients suffered long-term complications or died.

Eventually, it was determined that patients had likely contracted the organism

following the consumption of contaminated hamburgers (common ingredients ingested

by those who were ill included beef patties, onions, and pickles) sold at a local

McDonald's restaurants (1, 2, 202, 239). A frozen hamburger patty from the suspected

lot used in the Michigan restaurants tested positive for *E. coli* O157:H7 and had only 50

organisms per gram of the patty (310). This documentation of *E. coli* O157:H7 in an

incriminated hamburger patty lead to the conclusion that contaminated frozen beef patties improperly cooked before serving were likely the source of infection for these outbreaks (239). Such a conclusion was consistent with the knowledge at the time that salmonellosis could result from the ingestion of an improperly cooked contaminated hamburger (86).

Despite the identification of *E. coli* O157:H7 in stools of patients from these outbreaks, the mechanism of disease ("pathogenesis of the illness and the source of contamination of the raw meat") was not understood (239). The incriminated *E. coli* O157:H7 was neither enteroinvasive, nor did it produce a typical enterotoxin (i.e. heat-stable enterotoxin or heat-labile cholera-like toxin); however, it did induce nonbloody diarrhea in an infant rabbit model (239). At the end of their report, Wells *et al.* stated that "this organism may be the prototype of a group of *E. coli* strains which produce disease by a previously undescribed mechanism" (310). Furthermore, Riley and colleagues speculated that perhaps *E. coli* O157:H7 caused diarrhea via a previously undescribed enterotoxin (239); we now know that the production of Shiga toxin by *E. coli* O157:H7 is considered to be responsible for both the development of HC and the hemolytic uremic syndrome (HUS).

Following the U.S. outbreaks of *E. coli* O157:H7 in early 1982, there was a November outbreak in a nursing home in Ottawa. *E. coli* O157:H7 was isolated from the stools of 58% of the 31 residents who became ill. While a large order of hamburger had been purchased and used prior to the outbreak, no contaminating *E. coli* O157:H7 could be detected in the remaining uncooked meat. Additionally, person-to-person

transmission appeared to have been responsible for 3 subsequent cases of *E. coli* O157:H7 infection (267).

In addition to these outbreaks, sporadic cases of *E. coli* O157:H7-mediated illnesses were seen from late July of 1982 through 1983 throughout the United States. In total, 39 cases (as defined by CDC criteria) in 18 states were reported (70). As a result of the continuing spread of *E. coli* O157:H7, federal disease experts said they feared that the organism was "becoming established in the nation's food system" (302).

Jack-in-the-Box

E. coli O157:H7 did not gain nationwide notoriety as a food-borne pathogen until the Jack-in-the-Box (JITB) outbreak of 1993. That outbreak, which began in late 1992 and lasted through February of 1993, affected more than 700 people in western states (Washington, Idaho, Nevada, and California). About 25% of JITB outbreak victims were hospitalized, and the rate of HUS among infected individuals was 7.5%. Four children died in the outbreak (236).

During the JITB outbreak, a clear link was established between undercooked and contaminated ground beef patties and subsequent infection with *E. coli* O157:H7 (236). Furthermore, an epidemiological investigation by the Centers for Disease Control and Prevention (CDC) found that errors in both meat processing and cooking were to blame for the outbreak (21). JITB and its parent company, Foodmaker, Inc., recalled over a quarter of a million hamburgers (236) and sustained a net loss of over 160 million dollars (111). The response to the JITB outbreak was overwhelming with action taken by the federal government, the public, and the beef industry (111).

Largest Outbreak to Date

Between 1991 and 1995 Japan documented only six outbreaks of *E. coli* O157:H7 infection (outbreak defined here as involving 10 or more people). However, that number nearly tripled during the period from May to December of 1996; 16 outbreaks were reported for a total of 9,451 cases and 12 deaths. The largest of these outbreaks, and in fact the largest outbreak of *E. coli* O157:H7 to date, occurred in Sakai City, Japan during the summer of 1996. This outbreak affected over 7,000 elementary schoolchildren and was linked to the consumption of white radish sprouts that had been shipped from a specific farm and then served as part of a lunch program. During this same time, radish sprouts were implicated in two additional *E. coli* O157:H7 outbreaks. One of these clusters of illnesses occurred among 47 symptomatic individuals in a Kyoto business office and a second occurred in a nursing home in Habikino City among 98 symptomatic individuals (200). While the source of contamination of the sprouts was never determined, the presumption was that the seeds of the sprouts had become contaminated with *E. coli* O157:H7. This conjecture was based on previous reports of contaminated seeds as the cause of sprout-associated outbreaks of *Salmonella* as well as *E. coli* (197). Ironically, the sprout seeds inferentially linked to the Sakai outbreak were grown in the United States and shipped to Japan in early 1996 (200). This movement of contaminated foodstuff across international borders illustrates the necessity for improvements in global surveillance, communication, and control measures for food-borne pathogens such as *E. coli* O157:H7.

Odwalla Unpasteurized Juice Outbreak

In the fall of 1996, the same year as the huge outbreak of *E. coli* O157:H7 illness

in Sakai, Japan, another outbreak due to *E. coli* O157:H7 occurred in the western United

States and British Columbia. Eventually the source of infection was identified as

unpasteurized juice products (apple juice and apple-containing juice blends) sold by

Odwalla Incorporated. While the method of *E. coli* O157:H7 contamination of the

products was never discovered, the suspicion was that the bacterial contaminant was

present on the apples used to make some of the unpasteurized juice (54). A total of 65

persons were reported ill following the consumption of Odwalla's products, and one child

died from kidney failure (54). At the time, the company believed that pasteurization

resulted in an alteration in the fruit's natural flavor; since the outbreak, the company has

employed a flash pasteurization process to prevent bacterial contamination. Furthermore,

new federal regulations were instituted after this outbreak that required a warning label

on unpasteurized fruit and vegetable juices (54). Despite losses from fines, recall cost,

and settlements from several court litigations, Odwalla Incorporated eventually recovered

financially. The company was taken over by Coca-Cola in 2001.

Recent Outbreaks of **E. coli** *O157:H7*

Perhaps the most prominent recent *E. coli* outbreak occurred in 2006 when it was

discovered that fresh, bagged spinach and other spinach-containing products were a

source of *E. coli* O157:H7 infection. Spinach processed by Natural Selection Foods,

LLC (whose brands include Dole, O Organic, Trader Joe's, and Ready Pak to name a

few) was discovered as the infection source with the isolation of *E. coli* O157:H7 from

several spinach packages. The spinach leaves in these bags were harvested from farms in California. One theory on the source of contamination of the spinach was that the leaves on the ground either directly or indirectly came into contact with feral swine excrement, and the feral swine feces was contaminated with *E. coli* O157:H7 (135). During this outbreak, which lasted from August through to October 2006, there were reports of nearly 200 infected individuals from 26 different states and 3 confirmed deaths. The severity of infection was seemingly high in this outbreak: the hospitalization rate was ~51% and the HUS rate was ~16% (48). Subsequently, the *E. coli* O157:H7 strain responsible for the outbreak was identified as a member of *E. coli* O157:H7 clade 8, a group of highly related O157 strains that Manning and colleagues (186) recently suggested are more virulent than other clades (see *The Emergence of Clade 8* below for more details).

Later in 2006, an *E. coli* O157:H7 outbreak was reported in the northeastern states of NJ, NY, PA, DE, and SC. From November through December, 71 cases of illness were reported with a 75% hospitalization rate, an 11% HUS rate, and no reported mortality (44). The outbreak was linked to patronage of Taco Bell with potential vehicles that included lettuce, cheddar cheese, and ground beef; shredded lettuce was ultimately deemed the most likely source of *E. coli* O157:H7 (44).

More recently, *E. coli* O157:H7 was found as a contaminant in pre-packaged cookie dough sold by the Nestlé Corporation. Ingestion of the raw cookie dough lead to *E. coli* O157:H7 infection. Beginning in March 2009, and with the last cases reported in July, a total of 80 persons were infected in 31 states. Of these infected individuals, 35 were hospitalized (a rate of ~44%) and 10 developed HUS (a rate of ~13%) (45).

Following the discovery of *E. coli* O157:H7 in the dough, the FDA recommended that all pre-packaged Nestlé cookie products be discarded; baking the dough was not advised as it could lead to contamination of kitchen surfaces, utensils, and hands (76). *E. coli* O157:H7 was isolated from a lot of recalled cookie dough. However, after further laboratory analysis, the *E. coli* O157:H7 strain from the recalled lot was determined to be distinct from the one responsible for the outbreak (45).

Epidemiology of O157:H7

E. coli O157:H7 is responsible for an estimated 73,480 cases of illness, 2,168 hospitalizations, and 61 deaths annually in the United States (U.S.) according to data published by Mead *et al*. in 1999 (190). These figures include disease related to both sporadic illness and outbreak scenarios. While sporadic disease likely accounts for the majority of *E. coli* O157:H7 cases, most reported data on the epidemiology of infection tends to focus on evidence collected during outbreaks of *E. coli* O157:H7-mediated illness. The majority of such *E. coli* O157:H7 outbreaks in the United States are associated with food-borne transmission (236).

The incidence of *E. coli* O157:H7 infection seen in the United States varies with the season. Rates appear to peak in the warm summer months; indeed, the majority of outbreaks occur from May to November (236). Additionally, *E. coli* O157:H7 infection appears to occur more frequently in the Northern States and Canada; in fact, Canada reports increased rates compared with those seen in the U.S. (116, 289). Both the seasonality and regional differences in *E. coli* O157:H7 infection rates may be explained in part by an association with livestock. *E. coli* O157:H7 infection is generally acquired by the ingestion of contaminated food or water. Often, these sources of transmission are found to be contaminated with fecal material from *E. coli* O157:H7-infected cattle (Figure 2). Therefore, it is not surprising then that the epidemiology of *E. coli* O157:H7 is linked closely with the presence of cattle or their products.

The overall incidence of *E. coli* O157:H7 infection has declined since surveillance began in U.S.; the average number of outbreak cases per year from 2004-2006 was 353, a figure that is down from the 829 seen early in surveillance [1998-2000 (47)].

Figure 2: Modes of *E. coli* O157:H7 transmission to humans with emphasis on points and strategies for prevention/intervention

Wild and domesticated animals, especially cattle and other ruminants, serve as natural reservoirs for *E. coli* O157:H7. Thus, beef and dairy products may become contaminated with *E. coli* O157:H7 and infect people. Additionally, animal feces that contain *E. coli* O157:H7 may contaminate produce or water supplies (drinking or irrigatory) and thus lead to transmission of this low-infectious-dose organism to people. Other modes of transmission of *E. coli* O157:H7 have been reported and are discussed elsewhere.

- Cattle vaccines to reduce carriage
- Strategies to prevent direct/indirect contact

- Treatment of food to diminish bacteria (irradiation & cooking)

- Human vaccines to prevent infection/disease
- STmAbs
- Receptor analogs
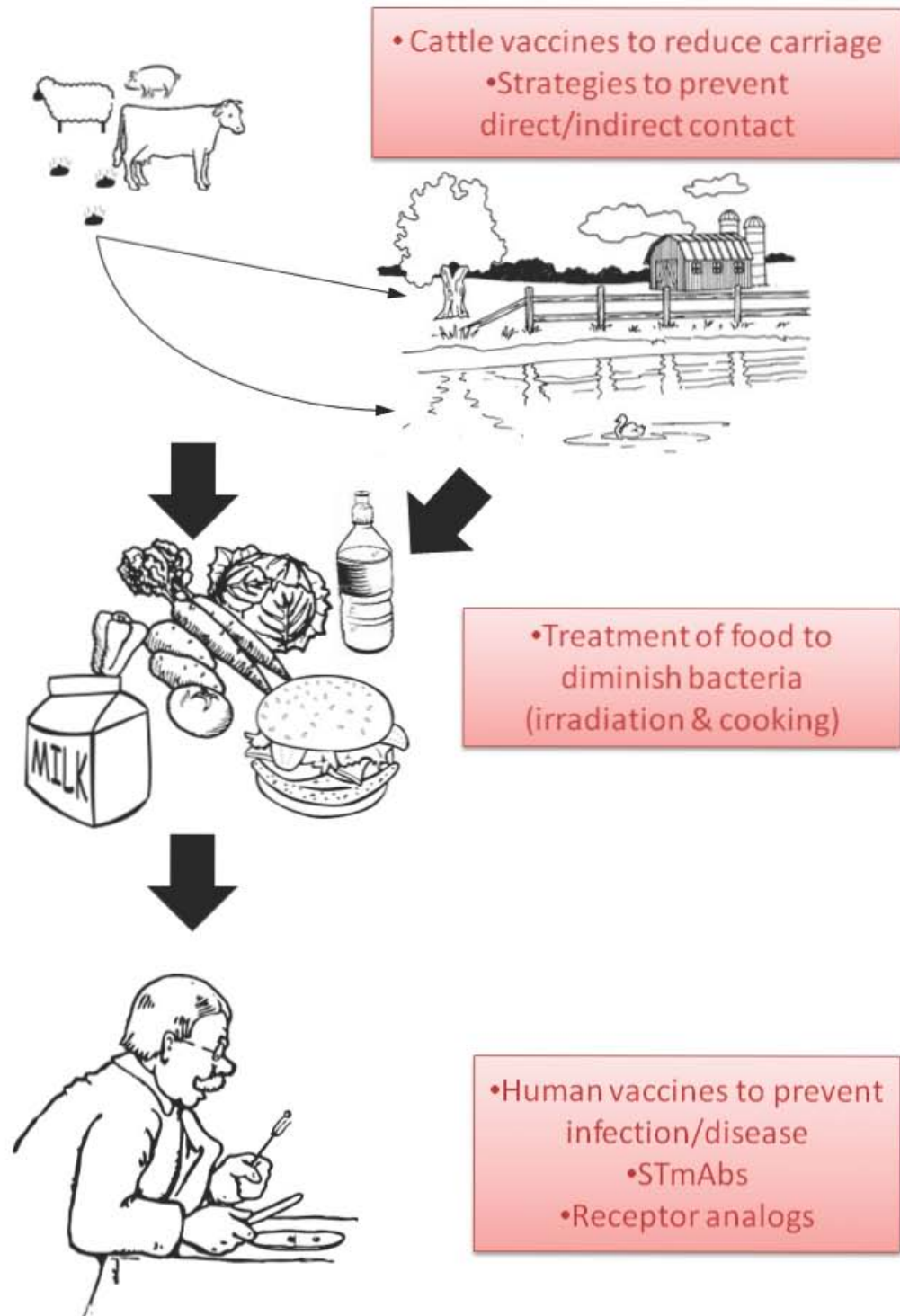
Unfortunately the downward trend in number of cases associated with *E. coli* O157:H7 outbreaks has recently stalled (46). The current plateau in disease incidence in conjunction with reports of large multi-state outbreaks has lead to speculation by some authorities that safety problems still exist within the food processing system. On-going governmental food safety efforts are focused on reducing the contamination of meat, poultry, produce, and other foods. Furthermore, in response to the 2006 spinach outbreak, the FDA has ruled to allow irradiation of lettuce and spinach (46).

       E. coli O157:H7 infection generally represents the largest of the STEC disease burden, both in the U.S. and worldwide. *E. coli* O157:H7 infection has been reported in more than 30 countries over 6 continents; high rates of infection are noted in Scotland, Canada, Japan, Argentina, and the U.S. (189). Overall, the five most common serotypes of STEC that are responsible for disease worldwide are O157:H7, O26:H11, O103:H2, O145:H⁻, and O111:H⁻ (147).

Transmission

       Since the first major outbreaks of *E. coli* O157:H7 were traced to contaminated ground beef, cattle were suspected as a reservoir for this pathogen. Therefore, investigators began culturing fecal specimens obtained from cattle on farms previously implicated in *E. coli* O157:H7 outbreaks. They found that *E. coli* O157:H7 could be recovered from heifers and calves (but not adult cattle) in Wisconsin, Washington, and Canada (116). The overall prevalence of *E. coli* O157:H7 carriage in cattle is now estimated to be somewhere between 0.5-2.0%; however, carriage rates can be much higher when *E. coli* O157:H7 is endemic within the cattle population (120). Moreover,

the rate of carriage of *E. coli* O157:H7 by cattle has been observed to fluctuate seasonally

(see below).

As a result of *E. coli* O157:H7 carriage in cattle, beef and dairy products often

become contaminated and serve as the source of infection in outbreaks of *E. coli*

O157:H7. However, many other vehicles for food-borne transmission of *E. coli*

O157:H7 have been identified and are described below (*O157:H7 in the Nation's Food*

Supply). In addition to transmission from contaminated food (or drink), person-to-person

and water-borne transmission [both likely facilitated by the low infectious dose of *E. coli*

O157:H7 infection (114, 116)] have also been described. Person-to-person transmission

can be exacerbated by asymptomatic and long-term carriage of the microbe by some

infected individuals (114, 189). Indeed, *E. coli* O157:H7 has been detected in the feces

of, in particular, children for weeks following resolution of their symptomatic infections

(189).   Other methods of *E. coli* O157:H7 transmission include occupational or

environmental exposure.

Reservoir in Nature

Cattle shed *E. coli* O157:H7 into the environment in their feces. Therefore, fecal

material can contaminate produce and water directly, or produce can be indirectly

contaminated by run-off from a farm or a contaminated water supply. While cattle are

the primary reservoir for *E. coli* O157:H7, the organism has been detected in the feces of

a wide array of animals that includes wild pigs [in the 2006 spinach outbreak (135)],

other ruminants (sheep, goats, buffalo, deer), horses, dogs, rabbits, and birds (gulls,

pigeons, chickens, turkeys) (41).

E. coli O157:H7 carriage by cattle is a common occurrence, although the carriage

levels may be transient or at a very low rate within a population. Nevertheless, the link

between carriage of *E. coli* O157:H7 by cattle and patterns of human disease is perhaps

best illustrated by both the regionality of outbreaks and the seasonality of *E. coli*

O157:H7 infection in humans. The precise reasons that *E. coli* O157:H7 outbreaks occur

predominantly in the northern states and Canada are unknown (116). However, one

explanation for this phenomenon is that larger livestock populations are present in

northern states, and, therefore, individuals who live in those areas have a greater chance

of exposure to the organism than do people who live in states with fewer cattle (50, 81).

In addition to this regionality of cattle distribution and human *E. coli* O157:H7 disease,

shedding of *E. coli* O157:H7 by cattle is more pronounced in the summer months (121).

As mentioned earlier, during the summer months there is also a concurrent spike in

number of reported *E. coli* O157:H7 outbreaks.

In addition to the seasonal nature of *E. coli* O157:H7 shedding by cattle, other

factors have been proposed to play a role in the levels of the organism excreted by these

animals. These variables include age, diet, stress, and husbandry conditions (25). For

example, younger animals, i.e. heifers and calves, have higher carriage rates when

compared with adult cattle (119, 121). Furthermore, husbandry conditions can have a

large impact on carriage as illustrated by the finding that *E. coli* O157:H7 can persist on

certain farms for prolonged periods (120). This persistence may relate to the

observations that *E. coli* O157:H7 can survive in the feces of cattle for up 99 days (29)

and can be recovered from the feed and drinking water of cattle (120). Other factors that

may play a role in *E. coli* O157:H7 carriage in cattle include the presence of super-

shedders among the cattle population (50) and the possibility that intermediaries (birds, insects, and rodents) may act as transmission vectors to spread the organism among neighboring farms (74, 120, 170, 217).

Previous reports based on experimental bovine models noted that "weaned calves, like neonatal calves, are susceptible to intestinal damage induced by EHEC O157:H7" (61). Additionally, diarrheal disease was manifest, to varying degrees, in some (but not all) infected animals; in fact, a few such infected calves displayed blood-tinged diarrhea (35, 59, 61, 63). On the other hand, most adult cattle appeared healthy following experimental infection with *E. coli* O157:H7 (59, 113), and on necropsy, no intestinal pathology was evident (59). However, in a more recent study of experimentally-infected cattle, Baines and colleagues challenged yearling steer with a mixture of *E. coli* O157: H7 strains that had been isolated from humans or cattle, and observed pathology in the intestines (18). Baines *et al*. reported that mild to severe pathological lesions were evident in the small and large intestines of cattle, with the most damage seen in the jejunum. These researchers also observed *E. coli* O157:H7 A/E lesion formation in the jejunum of the infected cattle. Furthermore, Baines and colleagues noted a link between the time of persistence of *E. coli* O157:H7 shedding by certain infected cattle and the period needed to resolve *E. coli* O157:H7-evoked intestinal pathologies. This association suggested to these investigators that the persistently shedding animals were more highly colonized than the non-persistently shedding animals, an assumption supported by the larger numbers of *E. coli* O157:H7 found in their feces over time compared to animals that cleared the challenge organism more quickly (18). Based on their findings, Baines *et al*. concluded that the heterogeneity among cattle in terms of degree of *E. coli* O157:H7

shedding is related to the extent of colonization of the small and large intestines by *E. coli* O157:H7: the higher the colonization load at those sites, the more persistent the shedding (18).

Petting Zoos

Animal contact was first reported as a mode of *E. coli* O157:H7 transmission and infection in 1996. Exposure to calves or cattle occurred either directly or indirectly. Such exposures have been noted at farms, county fairs, and petting zoos (236). As a result, handwashing following animal contact, especially at petting zoos, is recommended to deter the spread of *E. coli* 0157:H7 to visitors at such locales.

Water-borne Transmission

Transmission of *E. coli* O157:H7 by drinking and recreational water has been reported (236). In fact, the largest outbreak of *E. coli* O157:H7 in the United States occurred in 1999; the source of contamination was traced to drinking water provided at the Washington County Fair in upstate New York. The water was derived from a temporary, unregulated well that was found to be contaminated with *E. coli* O157:H7. In this outbreak, 781 cases were reported. Of these infected individuals, 9% were hospitalized, 2% developed HUS, and 2 died (236).

O157:H7 in the Nation's Food Supply

During the period from 1982-2002 food-borne outbreaks accounted for 52% of all *E. coli* O157:H7 outbreaks in the U.S. (236). The vehicle of *E. coli* O157:H7

transmission and infection in food-borne outbreaks can be one of a variety of sources

including beef (ground beef, roast beef, steak, salami, etc.), produce (unpasteurized apple

cider or juice, melons, grapes, lettuce, bean sprouts, spinach, etc.), or dairy (raw milk,

cheese, butter, etc.) (236). The spread of *E. coli* O157:H7 by these various food matrices

is facilitated not only by the pathogen's low infectious dose (116), but additionally by the

pathogen's capacity to grow over a broad temperature range and to survive both freezing

and acidic conditions (196).

The public outcry that followed the JITB outbreak that involved over 700 people

prompted major changes in the food industry. Notably, the Food and Drug

Administration (FDA) passed new regulations that raised the recommended internal

temperature for hamburgers cooked in restaurants. The Food Safety and Inspection

Service (FSIS) of the United States Department of Agriculture (USDA) declared *E. coli*

O157:H7 "an adulterant in raw ground beef" and launched several programs that included

those designed to increase the public's awareness of safe-handling procedures and the

importance of cooking meat properly (111). In 1994, *E. coli* O157:H7 became a

nationally-notifiable disease, with reporting mandated in 48 states (236). Moreover, the

CDC requested and received additional funds for the establishment of the Foodborne

Diseases Active Surveillance Network, also known as FoodNet (111). Members of the

public responded to the JITB outbreak by banding together and forming STOP (Safe

Table Our Priority), "the first consumer activist group devoted to food safety" (111).

The Emergence of Clade 8

Until the last 3 years, it appeared that the severity of *E. coli* O157:H7 infections in the United States was decreasing, as indicated by both the incidence of HUS and case-fatality rates. In a 2005 publication, Rangel *et al*. suggested that one explanation for this impression was that case reporting had increased and included patients with less clinically severe disease (236). However, a rise in both the hospitalization and HUS rates has been reported in association with more recent outbreaks of *E. coli* O157:H7. In data collected from outbreaks that occurred between 1982-2002, the average hospitalization rate was just over 17% and the average rate of HUS was ~4%. In two of the more prominent recent outbreaks, the spinach outbreak in 2006 and the cookie dough outbreak in 2009, increased rates of hospitalization and HUS were seen.

One hypothesis to explain the recent increase in rates of severe disease among those individuals infected with *E. coli* O157:H7 during recent outbreaks is that more virulent *E. coli* O157:H7 strains have emerged (186). In an attempt to address this hypothesis, Manning and colleagues performed single nucleotide polymorphism (SNP) analysis on large numbers of *E. coli* O157:H7 strains. In this manner, the *E. coli* O157:H7 strains they tested could be grouped into distinct SNP genotypes. Such genetic analyses, revealed 9 clusters or clades of *E. coli* O157: H7 clinical isolate strains (186). The spinach outbreak strain fell into clade 8, a clade that has been isolated more frequently since 2002. Members of clade 8 often carry the genes for both Stx2 and Stx2c (186). Additionally, members of this clade were not only responsible for the 2006 spinach outbreak but also another outbreak of severe disease in 2006 that was associated with produce (lettuce) (44, 48, 186). Furthermore, the *E. coli* O157:H7 strain associated

with the 2009 cookie dough outbreak appears to be an Stx2, Stx2c producer and thus

likely a member of clade 8 as well (P. Feng, personal communication). Thus, Manning *et*

al. surmised that a more virulent subpopulation (derived from clade 8) was emerging

among *E. coli* O157:H7 strains and that this evolutionary event was responsible for the

recent increase in overall severity of *E. coli* O157:H7 infections (186).

Disease, Diagnosis, and Treatment

Disease Features

       E. coli O157:H7 infection can manifest in a variety of ways. Some individuals

who are infected with the microbe remain asymptomatic, others experience diarrhea, but

most develop hemorrhagic colitis, the hallmark of *E. coli* O157:H7 infection.

Furthermore, children and the elderly appear especially susceptible to *E. coli* O157:H7-

mediated disease and, for reasons that are unclear, may develop hemolytic uremic

syndrome (HUS) and other systemic problems that include central nervous system (CNS)

impairment.

Asymptomatic Infection and Mild Illness

       The exact numbers of individuals who are afflicted with *E. coli* O157:H7-evoked

illnesses are difficult to determine because a portion of infected people experience only

mild disease. These individuals often do not seek medical attention, and, thus, their

illnesses are never reported or confirmed. In fact, for every one laboratory-confirmed

case of *E. coli* O157:H7, an estimated additional 4-8 symptomatic cases are missed (23).

Moreover, a population-based survey conducted in 1996-1997 determined that only 28%

of individuals with bloody diarrhea sought medical attention (23). Indeed, the incidence

of *E. coli* O157:H7 infection in the United States is estimated at ~20 times greater than is

reported (190). The consequences of not recognizing infection with this organism are

significant because individuals who have mild or asymptomatic *E. coli* O157:H7

infection can potentially transmit the infection to others. For this reason, following the

Sakai City outbreak in Japan, asymptomatic students infected with *E. coli* O157:H7 were prevented from attending school until their stools were free of the organism (197).

Hemorrhagic Colitis

Following ingestion *of E. coli* O157:H7, there is an incubation period of approximately 3-5 days before illness ensues. In a typical scenario, disease initially presents as non-bloody, watery diarrhea with severe abdominal cramping followed in a few days by the hallmark of *E. coli* O157:H7 infection, bloody diarrhea, or HC. However, as indicated above, a portion of those infected never progress to develop HC. These individuals generally have milder disease with a shorter duration of diarrhea and are less likely to manifest the accompanying symptoms, as described below (115).

The most common reported symptom of *E. coli* O157:H7 infection is bloody diarrhea. However, in addition to the bloody diarrhea and abdominal cramps, vomiting has been reported, albeit less frequently. While patients generally lack a fever, low-grade fevers have also been reported. The illness can be severe enough that people will seek medical attention. Most affected individuals will undergo spontaneous recovery from HC caused by *E. coli* O157:H7 within 5-7 days but upwards of 16% (although rates are lower in sporadic cases) will progress to develop the hemolytic uremic syndrome.

Hemolytic Uremic Syndrome

Hemolytic uremic syndrome or HUS is the most serious sequela of *E. coli* O157:H7 infection and can result in death. HUS is generally considered to include a triad of clinical manifestations: hemolytic anemia, thrombocytopenia, and renal failure (33).

These disease features are indicated by a decreased hematocrit with erythrocyte destruction evident in peripheral blood smears (hemolytic anemia), a reduction in platelets (thrombocytopenia), and an increase in urea and creatinine levels in the blood (renal dysfunction) (8). These latter blood chemistry findings occur because the kidneys can no longer function to properly excrete breakdown products in the urine. Approximately half of patients who progress to develop HUS will require dialysis, and 3-5% will succumb to the disease (189). Some but not all patients who recover from HUS suffer long-term complications (102), but the exact percentage of such individuals is unclear because such manifestations as the onset of high blood pressure may not be clearly ascribed to HUS (101).

Several factors can contribute to the likelihood that a patient will develop more severe disease after *E. coli* O157:H7 infection. These factors include: dose of *E. coli* O157:H7 ingested, age, and the characteristics of the infecting strain (116). Dose was suspected to play a role in disease in the very first outbreak of *E. coli* O157:H7; individuals who consumed Big Macs were more likely to develop disease in comparison to those who only ate regular hamburgers (239). The biggest risk factor for both the development of symptomatic disease and the progression to severe disease is age. Young children and the elderly are at an increased risk as illustrated by the numerous outbreaks that have occurred in connection with day-care centers and nursing homes. In fact, HUS is the primary cause of acute renal failure among children in the U.S, and ~90% of such cases follow diarrhea that is usually caused by *E. coli* O157:H7 infection (184).

Clinical Diagnosis

The initial presentation of *E. coli* O157:H7 is consistent with infection by a number of diarrheal agents and may alert medical providers to request collection of stool samples for enteropathogen screening. However, the severe abdominal pain that is a common feature of *E. coli* O157:H7 infection can mislead the attending physician. Indeed, the cause of such severe cramps in patients who have *E. coli* O157:H7 infection has been misdiagnosed as intussusception (kids), acute inflammatory bowel disease (young adults), or ischemic colitis (elderly). In some instances, unnecessary laparotomies or appendectomies have been performed.

Screening for *E. coli* O157:H7 from stool samples can be easily accomplished as this organism ferments sorbitol slowly; when grown on sorbitol-containing MacConkey (SMAC) agar *E. coli* O157:H7 will appear as colorless, sorbitol-negative colonies after 24 hours. SMAC-negative colonies can be further screened with anti-O157 typing sera to agglutinate bacteria that express the O157 O-antigen polysaccharide. Additionally, toxin detection assays are available to screen for *E. coli* O157:H7 and other STEC (Vero cell cytotoxicity assay, toxin ELISA). Since *E. coli* O157:H7 can be easily recovered from the feces of infected individuals within the first week of symptomatic presentation, stool specimens obtained from patients can be cultured and screened by the methods described above to confirm a diagnosis of *E. coli* O157:H7.

Treatment and Prevention

Treatment of *E. coli* O157:H7 infection in the United States is limited to supportive care because antibiotics and anti-motility drugs are contraindicated as

therapies. This restriction on the use of antibiotics to treat *E. coli* O157:H7 infection in

particular is based on retrospective studies that showed that the chance of an infected

patient progressing to HUS is greater if so treated (277). One explanation for this finding

is that certain antibiotics, such as ciprofloxacin, can cause induction of lysogenic Stx-

converting phage as has been demonstrated *in vitro* and *in vivo* in mice (318). As a result

of the limited treatment options, patients who have documented *E. coli* O157:H7

diarrheal illness and who may be developing HUS require hospitalization because the

management of this disease (rehydration therapy) must be carefully supervised.

The most promising prevention strategies for *E. coli* O157:H7 focus on

minimizing exposure to this pathogen (Figure 2). Some of these measures were

described elsewhere (*Petting zoos* and *O157:H7 in the Nation's Food Supply*). Others

are still to be discussed in the section entitled "Therapeutics and Vaccines."

Pathogenic Determinants of *E. coli* O157:H7

E. coli O157:H7 colonizes the intestines and causes disease in the absence of enteroinvasion. *E. coli* O157:H7 shares a number of virulence determinants, and hence pathogenic properties, with EPEC (another member of the pathogenic *E. coli*). In fact, Whittam and colleagues proposed that *E. coli* O157:H7 evolved from EPEC O55:H7 (168, 312). These investigators further hypothesized that the acquisition of toxin-carrying phage, the pO157 plasmid, as well as additional acquisitions, mutations, losses, and further host adaptations by EPEC O55:H7 resulted in the evolution of the *E. coli* O157:H7 lineages we know today (168, 312).

E. coli O157:H7 is well adapted to cause disease in humans. It has a number of virulence factors that contribute to its pathogenicity, the most important of which is Shiga toxin. Indeed, Stx is considered to be responsible for the severe complications of *E. coli* O157:H7 infection, including HUS. In addition to Shiga toxin, *E. coli* O157:H7 expresses several other important virulence factors that include intimin, the translocated intimin receptor (or Tir), a type three secretion system (TTSS), and enterohemolysin (located on the pO157 plasmid) (Figure 3). The genes for many of these factors are located within a 44 kb pathogenicity island known as the locus of enterocyte effacement, or the LEE locus.

Shiga toxin

Shiga toxin (Stx) was first discovered in extracts of *Shigella dysenteriae* type 1 in the early 1900s (156). These extracts caused limb paralysis and death in experimental animals, and, hence, the toxin within these extract was originally named Shiga

Figure 3: *E. coli* O157:H7 virulence factor expression and interaction with host cells

       E. coli O157:H7 possesses a large plasmid (pO157), carries the LEE PAI (and thus is intimin-positive), and expresses Shiga toxins. The LEE locus encodes a TTSS and TTSS effector proteins. One of the TSSS proteins, EspA, forms a filament that serves to translocate TTSS effector proteins from the bacterium into the host cell by way of a pore created by EspB and EspD. One of these effectors, Tir, serves as the receptor for the major adhesin, intimin, and thus allows adherence of the bacterium to the host cell. The intimin-Tir interaction, coupled with the effects of other TTSS effectors, induce formation of the characteristic A/E lesion.
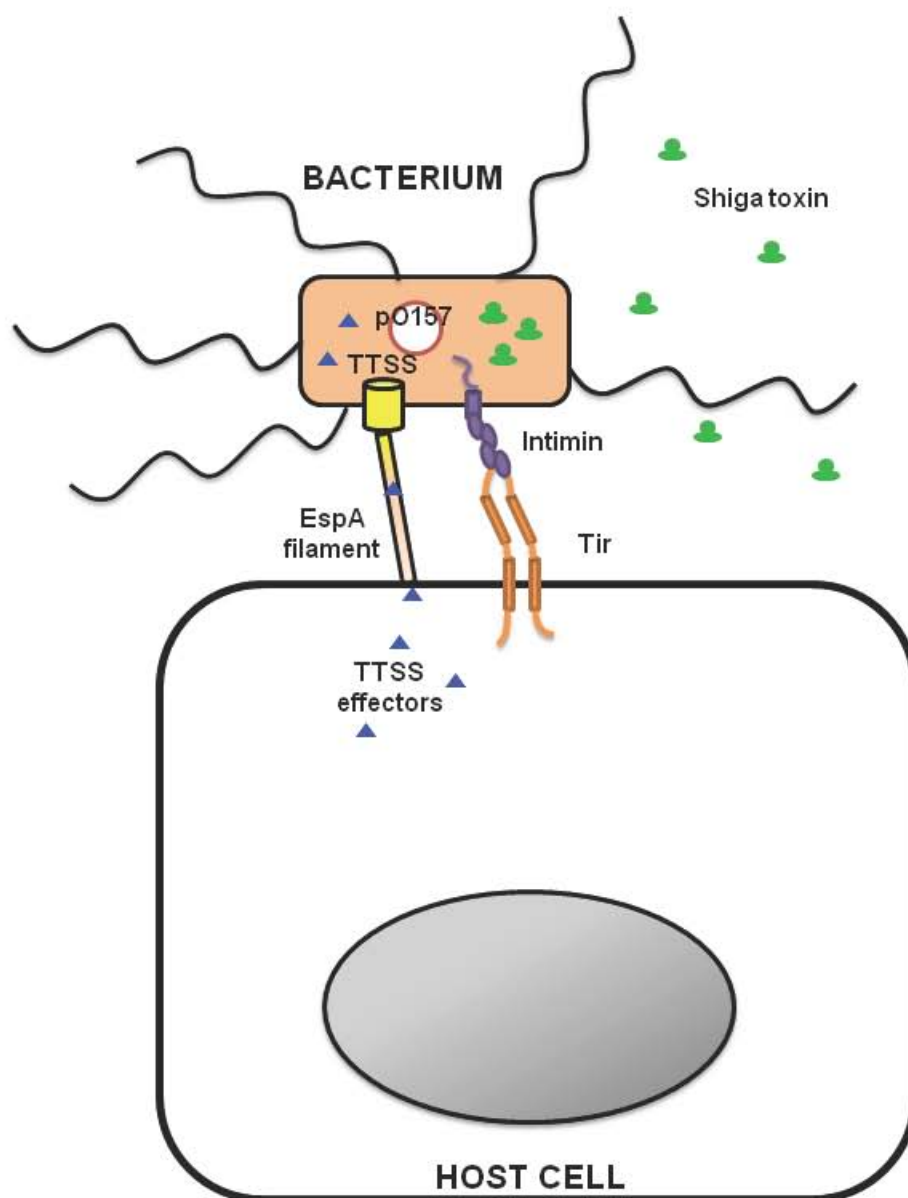
"neurotoxin" (now called Shiga toxin), after the discoverer of *Shigella dysenteriae*, Dr. Kioshi Shiga (156, 222). The discovery that *E. coli* O157:H7 made similar toxins did not occur until many decades later, and this finding was dependent on work done by Konowalchuk *et al.* in 1977. Konowalchuk *et al.* were the first to observe that certain strains of diarrheagenic *E. coli* make a cytotoxin that can kill Vero cells (164). Following this initial report, O'Brien *et al.* purified and characterized the cytotoxin from one of Konowalchuk's *E. coli* strains [noting its similarity to Shiga toxin of *Shigella dysenteriae* type 1, O'Brien *et al.* named this toxin Shiga-like toxin or SLT (223)] and demonstrated that this toxin could be neutralized by anti-*Shigella dysenteriae* type 1 toxin antibody (224).

Concurrently, *E. coli* O157:H7 was emerging as a pathogen responsible for HC and HUS. Karmali *et al.* detected a cytotoxin for Vero cells in the feces of patients who presented with HUS and who were infected with one of several serotypes of *E. coli* (including *E. coli* O157:H7). Karmali *et al.* called this toxin Vero toxin or VT after Konowalchuk's discovery (151). O'Brien *et al.* subsequently reported that Shiga-like toxin and Vero toxin were in fact the same toxin and, furthermore, that this toxin was produced by *E. coli* O157:H7 (225). While the nomenclature SLT and VT are still in use today, this apparent distinction (when in fact none exists) can be confusing. In 1996, the nomenclature was revised (39), and, for the remainder of this discussion, SLT, VT, and Stx will be referred to together as Shiga toxin.

Not long after the discovery that *E. coli* O157:H7 makes Stx, Karmali *et al.* reported an epidemiological link between STEC and the development of HUS; namely, STEC, Stx, or both were detected in the feces of pediatric HUS patients but not in

controls (150, 151). In their latter report, Karmali *et al.* proposed that Stx is involved in the pathogenesis of HUS. This seminal concept has been corroborated with additional epidemiological evidence (4, 220, 230) and extensive animal studies (79, 100, 182, 245, 258, 280, 321).

Toxin serotypes

Following the discovery that *E. coli* O157:H7 produced Stx, evidence began to mount that in fact two antigenically distinct toxins exist. In 1986, Strockbine *et al.* reported that *E. coli* O157:H7 strain 933 (an isolate from the original outbreak in 1982) did encode two similar, yet serologically distinct Stxs and that the genes for these toxins were carried on different toxin-converting phages (269). Strockbine *et al.* proposed that the originally reported toxin, which is neutralized by anti-*Shigella dysenteriae* type 1 toxin antibody and is associated with induction of 933J phage, be referred to as Stx1 and the other toxin, which is expressed after induction of the 933W phage, be called Stx2 (269). Thus, the Stx family of AB_5 toxins (A for enzymatic activity and B for binding) contains two subgroups, Stx1 and Stx2. In addition to these two Shiga toxin serotypes, variants of each have been described: Stx1c, Stx1d, Stx2c, Stx2d, Stx2d-activatable, Stx2e, and Stx2f [reviewed in (195)].

Stx1 and Stx (of *Shigella*) are nearly identical; the sole difference between the toxins is a single amino acid residue change in the A subunit (193). While Stx1 and Stx2 have the same overall structure (see below), these toxins are antigenically distinct such that antibody against Stx1 will not neutralize Stx2, and vice versa (311). On the amino acid level, Stx1 and Stx2 share 61% overall identity and 75% overall similarity, with the

B subunits of these toxins more highly conserved than the A polypeptides (265). Stx1 and Stx2 also have the same mode of action, but they differ in both their activities *in vitro* and in toxicity for mice. The cytotoxic dose 50% (CD_{50}) of Stx1 for Vero cells is lower than that seen for Stx2 (266). However, the lethal dose 50% (LD_{50}) of purified Stx1 for parenterally or intravenously inoculated, adult CD-1 mice is ~125 ng compared to ~1 ng for Stx2 (264). Moreover differences in toxicity are also evident when human renal endothelial cells are treated with purified Stx1 or Stx2; Stx2 is about 1,000-fold more toxic (179). Finally, epidemiological data suggest a difference in Stx1 and Stx2 toxicity in people; Stx2-producing *E. coli* O157:H7 strains are more frequently associated with HUS than are strains that produce Stx1 (28, 228, 251).

Structure and Mode of Action

As mentioned above, Shiga toxins are AB_5 toxins (Figure 4); they are composed of a single A or active subunit with a molecular mass of ~32.2 kDa that is noncovalently associated with a pentameric ring of B or binding subunits, each with a molecular mass of ~7.7 kDa (221). The B subunits function to bind the cellular toxin receptor, globotriaosylceramide (also known as Gb_3 or CD77), a Pk blood group antigen common on a variety of cells (175, 176). Upon binding of the B pentamer to Gb_3 [~ 3 binding sites per B subunit (174)] on a host cell, the toxin becomes internalized via receptor-mediated endocytosis, undergoes retrograde transport to the endoplasmic reticulum, and the A_1 fragment (see below) is eventually released into the cytoplasm to exert its activity (244) (Figure 5). Once the toxin is taken into the cell within an endocytic vesicle, the A subunit of Stx is cleaved by furin and then reduced so as to form an enzymatically active

Figure 4: Crystal structure of Stx2

The crystal structure of Stx2 as adapted from Fraser *et al*. 2004 (91). The A_1 fragment, A_2 fragment, and B pentamer are indicated. The A subunit consists of an A_1 fragment and an A_2 fragment. The B pentamer is comprised of five identical B subunits. The A_1 fragment is the active portion of the A subunit that inhibits protein synthesis in the host cell. The A_2 fragment serves to link the A_1 fragment with the B pentamer during binding of the toxin to the host cell surface and during subsequent translocation of Stx2 into the cell.

A₁ fragment

B pentamer
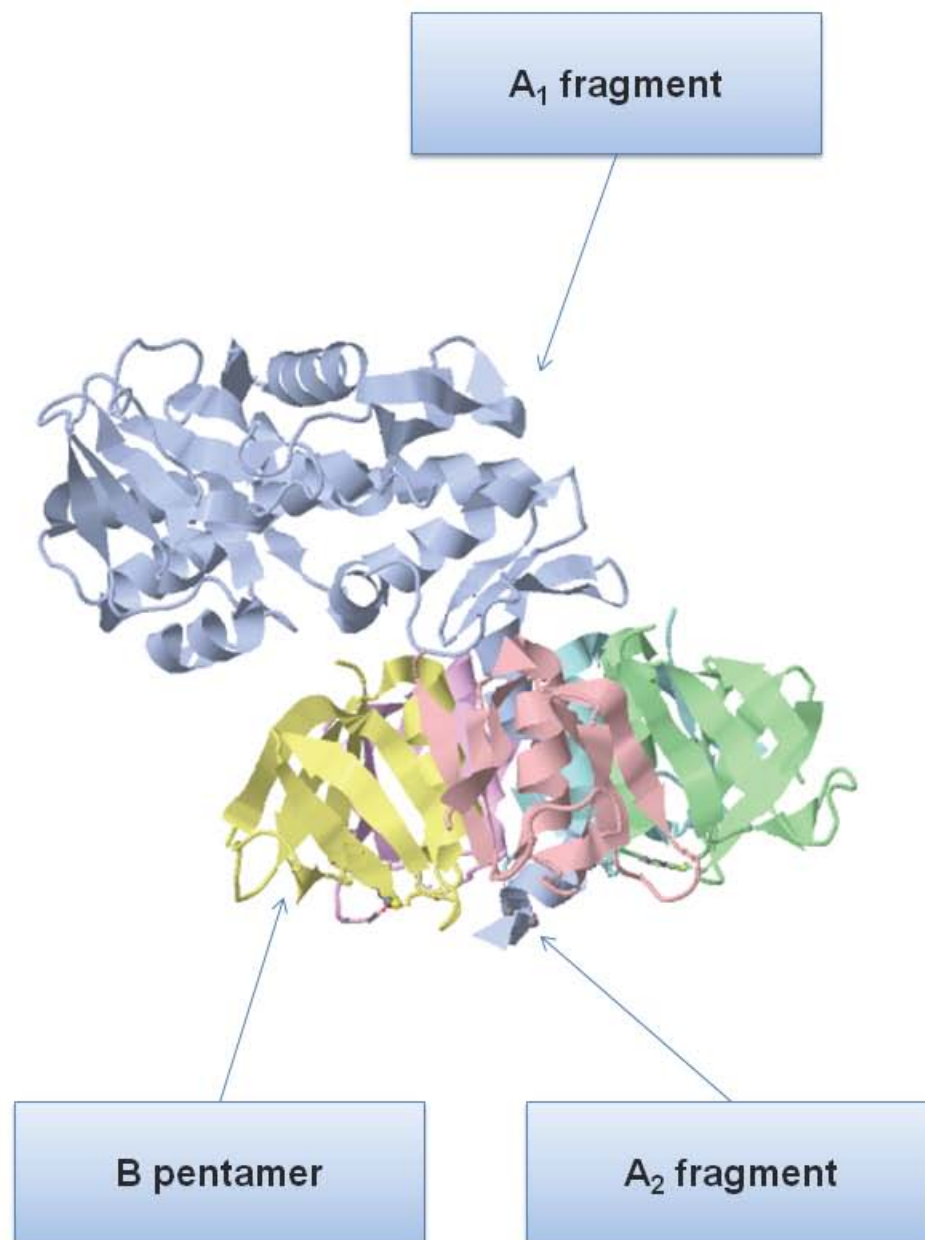
A₂ fragment

Figure 5: Trafficking of Stx in a host cell

Shiga toxin binds to the glycolipid globotriaosylceramide (Gb_3) and is endocytosed in a receptor-mediated fashion. Cleavage of the A subunit into the A_1 and A_2 fragments occurs in either the endocytic vacuole or the *trans*-Golgi network (TGN). The toxin is trafficked in a retrograde fashion to the TGN and then the endoplasmic reticulum (ER). In the ER the disulfide bond that holds the A_1 and A_2 fragments together is reduced, a reaction that allows the A_1 fragment to dissociate from the A_2 fragment and B pentamer. The A_1 fragment can then be released into the cytoplasm where it acts on the 28S ribosomal subunit to inhibit protein synthesis by preventing peptide chain elongation. Adapted from Johannes and Romër 2010 (138).

Receptor-mediated endocytosis

Retrograde traffic to *trans*-Golgi network

Retrograde traffic to ER

Translocation to cytosol

Inhibition of protein synthesis
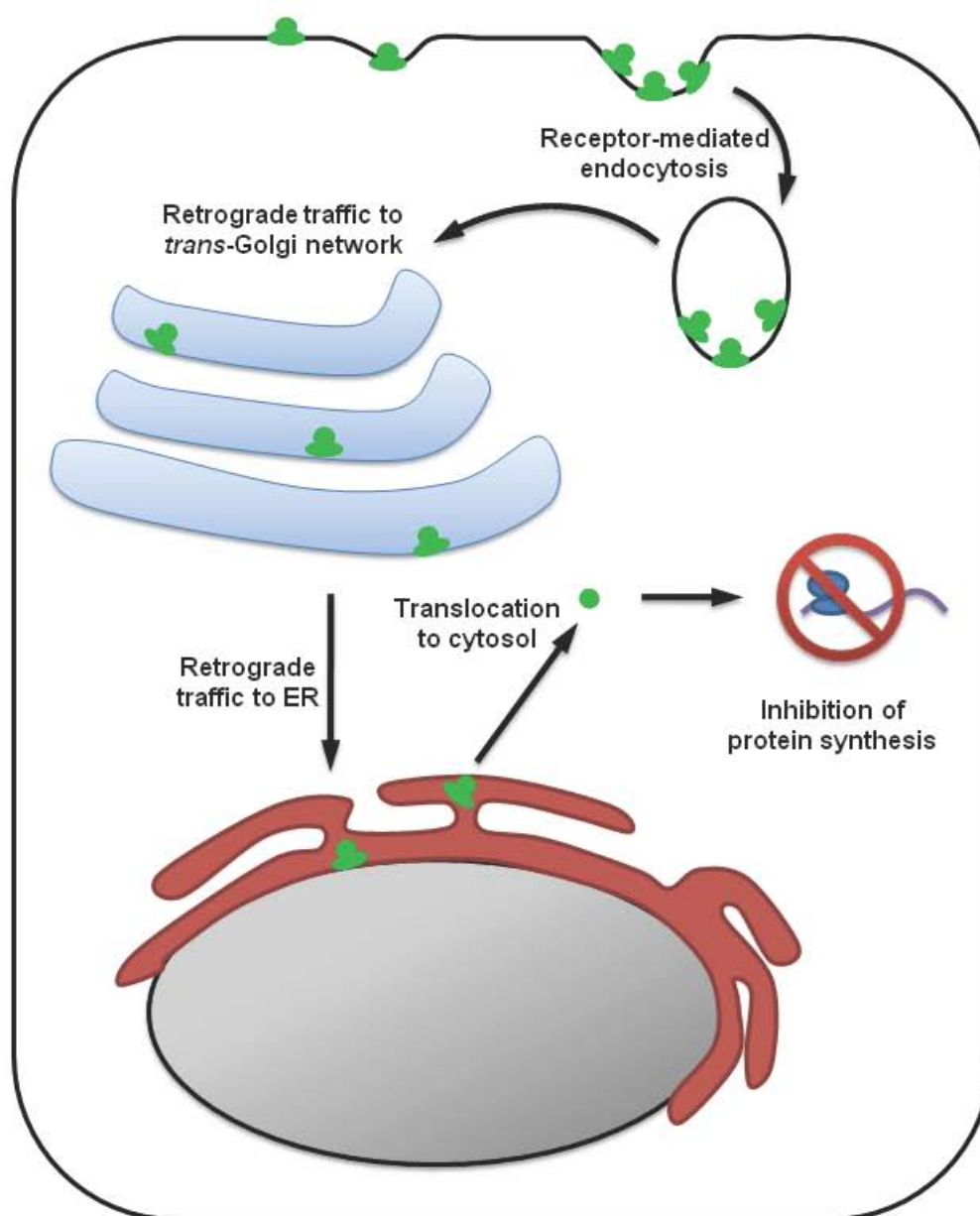
A_1 polypeptide and an A_2 fragment that serves to connect the A subunit to the B subunits (15, 104). While this cleavage event results in the activation of Stx, it may not be absolutely dependent on furin; in some cell types, such as Vero cells, Stx1 and Stx2 are readily nicked by other cellular proteases (103, 104, 243). The A_1 fragment in the host cell cytoplasm acts as an N-glycosidase to remove a single adenosine residue on the 28S ribosomal RNA of the 60S ribosome (75, 248). This action then inhibits protein synthesis by preventing the binding of elongation factor to the ribosome (226). The result of this Stx-mediated inhibition of cellular protein synthesis is generally death of the intoxicated cell by apoptosis.

pO157 plasmid

The pO157 is a 93 kb plasmid (~60 MDa) found in most *E. coli* O157:H7 isolates as well as other EHEC strains (172, 215). While the plasmid is thought to be important for virulence [studies have correlated pO157 with hemolytic activity (250) and intestinal adherence (145)], its exact role in pathogenesis is unclear [reviewed in (215)]. The plasmid has been sequenced and contains 100 open reading frames, only 19 of which have confirmed functions (36). One important product encoded for by the pO157 plasmid is an enterohemolysin. This enterohemolysin, or EHEC-hemolysin, is encoded by the gene locus *ehxCABD* and shares >60% homology with the chromosomally encoded alpha-hemolysin present in a number of *E. coli* (250). Enterohemolysin is a pore-forming RTX (repeats in toxin) toxin that can lyse washed sheep erythrocytes (26). Enterohemolysin is expressed *in vivo*, since it reacts with sera from HUS patients (249). The pO157 plasmid also encodes ToxB, a protein shown to promote adherence of *E. coli*

O157:H7 to tissue culture cells (278). ToxB shares sequence similarity to the adhesins

EHEC factor for adherence (Efa-1) and lymphostatin (LifA or lymphocyte inhibitory

factor) of other EHEC and EPEC, as well as homology to members of the large

Clostridial toxin family (144). In addition, the pO157 plasmid encodes a type two

secretion system (36); its effectors (StcE and YodA) have been proposed to have a role in

promoting *E. coli* O157:H7 adherence (123).

Locus of Enterocyte Effacement

The locus of enterocyte effacement, or LEE, is a chromosomally-located

pathogenicity island (PAI) that encodes a variety of pathogenic determinants; these

virulence-associated factors include an adhesin (intimin), a type three secretion system

(TTSS), and TTSS effector proteins. A similarly arranged LEE PAI can be found in the

related enteropathogen EPEC, although it is larger in EHEC (44 kb) than in EPEC (35

kb). Feng *et al*. speculate that some form of a LEE PAI was present in the EPEC-like

strain that likely served as a common progenitor for both the atypical EPEC O55:H7

group and an EHEC lineage (including *E. coli* O157:H7) (77); however the presence of

the LEE at a different insertion site in other STEC members indicates alternative

evolutionary pathways for these strains [reviewed in (168) and (67)]. The LEE can be

divided into three regions: a region furthest upstream that encodes the TTSS, a middle

region that encodes intimin and Tir, and a downstream region that encodes the effector

proteins of the TTSS (90).

Intimin

Intimin is an outer membrane protein found on the surface of EHEC, EPEC, and *Citrobacter* organisms and is encoded by the *eae* gene located within the LEE. Intimin was first discovered by Jerse *et al.* in EPEC in 1990 (137). The *eae* locus was so named because it was associated with the <u>E</u>. *coli* <u>a</u>ttaching and <u>e</u>ffacing phenotype (137). Intimin is a 97 kDa protein that is the major adhesin of *E. coli* O157:H7. In *E. coli* O157:H7, intimin allows the bacterium to intimately adhere to an epithelial surface (thus the name intimin); *E. coli* O157:H7 lacks a <u>b</u>undle-<u>f</u>orming <u>p</u>ilus (BFP) that is required for full virulence of EPEC (90). Although STEC strains that lack intimin can cause human disease (78, 233), *E. coli* O157:H7 appears to require intimin to establish colonization (56, 66, 140, 188, 259, 295, 314).

Intimin can be dissected structurally into three parts: a periplasmic tail at the amino terminus, a conserved porin-like transmembrane domain, and an extracellular domain at the carboxy terminus. The extracellular domain of intimin interacts with various receptors to facilitate *E. coli* O157:H7 adherence. Sequence variations within this extracellular domain among intimin-expressers serve as the basis for the classification of intimin into subtypes; *E. coli* O157:H7 expresses γ intimin, EPEC generally express α intimin, and *Citrobacter* has intimin type β. Indeed, Oswald *et al.* postulated that the slight sequence variations seen in α, β, and γ intimin gave rise to the various tissue tropisms (small intestine and/or large intestine) of the corresponding intimin-expressing organisms (229). Furthermore, several groups attempted to modify the pathogenicity and tropism of an organism by altering the type of intimin it expresses with varying success (66, 295).

Several investigators have speculated that *E. coli* O157:H7 colonization initially proceeds via the interaction of the outer membrane protein intimin with cell-surface-expressed factors (89, 260, 261). In fact, Frankel *et al.* showed that intimin can bind to β1 integrins (89), and Sinclair *et al.* established that intimin can adhere to nucleolin (260, 261). Thus, one model of the mechanism of *E.* coli O157:H7 colonization purports that host molecules may serve as an initial point of *E. coli* O157:H7 adherence prior to the formation of the highly avid interaction between intimin and Tir, the bacterially encoded intimin receptor (260). The tight interaction of intimin with Tir, perhaps in conjunction with the effects of the TTSS effectors, then induces cytoskeletal rearrangements that eventually culminate in the formation of an actin-rich pedestal that is characteristic of both of EHEC and EPEC (90). Furthermore, data suggest that once the bacterium has firmly adhered to the epithelial surface, intimin expression is down-regulated (159, 260). This apparent reduction in intimin expression appears to occur despite the prominent appearance of Tir underneath the bacterium (259, 260).

Translocated Intimin Receptor

E. coli O157:H7 is unique among bacterial pathogens (with the exception of other LEE-encoding bacteria) in that it encodes its own receptor, Tir, which is injected into target host cells by way of the TTSS. The *tir* locus is located on the LEE slightly upstream of *eae* and encodes a 72 kDa protein that is comprised of three functional domains: extracellular, transmembrane, and cytoplasmic (65, 90). Once Tir is inserted into the host cell plasma membrane, it adopts a hairpin-loop structure wherein both the amino and carboxy termini reside within the host cell cytoplasm (65) and the central

region of the protein is presented as the extracellular domain (90). The extracellular

domain of Tir binds to intimin (Figure 3). The host cell cytoplasmic regions of Tir

participate in a signaling cascade that leads to pedestal formation [(40); also described

above for intimin].

Type Three Secretion System and Effectors

The type three secretion system (TTSS) of *E. coli* O157:H7 is a contact-

dependent system for transport of bacterial proteins, or effectors, into host cells. The

genes for both the TTSS and the TTSS effectors are located on the LEE; the TTSS loci

are located upstream of those for the effectors. The TTSS effectors consist of EspA (for

E. coli secreted product A), EspB, EspD, EspF, and EspG, as well as Map (mitochondria-

associated protein) and Tir. EspA is a protein that forms a filamentous structure through

which the TTSS effectors are secreted (72, 161, 252) (Figure 3). EspB and EspD

function together to form a pore in the target host cell membrane that then allows entry of

the other effectors (127, 165, 254, 308, 313).

Pathogenesis of *E. coli* O157:H7

Knowledge of the pathogenesis of *E. coli* O157:H7-mediated disease in humans is quite limited. The use of human subjects to investigate the steps required for wild-type *E. coli* O157:H7 to evoke intestinal pathology is considered unethical because of the possibility that a volunteer could develop HUS. Thus, numerous animal models have been developed in an attempt to mimic various aspects of *E. coli* O157:H7 disease in humans. However, no one model has yet to fully recapitulate the spectrum of *E. coli* O157:H7 disease in people, and the models themselves sometimes provide contradictory results. The state of our understanding of *E. coli* O157:H7 pathogenesis in humans is summarized below.

Establishing Infection

As mentioned earlier, *E. coli* O157:H7 infection most commonly occurs following ingestion of contaminated food (although other sources of infection have been described). Ingestion of as few as 50 organisms appears to be sufficient to cause disease (288); this low infectious dose makes *E. coli* O157:H7 a particularly virulent pathogen. The fate of *E. coli* O157:H7 following ingestion is less clear. The recovery of the organism both from intestinal tissue (185, 270, 271) and from the feces of infected individuals indicates that *E. coli* O157:H7 is able to survive passage through the gastrointestinal tract. Since prolonged shedding of the organism has been reported, particularly in young children (114), *E. coli* O157:H7 clearly has the capacity to colonize the human gut. However, the preferred site of *E. coli* colonization within the human gastrointestinal tract is unclear at present (Figure 6).

Figure 6: Sections of the human gastrointestinal tract

The human gastrointestinal tract is composed of the oral cavity, the esophagus, the stomach, the small bowel [duodenum, jejunum, and ileum (indicated in red)], the ileocecal junction [separating the small bowel from the lower bowel (indicated in both red and blue)], the large bowel [cecum and colon (indicated in blue)], and the rectum. The diversity and quantity of organisms in the gastrointestinal tract varies by location with relatively few normal flora microbes in the stomach and small bowel, and much larger numbers of such organisms in the large bowel. Thus, the ileum acts as a transitional zone between the relatively sparsely colonized small intestines and the more highly colonized lower bowel.

Oral cavity

Stomach

Transverse colon

Duodenum

Ileum

Jejunum

Ileocecal valve

Descending colon

Ascending colon

Cecum
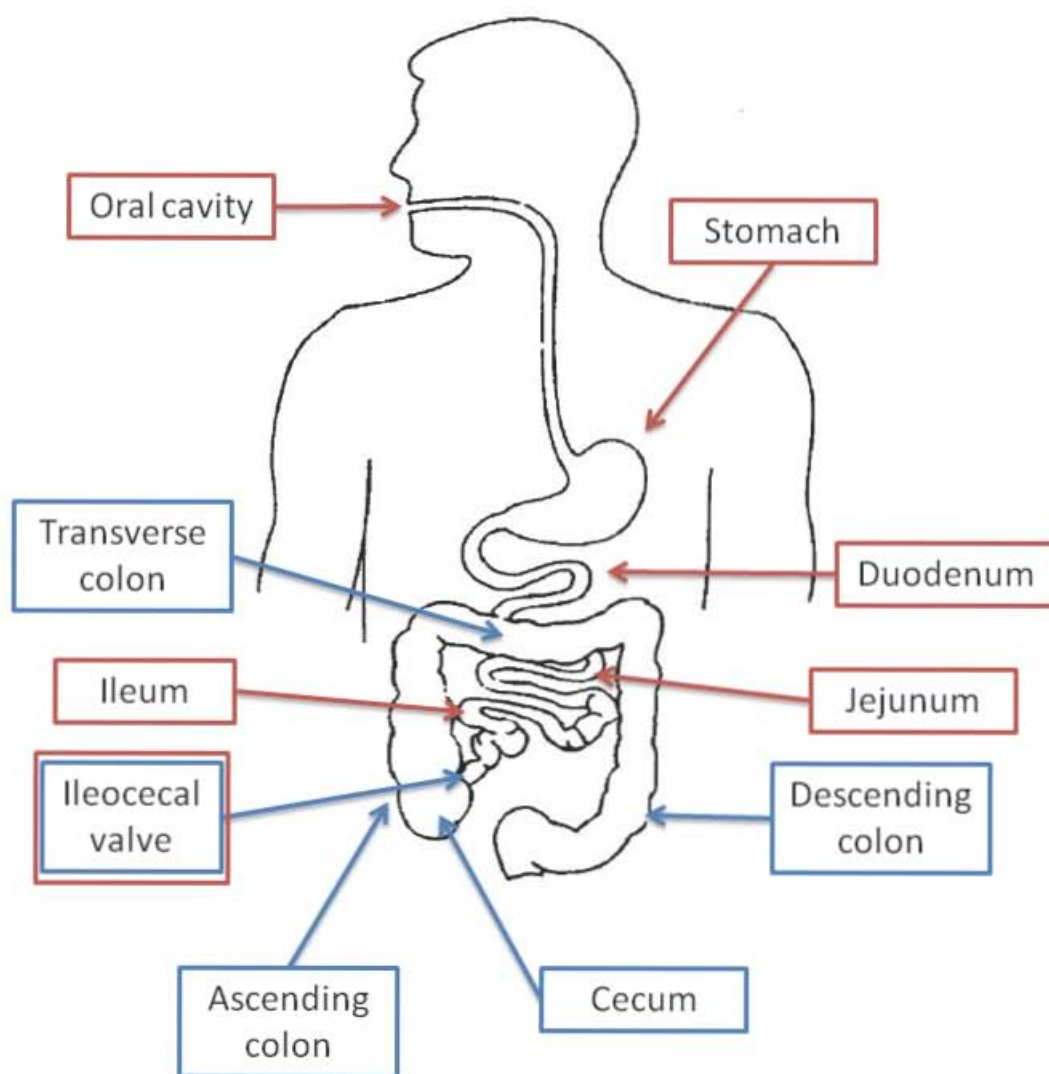
Early data, particularly from barium-enema radiograms of *E. coli* O157:H7 infected individuals' intestines, demonstrated what is now described as the characteristic "thumb-printing" pattern in areas of edema in the ascending and transverse colon (239). Thus, investigators surmised that *E. coli* O157:H7 was adhering to and colonizing in the colon. Indeed, upon endoscopy the colonic mucosa appeared edematous and hemorrhagic, with ulcerations or pseudomembranes seen in some instances (189). Furthermore, sigmoidoscopic and colonscopic examinations revealed a gradation of mucosal abnormalities, and these pathological lesions occurred more frequently in the cecum compared to the rectum (115).

For over two decades the dogma in the field remained that *E. coli* O157:H7 colonized the colon of people, despite the inability of researchers to convincingly and routinely demonstrate A/E lesion formation in that particular site (51, 143, 215, 253). Studies *ex vivo* on pathological sections from *E. coli* O157:H7-infected individuals have been used in attempts to decisively demonstrate the site for *E. coli* O157:H7 colonization. Phillips *et al.* were the first to report the application of *in vitro* organ culture (IVOC) as a method for evaluating how well exogenously added *E. coli* O157:H7 adhered to different types of human intestinal tissues. In fact, Phillips and colleagues found that a Stx-negative *E. coli* O157:H7strain (used to preclude destruction of the epithelium by toxin) adhered to the follicle-associated epithelium (FAE) of the Peyer's patches of the terminal ileum; no adherent bacteria were detected in the proximal small intestine, distal ileum, or transverse colon (235). Chong *et al.* extended these findings through evaluation of the adherence of additional *E. coli* O157:H7 isolates in an IVOC system. As was seen

previously, the *E. coli* O157:H7 strains bound to the FAE on Peyer's patches (51).

However, three strains (of the additional 5 tested) also bound with similar frequency to

the villous surface of the terminal ileum. The binding of the organism to the distal

duodenum was rarely observed, and of the 40 transverse colonic samples tested by IVOC,

A/E lesion formation occurred in only one (51). These reports suggest that the primary

site of colonization may be the FAE on Peyer's patches of the terminal ileum. Whether

subsequent colonization of the colonic epithelium can occur, presumably from these

initially seeded sites, remains to be determined.

Recently, Malyukova *et al.* analyzed historical intestinal tissue samples obtained

during the 1993 Jack-in-the-Box outbreak from *E. coli* O157:H7-infected individuals

with severe disease at biopsy or on autopsy (185); the clinical course of infection in the

patients was available and colonic damage was reported (211). Malyukova *et al.* stained

a portion of these tissue specimens and reported detection of *E. coli* O157:H7 at the

apical epithelial surface of a sample of the ileocecal valve; no stained bacteria were

present in the colonic samples tested (185). Malyukova and colleagues also

demonstrated the presence of Stx1 and Stx2 in the colonic tissue despite the absence of

detectable bacteria (185). This latter finding lends support to the theory that the colonic

damage reported in association with *E. coli* O157:H7 may be due to the presence of Stx

in the colon as opposed to destruction by the bacterium itself (51, 155, 235).

Systemic Manifestations

E. coli O157:H7 is not considered to be an enteroinvasive pathogen since it does

not breach the mucosal barrier and multiply submucosally. Furthermore, *E. coli*

O157:H7 bacteremia is rarely observed in patients (153, 277). In addition, few extra-intestinal *E. coli* O157:H7 infections have been reported, and these positive cultures (such as from the urine) were primarily from individuals who also had diarrhea (116). Thus, the systemic manifestations that arise from *E. coli* O157:H7 infections are likely the result of transit of bacterial factors from the site of colonization within the gastrointestinal tract. Shiga toxin is viewed as responsible for the severe systemic injury that may follow *E. coli* O157:H7 infection, since production of the toxin by the infecting organism is so closely linked epidemiologically to cases of HUS (150, 151).

The detection of free toxin in the feces of *E. coli* O157:H7-infected individuals supports the notion that toxin is produced locally within the gastrointestinal tract (150, 151, 230). While it is not fully understood how toxin translocates from the lumen of the gastrointestinal tract to the blood stream, the alleged detection of toxin in the blood and serum of infected patients (282, 283), in addition to the detection of toxin in the kidney (49, 298), though rare occurrences, indicate that such movement of Stx does occur. Stx in the circulation is proposed to be the cause of the observed injury to microvascular endothelial cells that results in thrombotic microangiopathy, a characteristic feature of HUS. The presence of such thrombi in individuals with HUS is evidenced by increases in the patients' plasminogen activator inhibitor 1 activity (acts to inhibit fibrinolysis), D-dimer concentrations (indicative of increases in fibrin generation), and fragment 1+2 concentrations (thrombin generation) [reviewed in (277)]. In addition, toxin is presumably trafficked to the kidneys by means of the circulatory system although how this occurs is unclear. While toxin has been detected in the circulation associated with

polymorphonuclear cells (PMNs) (34, 281-283), whether such PMNs serve as vehicles to transport Stx to the kidney (or elsewhere) remains a subject of controversy (84, 105).

The primary histopathological feature in HUS patients is the presence of glomerular lesions in the kidneys (237, 242), although tubular damage can be seen as well (49, 141, 275). Additionally, Stx can be detected bound to renal sections taken after death from *E. coli* O157:H7-infected HUS pediatric patients (298). That HUS is mediated by the impact of Stx on the kidney is indicated by the detection of toxin bound to renal sections coupled with the concurrent histopathological changes in that organ (49). These renal lesions are reflected in reduced kidney function as evidenced by increases in the patient's clinical laboratory values for blood urea nitrogen (BUN) and creatinine. Additional toxin-mediated systemic effects of *E. coli* O157:H7 infection are discussed elsewhere in association with animal data (*CNS Manifestations*).

Modeling *E. coli* O157:H7 Infection and Pathogenesis

In vitro *and* Ex vivo

In vitro systems such as cell monolayers, transwells, organoids, and *in vitro* organ culture (referred to as IVOC) as well as *ex vivo* cultures of biopsies are useful for the study of several aspects of *E. coli* O157:H7 pathogenesis such as adherence of the microbe to eukaryotic cells and the impact of Stx on those cells. In addition, the type of cells used often depends on the specific aspect of infection and/or pathogenesis under investigation. For example, epithelial cells are commonly used to evaluate *E. coli* O157:H7 adherence mechanisms while endothelial cells may be employed to examine the effect of Stx on vascular cell integrity and cytokine response.

Epithelial cell lines derived from different sources have been used to study adherence of pathogenic *E. coli* such as *E. coli* O157:H7. These cell lines include: HeLa (human cervix), HEp-2 (human larynx), HEL (human embryonic lung), Henle 407 (human small intestine), Caco-2 (human colon), HCT-8 (human ileocecum/colon), and T84 (human colon) (188, 276). The original reports that described *E. coli* O157:H7 adherence mechanisms used assays modeled after work done with EPEC because, like EPEC, *E. coli* O157:H7 evokes a characteristic A/E lesion when bound to the intestines (297). Moreover, both types of organisms evoke a FAS (fluorescent actin stain)-positive signal when adherent to HEp-2 cells (160). *E. coli* O157:H7 also adheres to intestinal tissue (both of animals and humans) *ex vivo* (18, 109, 235). In fact, pediatric intestinal biopsy samples have been used to demonstrate intimin-dependent (and specific) adherence of *E. coli* O157:H7 for the follicle-associated epithelium over Peyer's patches (82).

In an attempt to model not only adherence but also the early steps in *E. coli* O157:H7 pathogenesis, transwell systems were utilized to create polarized monolayers. Thus, researchers were able to explore the mechanisms of both *E. coli* O157:H7 binding to and transit of Stx across the epithelium (6, 133). In this manner, researchers demonstrated the translocation of both Stx1 and Stx2, albeit at varying rates, across CaCo-2A, T84, and HCT-8 cells, all of which are of intestinal origin (7). A key feature of the transwell approach is that it allows for the development of polarized cell monolayers that express features more characteristic of differentiated cells, such as the formation of tight junctions and the expression of unique cellular factors (183). Another tissue culture model that has been used to explore *E. coli* O157:H7 adherence and any subsequent host cell damage is the organoid system in which cells grown on a scaffold under microgravity conditions form pieces of tissue-like material (118, 216, 300). When an organoid derived from HCT-8 colonic cells was infected with *E. coli* O157:H7, A/E lesion formation was evident as was some slight tissue damage, the latter of which may have been due to the Stx2 produced by the organism (42). In addition, *in vivo*-grown organ cultures of intestinal samples have been applied to the study of both adherence of *E. coli* O157:H7 to gut mucosal cells and any resultant damage to those intestinal cells (19, 51, 108, 109, 235). Finally, excised intact murine intestinal segments placed into Ussing chambers were employed to demonstrate the movement of Stx1 across the mucosa by Acheson *et al.* (7).

In an effort to understand the pathogenesis of HUS, the effect of Stx has been studied on human endothelial cells (mainly from the umbilical vein and human glomeruli) (204). Studies with human umbilical vein endothelial cells (HUVECs)

revealed that in response to various stimuli (for example, TNF-α), the amount of Gb$_3$

expressed on the surface of the cells increased which, in turn, rendered these HUVECs

sensitive to the effects of Stx1 (303).   Experiments done with human glomerular

microvascular endothelial cells (GMVECs) from different sources revealed a range of

responses to Stx. Thus, GMVECs isolated from rejected human kidney transplant

specimens responded to toxin in a similar manner to that described for HUVECs above

(304). Conversely, Obrig *et al.* found that human glomerular endothelial cells could

express Gb$_3$ and were sensitive to Stxs without addition of exogenous factors (227).

Additional cell types that have been examined *in vitro* for response to toxin include

epithelial cells, mesangial renal cells, monocytes, macrophages, and PMNs to name a few

(193, 204). Nevertheless, the consensus in the field among researchers who study STEC

is that the primary targets for Stx-evoked damage *in vivo* are cells of the microvasculature

in the renal glomeruli and at other sites in the body (134, 148, 212).

Animal models

Many animal models have been developed to facilitate study of EHEC

pathogenesis *in vivo*. In general, these models exist in two varieties: those solely focused

on the effects of Stx (in the absence of bacteria) and those that explore *E. coli* O157:H7

infection. Models that evaluate toxicity rely on injection of Stx (with or without LPS)

and often measure the extent of mortality (or mean time to death) as the endpoint of the

particular investigation. Such *in vivo* assays have been used to explore differences in

relative toxicity among Stx toxin types (285), as a means to assess the protective capacity

of some factor (12, 24, 256), as a way to model the pathogenesis seen in HUS (245), or

for a variety of other reasons (A. Melton-Celsa, unpublished)(80, 205). While each

applicable model, whether an intoxication alone or an infection, can be used to study one

or more components of the steps in the pathogenesis of *E. coli* O157:H7- or other STEC-

mediated disease (from initial colonization to mortality), no one animal model system to

date has been described that mimics the full spectrum of STEC-evoked illness in people

(to include the development of HC and HUS) [reviewed in (192)].

Small animals that have been employed as models for EHEC infection and

disease include mice (71, 152, 166, 257, 305), rats (321), and rabbits (99). Larger

animals that have also been so used, albeit less frequently, include: chickens (20, 272),

pigs (297), cows (64), dogs (79), baboons (280), and macaques (142). The presence of

characteristic attaching and effacing (A/E) lesions in the gastrointestinal tract of *E. coli*

O157:H7-infected animals has been described for gnotobiotic piglets, infant rabbits,

calves, chickens, and macaque monkeys [reviewed in (206) and (62)].

Naturally occurring HUS-like diseases have been described in greyhounds

[known as idiopathic <u>c</u>utaneous and <u>r</u>enal glomerular <u>v</u>asculopathy of <u>g</u>reyhounds

(CRVG) or "Alabama rot" (58, 79)] and rabbits (100), and to a lesser degree, cats, dogs

other than greyhounds, horses, and cattle (79). The CRVG is a fatal syndrome that is

similar to STEC-evoked HUS in the pathophysiology of disease; however, the illness

affects not only the kidney but also the skin (unlike STEC infection) (79). While there is

a large body of evidence that suggests that CRVG results from STEC-infection, no

studies have been published that prove the link in the form of Koch's postulates (79). In

addition to CRVG, an outbreak of diarrhea and sudden death in Dutch belted rabbits was

reported by Garcia *et al.* in 2002 (100). These investigators discovered that the rabbits

were coinfected with an EPEC O145:H⁻ strain and Stx1-producing EHEC O153:H⁻

isolate. Most importantly, the clinical presentation in the animals and pathology on

necropsy were consistent with many features of both HC and HUS described for humans

(100). Because of the gastrointestinal manifestations of disease and renal lesions seen in

these STEC-infected animals, Garcia and colleagues developed a model for the study of

E. coli O157:H7 pathogenesis in Dutch belted rabbits (99). Piglets are also naturally

susceptible to the effects of STEC proliferation within the gut and display both systemic

manifestations (known as edema swine disease) and diarrhea (296). In fact, A/E

intestinal lesions evoked by *E. coli* O157:H7 were first described *in vivo* in gnotobiotic

piglets (88). Moreover, the gnotobiotic piglet model is quite well established and has

been applied by several groups for the study of *E. coli* O157:H7 pathogenesis. Indeed,

gnotobiotic piglets are favored by some investigators as a model for preclinical

evaluation of STEC therapeutics (296).

Mouse Models

Although large animals models such as the gnotobiotic piglet exhibit a number of

features of *E. coli* O157:H7 pathogenesis, the derivation of such animals and their

maintenance require considerable veterinary skill. Moreover, the space needed for

housing gnotobiotic piglets (or calves, baboons, or dogs) is not routinely available to the

average investigator. Thus, small animal model systems are preferable for general use

by researchers. Mouse models in particular offer a number of benefits that include:

relative costs for purchase and maintenance, ease of care and handling, ready availability

of numerous immunological reagents, variations in genetic backgrounds among inbred

mouse strains as well as access to transgenic and recombinant inbred animals, and, very importantly, the feasibly of using sufficient numbers of animals in a single study to accrue meaningful statistical analyses on the resultant data. Popular mouse models of *E. coli* O157:H7 infection include axenic mice (which lack an indigenous intestinal flora) or streptomycin-treated mice (which have a reduced normal flora) because these animals have proven amenable to EHEC colonization. A summary of the main mouse models that have been used for *E. coli* O157:H7 oral infection studies is presented in Table 1.

Streptomycin-treated Mouse Model

The first mouse system described for study of the pathogenesis of *E. coli* O157:H7 was the streptomycin-treated murine model developed by Wadolkowski *et al.* (305). This *E. coli* O157:H7 mouse model incorporates streptomycin (str) treatment of animals via their drinking water as a means of reducing the animals' normal intestinal facultative flora so as to decrease bacterial competition for the infecting EHEC strain. This methodology was based on the work of Myhal *et al.* as described in 1982 (213). The goal of the original study by Myhal and colleagues was to assess the relative colonizing capacities of different *E. coli* isolates. Prior to the report by Myhal *et al.*, *E. coli* and several other bacteria (to include *Salmonella* and *Vibrio*) were shown to have the capacity to colonize mice if the animals had been antibiotic-treated or were axenic (198, 201). However, as Myhal *et al.* went on to demonstrate, even a laboratory-adapted *E. coli* K12 given orally as a single strain challenge was capable of colonizing str-treated mice to levels equivalent to those observed for human fecal *E. coli* isolates.   Thus, as the authors suggested, str-treated mice are best used to evaluate the relative colonization

Table 1: Summary of the commonly used mouse models of STEC colonization and/or disease

       The most widely used and thoroughly described mouse models of STEC infection and disease are listed by model name in the table below. The table also summarizes such parameters of the model as the inoculation method, the required inoculum size, the disease features evaluated, and any histopathology reported. In addition, the references for the models are presented.

Model	Inoculum (CFU)	Inoculuation method	Features	Histopathology	Reference
Str-treated	10^10	Feeding	Colonization; morbidity and mortality with 933cu-rev	Kidney	Wadolkowski 1990
Str-treated (O91:H21)	LD50 <10	Feeding	Colonization, morbidity, mortality	Kidney	Lindgren 1993
MMC & str (O157:H-)	>10^9 (req. for mortality)	IG	Colonization, morbidity, mortality	Kidney, brain	Fujii 1994
Conventional	10^7, 10^8	IG	Morbidity and mortality	Kidney, intestines	Karpman 1997
Germfree	2X10^2 (colonization), 2X10^9 (disease, mortality)	IG	Colonization, morbidity, mortality	Kidney, intestines, brain	Isogai 1998
PCM	2X10^5-7	IG	Colonization, morbidity, mortality	Kidney, intestines, brain	Kurioka 1998
Conventional	~2X10^10	IG	Colonization		Conlan & Perry 1998
Germfree	5X10^7	IG	Colonization, morbidity, mortality with hyper-toxigenic strain	Kidney, intestines, brain	Taguchi 2002
Conventional	10^11 CFU/kg	IG	Colonization		Nagano 2003
MMC & str	5X10^3	IG	Colonization, morbidity, mortality	Kidney, other	Shimizu 2003
Germfree	10^2-6	IG	Colonization, morbidity, mortality	Kidney	Eaton 2008
Conventional (weaned)	6X10^9 CFU/kg	IG	Morbidity and mortality (% shedding)	Kidney, intestines	Brando 2008

capacity of an *E. coli* strain if given with another isolate in a competitive infection study (213).

For their work, Myhal and colleagues used 5-6 week old, male CD-1 (out-bred mice, also known as ICR) mice (213). Myhal *et al.* demonstrated that addition of 5 g/L of streptomycin sulfate to the animals drinking water, for as little as one day, reduced the numbers of facultative anaerobes shed (from 10^8 CFU/g feces prior to streptomycin treatment down to $<10^2$ CFU/g feces) but had little or no effect on the numbers of obligate anaerobic bacteria present within the gastrointestinal tract (10^9 CFU shed/g feces). The authors selected streptomycin as the antibiotic treatment of choice as they reasoned that a mutation that rendered the bacteria resistant to streptomycin (a presumed alteration to the ribosomes) should have little effect on the bacterial surface and thereby on colonization. In their report, str-treated mice that had been deprived of food/water were infected with 10^{10} CFU of str-resistant *E. coli* in a solution of 20% sucrose. Colony counts of feces collected daily indicated that high levels of colonization were achieved by all of the challenge strains when given alone (~10^8 CFU/g feces), whereas in co-feeding or competition experiments varying colonizing capacities of the strains were revealed. Furthermore, the authors found that in this model human fecal isolates colonized both the cecum and the large intestines in high numbers. They concluded that the large intestines, which had slightly more bacteria adhered to plated intestinal segments than were seen in the cecum, was the main site of *E. coli* colonization in their str-treated model (213). This site of primary colonization was consistent with what had previously been reported for *E. coli* colonization in the untreated mouse model (246).

Wadolkowski *et al.* followed a very similar methodology to that described by Myhal *et al.* to establish str-treated mice for *E. coli* O157:H7 infection studies (213, 305). Specifically, these investigators used CD-1 male mice exclusively and provided them streptomycin sulfate in their drinking water at a concentration of 5g/L (305). Following a decline in the normal flora (reduced facultative anaerobes), Wadolkowski *et al.*, fasted mice overnight and then inoculated them with 10^{10} CFU of the strain/s of interest in 20% sucrose (w/v). Food was returned after infection, and the animals were then housed individually. Colonization levels of the infecting *E. coli* O157:H7 strain were monitored in shed feces through determinations of CFU/g feces as measured by plate counts of str-resistant *E. coli* O157:H7 present in serially-diluted homogenates of weighed fecal pellets.

Wadolkowski, *et al.* tested three *E. coli* O157:H7 strains [n=3 per strain; experiments done in triplicate (at least)] in their initial experiments: the WT strain 933, a plasmid-cured mutant 933cu, and an additional cured mutant recovered from coinfection (or competition) studies named 933cu-rev (305). Not surprisingly, 933 and 933cu colonized to similar levels (10^7 CFU/g feces for 25 days) in single infection experiments with no observed disease manifestation. When competition assays were conducted, 933 outcompeted 933cu in 2/3 of the mice. In 1/3 of the mice, after a decline in the load of 933cu, there was a steady increase in levels of 933cu to those of WT levels. An isolate of 933cu that was recovered from that coinfected mouse was labeled 933cu-rev ("rev" meaning "revertant" to wild-type or virulent colonization levels). Although no signs of illness were apparent in the coinfected mouse with the high levels of 933-cu rev, disease manifestations were evident in mice subsequently infected with 933-cu-rev alone. The

933cu-rev-infected mice shed loose stools, were anorexic and lethargic, and died within a few days of disease presentation. Extensive necropsies (included the liver, brain, heart, stomach, SI, cecum, LI, spleen, and kidneys) of singly-infected animals revealed that only the kidneys from animals infected with strain 933cu-rev demonstrated pathology of any kind. Evaluation of the histopathological slides of kidneys from 933cu-rev infected animals indicated widespread bilateral acute renal cortical tubular necrosis despite apparently normal glomeruli with no evidence of fibrin deposits, elastic fibers, or bacteria (as detectable by tissue Gram stain.). The authors concluded that this pathology was more indicative of insult from a toxin rather than a result of dehydration that might be expected in an anorexic animal with loose stools (305).

In this same report, Wadolkowski *et al.* also assessed the location of bacterial colonization within the gastrointestinal tract, as well as the role of mucus from these areas in bacterial growth (305). The results indicated that all strains had the greatest level of epithelial cell colonization within segments from the cecum followed by those of the proximal large intestines. However, 933cu-rev also demonstrated an increased capacity to colonize the small intestines and, additionally, other sections (mid and distal) of the large intestines. This latter finding was consistent with the increased capacity of strain 933cu-rev to multiply within mucus obtained from these various intestinal segments [note: Wadolkowski *et al.* had previously shown that multiplication in cecal mucus was required for a human fecal isolate of *E. coli* to colonize the LI (306)]. Wadolkowski and colleagues went on to speculate that the increased virulence of 933cu-rev may in part have been attributable to its broader range of colonization locales and, more specifically to the increased capacity of the distal small intestines (versus the cecum or LI) to absorb

Stxs produced at that site (305). Furthermore, the link between site of colonization and extent of disease was also suggested by Tzipori in relation to EPEC colonization (294). Strains that caused more severe disease colonized the proximal small intestines of piglets, whereas strains that caused milder pathogenesis tended to colonize distal regions of the small intestines as well as distal portions of the large intestines.

Expansion of the Streptomycin-treated Mouse Model

Since the report by Wadolkowski and colleagues that described an *E. coli* O157:H7 model for colonization *in vivo* (305), several other investigators have made use of streptomycin treatment of mice to attain STEC colonization (14, 16, 93, 94, 97, 117, 139, 173, 191, 194, 284, 299). The utility of the model was extended from the original report of *E. coli* O157:H7 infection and colonization to include the evaluation of non-O157 STEC strains (173). Moreover, in 1994 Fujii *et al.* expanded the features of STEC (*E. coli* O157:H⁻) pathogenesis, specifically the development of neurological manifestations of disease, seen in infected str-treated mice by incorporation of mitomycin C (MMC) in the treatment regimen (93). MMC treatment results in induction of phage expression by the bacterium, which concurrently leads to increases in toxin production. Agents that induce toxin expression, to include MMC or ciprofloxacin treatment, are frequently used in mouse models of STEC infection (14, 93, 257, 284). Other minor modifications to the str-treated mouse model methodology (alone or in combination) have been applied by various groups. These alterations to the protocol include a reduction in the amount of bacteria fed (191, 257); a lengthening of the period of streptomycin treatment prior to infection (94, 97); the application of intragastric

inoculation of the organism (97); and finally, changes in the sex, age, or strain of mouse employed (16, 94, 97, 117, 299).

Protein Calorie Malnutrition Mouse Model

While the str-treated mouse model has proven particularly useful for reproducibly attaining high levels of STEC colonization, this animal system has limitations. For example, very high inocula of *E. coli* O157:H7 are required to cause morbidity or mortality in even a portion of str-treated animals even though low to moderate doses of STEC strain B2F1 (*E. coli* O91:H21) have been used successfully to achieve colonization of str-treated mice and induce disease (173). In an effort to reduce the inoculum of *E. coli* O157:H7 required to evoke disease in mice and to thus bring the mouse dose closer to that speculated to result in disease following human exposure (thought to be around 50 organisms), other mouse model systems have been developed. One such system described by Kurioka *et al.* was based on the observation that some children who contract STEC infections subsisted on an unbalanced diet prior to infection (166). Therefore, the authors theorized that protein calorie malnourished (PCM) mice would be more readily infected by low doses of *E. coli* O157:H7 than conventional mice. The authors' hypothesis proved correct in that the minimal infectious dose of *E. coli* O157:H7 in PCM mice was over 3 logs lower than that of control mice. The authors reported pathological changes in the intestinal tract of PCM mice infected with *E. coli* O157:H7 (underdevelopment of the intestinal epithelium in response to PCM, likely resulting in the animals' predisposition to *E. coli* O157:H7 colonization) and a slight increase in TNF-α in the blood of PCM mice compared to controls. However, they did not observe

significant renal pathology [as was seen by Isogai *et al.* (131)] but did note minimal

degeneration of renal tubules and weak staining of the cortical tubular epithelium for Stx

(166). Kurioka *et al.* also postulated that retardation of intestinal development by PCM

might facilitate Stx and LPS transit across the intestinal barrier. That Stx likely did cross

the mucosal barrier more readily in PCM-infected animals was strongly suggested by the

CNS findings in the PCM mice; cerebral hemorrhage was evident and toxin was detected

in the hippocampus. Thus, this report confirmed that Stx can affect the CNS of mice

(leading to death of the infected PCM mice within 10 days). Moreover, these CNS

findings in *E. coli* O157:H7-infected PCM mice are consistent with the observation

noted by Kurioka *et al*. (166) that up to 30% of STEC-infected children display

neurological manifestations of disease (52).

Gnotobiotic Mouse Models

In an attempt to expand the renal pathology evident in str-treated, STEC-infected

mice (acute renal cortical tubular necrosis) to include presentation of glomerular lesions,

Isogai and colleagues infected germ-free mice with *E. coli* O157:H7 (technically

gnotobiotic once they were infected with *E. coli*) (131). Although these animals became

colonized with *E. coli* O157:H7 following a low-dose challenge, the animals did not

display signs of disease. Indeed, high inocula (comparable to those reported for the str-

treated model) were necessary to achieve histopathology (of the colon, kidneys, and

brain) and disease (lethargy, paralysis, anorexia, dehydration, death within 7 days).

However, when these gnotobiotic mice were treated with TNF-α and then infected with a

low-dose of *E. coli* O157:H7, systemic disease, neurological manifestations, and glomerular pathology were all seen (131).

Since this original report by Isogai *et al.*, other groups have employed germ-free animals to evaluate *E. coli* O157:H7 pathogenesis. Specifically, Sawamura *et al.* explored the effects of antibiotic treatment on *E. coli* O157:H7 infection in a germ-free mouse model they developed (247) and later used to investigate the role of epithelial cell bacterial internalization in STEC pathogenesis (9). Isogai and colleagues subsequently used germ-free mice to explore the effects of antibiotic treatment and green tea extract on *E. coli* O157:H7 infection (128-130, 132). A variant of the germ-free mouse model was developed by Taguchi *et al.* who combined it with challenge by a hyper-toxigenic strain of *E. coli* O157:H7 to achieve 100% mortality among infected animals (273). Takahashi *et al.* went on to use this later model to investigate the effect of probiotics on *E. coli* O157:H7 infection (274). In 2007, Jeon *et al.* used a germ-free mouse model to assess the virulence of a mutant strain of *E. coli* O157:H7 (136). Furthermore, Eaton and colleagues exploited germ-free Swiss Webster mice to explore the roles of the infecting *E. coli* O157:H7 strain type [including Stx type(s) expressed by the strain] and such host factors as age and gender of the mouse (71). Eaton *et al.* reported that while *E. coli* O157:H7 was present (as assessed by colony counts per g tissue) throughout the lower intestines, gut adherent organisms (as assessed by histological evaluation) were seen in the ileum and cecum but not the colon. This failure to detect mucosally-adherent *E. coli* O157:H7 in the colon of germ-free mice is in contrast to the observations of Wadolkowski *et al.* in the str-treated model (305).

Conventional Mouse Models

While models that make use of streptomycin-treated or axenic animals are useful for assessing disease outcome, they rely on the absence of colonization resistance. The term "colonization resistance" was originally coined by van der Waaij in 1971 to explain a phenomenon whereby "a complex intestinal microflora provides protection against colonization by many pathogenic infectious agents" (14). As a result of the partial or complete absence of a competing microbiota in antibiotic-treated or axenic animals, the inoculated microorganism has a colonization advantage (it no longer encounters colonization resistance). Thus, studies that make use of these models are of limited utility for the assessment of the colonizing capacity of an STEC strain in the face of the physiologically more relevant situation where normal bowel flora are present.

However, in 1997 an alternative model was described that no longer required the alteration of the indigenous flora of the mice. Karpman and colleagues intragastrically administered high doses of *E. coli* O157:H7 to C3H/HeN and C3H/HeJ mice and reported significant morbidity and mortality in the infected animals (152). Mice in this model, which is discussed more extensively in Chapter 2, developed gastrointestinal, neurological, and systemic disease manifestations. Renal pathology included both glomerular mesangial changes and tubular necrosis. Focal areas of colonic necrosis were also evident. Of note, the administration of anti-Stx2 antibodies protected animals from disease symptoms and pathology (152).

Like Karpman *et al.*, Conlan and Perry investigated conventional mice as a model for *E. coli* O157:H7 infection (55). In their report, Conlan and Perry considered three strains of female mice: CD-1 (outbred), BALB/c (inbred), and C57BL/6 (inbred).

Ultimately, the authors were seeking a mouse model to screen potential vaccine candidates. Following intragastric administration of ~10^{10} *E. coli* O157:H7 CFU to conventional mice of these strains, fecal shedding of the organism was monitored as a surrogate for colonization (fresh fecal pellets were collected from individual animals). Although all mouse strains were colonized after infection (generally for 1-2 weeks), no morbidity or mortality was observed. Interestingly, only BALB/c mice seemed to be relatively resistant to re-infection; they shed *E. coli* O157:H7 for a shorter duration than did other mouse strains that had also received a second challenge with the microbe. Also, BALB/c mice, in comparison to C57BL/6 mice demonstrated higher titers of O157-specific IgA (both serum and fecal) in response to both primary and secondary infection despite significantly lower serum anti-O157 IgG titers following secondary infection. Thus, BALB/c mice can serve as models for *E. coli* O157:H7 vaccine studies, despite their reported lack of mortality and/or morbidity in the study by Conlan and Perry (55).

The reports by Conlan and Perry and Karpman *et al.* indicated that *E. coli* O157:H7 could colonize conventional mice (55, 152). Nagano and colleagues then sought to build on these findings and determine the functionality of specific pathogen free (SPF) mice as models for *E. coli* O157:H7 colonization (214). In a similar fashion to Conlan and Perry (55), Nagano *et al.* explored the susceptibility of various mouse strains to colonization after infection with *E. coli* O157:H7 (214). The mouse strains they examined included ICR (also known as CD-1), BALB/c, C3H/HeN, C3H/HeJ, and A/J (all female SPF). At one week post-infection, even with a high inoculum of *E. coli* O157:H7, only the ICR animals remained colonized as a group. In fact, the majority of ICR mice stayed colonized and shed *E. coli* O157:H7 in their feces for the duration of the

study (day 28 post-infection). Moreover, Nagano *et al.* detected *E. coli* O157:H7 in both the cecum and the colon later in infection. However, they concluded that the cecum was the primary site of colonization because only at that location were *E. coli* O157:H7 colonies found adherent to epithelial cell surfaces (with F-actin accumulation beneath the bacteria). In spite of this persistent colonization of ICR mice by *E. coli* O157:H7, and as was the case with Conlan and Perry's study (55), Nagano *et al.* were unable to demonstrate morbidity or mortality following *E. coli* O157:H7 infection of these animals (214).

Recently, Brando *et al.* used weaned BALB/c mice to investigate *E. coli* O157:H7 pathogenesis (32). Weaned mice were selected over adult mice as a model in an attempt to render the animals more susceptible to *E. coli* O157:H7 infection; indeed, age has been described as an important factor in susceptibility of animals to EHEC disease (71). In Brando and colleagues' model, only mice <21 days of age demonstrated systemic manifestations of disease: mortality (within 96 hours) and increased plasma urea levels (32). Brando *et al.* noted the presence of occult blood in the stools of mice that succumbed to infection. Histologically, they observed tubular necrosis (consistent with other STEC models) as well as alterations of the glomeruli of infected mice. Colonization was assessed at 48 and 72 hours by rectal swab; it was noted that only a portion of infected mice were shedding bacteria by 72 hours post-infection, and all survivors had cleared infection by day 7. Furthermore, in their model, only transient colonization of the small and large intestine (the cecum was not analyzed) was observed. However, histological analysis revealed damage to the intestinal epithelium and the presence of inflammatory infiltrates. Manifestation of these pathological features was

likely facilitated by the incompletely developed intestinal epithelium in weanling mice; this intestinal characteristic of young mice was also likely to have aided in the systemic absorption of toxin as was seen in PCM-infected mice (32, 166).

Other studies that used conventional mice to study STEC pathogenesis can be found in the literature, although they are less thoroughly described (96, 162, 169, 210, 255). However, a common feature among many of these conventional mouse model investigations of *E. coli* O157:H7 infection was that they either assessed colonization or monitored the development of disease. For example, in the studies by Nagano *et al.* and Conlan and Perry, colonization was followed but morbidity of infected mice was not observed (55, 214). Conversely, in the study by Karpman and colleagues, morbidity and mortality was seen as an outcome of *E. coli* O157:H7 infection of mice but colonization was not monitored (152). Thus, until our report of a new conventional BALB/c mouse model of *E. coli* O157:H7 infection [described in Chapter 2 and published as (203)], no adult mouse studies with conventional animals had been reported that explored in detail both colonization and disease after *E. coli* O157:H7 oral infection.

Citrobacter rodentium as a Surrogate for E. coli *O157:H7*

Another mouse model system that has been developed to evaluate the virulence mechanisms of EHEC employs the natural mouse pathogen *Citrobacter rodentium* as a surrogate for *E. coli* O157:H7. *C. rodentium* is similar to both EPEC and EHEC in that it carries a homolog of the LEE PAI of EPEC and EHEC and has the capacity to evoke attaching and effacing (A/E) lesions. Thus, *Citrobacter* has been used to study the molecular basis for AE lesion formation in mice because it is the only known LEE-

positive organism that is naturally pathogenic for rodents (181). *C. rodentium* causes

transmissible murine colonic hyperplasia (TMCH) (181). While the organism is useful

for assessing the contribution of various genes/proteins to virulence, this model fails to

recapitulate the pathogenesis seen by EHEC as a whole because it does not make Shiga

toxin [although there was one report of a strain of *Citrobacter freundii* that produces Stx2

(292)].

Alternative Models to Oral Infection

In studies of mice infected with *E. coli* O157:H7, the oral route of challenge is

used most frequently. Nevertheless, alternative means of introducing STEC into mice,

although infrequent, have been described. For instance, one of the earliest published

murine models of STEC infection relied on the subcutaneous injection of large quantities

(10^8 CFU) of *E. coli* O157:H7 into SPF mice (167). Since then, other venues of

administration of STEC have been used for various purposes. For example, intravenous

injection of mice with spontaneously-derived motility mutants of *E. coli* O157:H7 was

explored as a system to assess potential differences in virulence of the strains (290).

Most recently, Gao *et al.* evaluated the protective impact of an Stx fusion protein vaccine

on challenge with *E. coli* O157:H7 given intraperitoneally (98).

For our experiments, we deemed that oral infection of mice with *E. coli* O157:H7

(by feeding or gavage) was the most desirable route by which to introduce the inoculum

because it was most like natural exposure. Furthermore, single infections were necessary

in our studies to prevent *in vivo* complementation of the stx_2 mutant strain by toxin

produced by the WT, as was discussed in the study by Robinson *et al.* (241). As a result

of the need for single strain infections and a desire for the requirement of bacterial competition with an indigenous flora (as would be seen in human infections), we elected not to use antibiotic-treated or axenic mice as our model system. Thus, we considered that the development of a murine model of infection in the presence of the animal's intact commensal flora was essential for our purposes.

Role of Shiga toxin in Pathogenesis

The most severe manifestations of disease seen after infection with *E. coli*

O157:H7 in particular but with and other STEC isolates as well, i.e. HC and HUS, have

been attributed to the production of Stx by the organisms. Reviewed below are the

proposed mechanisms by which toxin exerts its effects systemically and locally within

the gastrointestinal tract. Additionally, the role that toxin may play in increasing

adherence *in vitro* and colonization *in vivo* is described.

Systemic Manifestations of Disease

Several years after the emergence of *E. coli* O157:H7 as pathogen, investigators

epidemiologically linked the production of Shiga toxin by this organism (as well as other

STEC) to the development of HUS (150, 151). Moreover, a study done in macaques with

a Shiga toxin mutant of *Shigella dysenteriae* type 1 demonstrated that in the absence of

Stx , systemic vascular damage was not observed (85). Furthermore, administration of

Stx-neutralizing antibody to STEC-infected animals precluded their development of renal

pathology (152, 305). Other studies have shown that the neurological manifestations of

E. coli O157:H7 infection are also likely the result of Stx production (93, 166, 320).

While *E. coli* O157:H7 itself is not an invasive organism, and infected individuals

almost never become bacteremic (153, 277), Stx produced by the bacteria has been found

systemically both in animal models of infection (9, 57, 166), as well as in biopsies taken

from infected individuals (298). The exact mechanism by which toxin transits from the

gastrointestinal lumen to the blood stream is not known. Furthermore, the means by

which toxin traffics to various organs remain to be elucidated. While it has been

postulated that toxin binds to polymorphonuclear (PMN) cells or monocytes that then carry the toxin to the renal endothelium (34, 281-283), conflicting data make this hypothesis a subject of controversy (84, 105, 106).

Systemic toxemia results primarily in damage to the blood vasculature; when this injury occurs within the bowel it can manifest as HC and when this damage occurs to the renal endothelium it can result in HUS (176). Shiga toxin targets small vessel endothelial cells, irrespective of location. Stx binds to receptor-rich cells that are present in the renal endothelium, mesangium, and tubular epithelium (277). While generally thought of as a protein synthesis-inhibiting toxin, Stx can have dramatic effects on gene expression; specifically, the toxin can increase endothelial cell expression of proadhesive, prothrombotic, and inflammatory genes and thereby exert effects on fundamental cellular pathways (234). Thus, when Stx damages endothelial cells, a prothrombotic response can occur that can then lead to fibrin deposition in and around the injured cells. The amount of fibrin that accumulates at the site of Stx-mediated damage to these cells is enhanced by increases in plasminogen activator inhibitor 1 (PAI-1), a substance that functions to prevent fibrinolysis (fibrin lysis leads to the production of D-dimers) (277). Fibrin accumulation in the presence of platelets produces a thrombus, or clot, that can occlude the local vasculature. In turn, this occlusion can result in an infarct and small blood vessel rupture. However, based on the work of Cunningham and colleagues (60), Tarr *et al*. have stated that even without vascular occlusion "thrombosis-independent thrombin-mediated host mechanisms" may be responsible for renal injury (277).

The effects of Stx are not solely limited to the kidneys. Systemic toxemia can result in the presence of microthrombi in a variety of organs and may play a role in

determining a patient's ultimate prognosis (301). Early studies into nonrenal

involvement of HUS revealed that among patients with a poor disease outcome,

manifestations of neurological symptoms (hallucinations, disorientation, seizures, stupor,

and coma) were evident (301). In fact, among patients admitted to the hospital following

E. coli O157:H7 infection, up to 30% develop CNS involvement (52). Additionally,

animals injected with Stx may display such evidence of neurological system involvement

as hind-leg paralysis. In a study of the effects of Shiga toxemia in rabbits, the spinal

cords of the animals showed evidence of microvascular thrombosis, edema, and

hemorrhage at necropsy (238). The investigators in that study postulated that the

ischemic necrosis observed by histology in sections of the grey matter of the rabbits'

spinal cords caused the limb paralysis observed in the intoxicated rabbits. Furthermore,

the severity of CNS histopathology diminished from posterior to anterior brain sections

(238). In mice, fatal acute encephalopathy can result (93) from infection with STEC.

Finally, autopsies of HUS patients have revealed that in addition to the kidneys and brain,

the heart and pancreas may also be affected by thrombotic microangiopathy (211) that is

presumably mediated by Stx.

Intestinal Damage

In addition to the widespread systemic vascular disease caused by Stx and the

resultant complications of such damage (HUS and CNS involvement), Stx may induce

damage to the microvasculature of the intestines that can result in HC. While not as

widely characterized as the effects of Stx on the kidneys of patents with HUS, the

gastrointestinal pathology of STEC-infected individuals who present with HUS has been

examined (211, 237). In general, gastrointestinal findings from these individuals include thrombotic microangiopathy (TMA; leading to ulceration or necrosis), thickening of the intestinal wall, and pseudomembrane formation (237). While the predominant pathology is seen in the colon (in part because this gastrointestinal site if often exclusively examined), affected areas can extend proximally into the terminal ileum (either focally or in a more dispersed manner) (211, 237).

The extensive presence of TMA within the gastrointestinal tract of infected individuals is supportive of the hypothesis that the gastrointestinal involvement is in part mediated by toxemia. While Stx is not generally considered to act directly on intestinal epithelial cells [rather the colitis is thought to result from vascular injury as a consequence of systemic toxemia (8)], apoptosis of the intestinal epithelium as a result of Stx and STEC infection has been described in a rabbit intestinal loop model (154), an infant rabbit model (231), and in at least one instance of human infection (237). Some investigators have also suggested that circulating Stx may result in mesenteric ischemia that then causes diarrhea, bloody or otherwise, as opposed to Stx acting directly on the intestinal vasculature (277). Irrespective of the mechanism by which Stx-induced colitis occurs, it has been observed in a number of animal models after parenteral administration of toxin (240, 258). Studies done in rabbits showed that intravenously administered Stx alone was sufficient to evoke vascular damage to the colon [and the brain (238)]. Furthermore, in an infant rabbit model, toxin-producing STEC strains were more virulent than were non-Stx-producing strains and, most importantly, feeding Stx alone recapitulated both the clinical symptoms and intestinal histopathology seen in STEC infection (231). Finally, Fontaine *et al.* demonstrated that Stx production by *S.*

dysenteriae type 1 was required for the development of bloody diarrhea, but not watery diarrhea, in infected macaques (85).

Stx expression by STEC in the gastrointestinal tract is proposed to evoke increases in IL-8 production as inferred from studies done *in vitro* (287, 316). On the other hand, the bacterium itself may dampen the inflammatory response against the toxin (22). Nevertheless, one current hypothesis is that the effect of toxin on IL-8 superinduction and the resultant cytokine synthesis may facilitate toxin spread systemically by "enhanced recruitment and activation of inflammatory cells with subsequent compromise of the intestinal barrier" (287). Although the mechanism by which Stx penetrates the intestinal epithelium is unclear, the consensus among researchers in the field is that once the toxin has entered into the intestinal mucosa and submucosa it damages the local intestinal vasculature. Additionally, there is evidence to suggest that Stx may promote edema via destruction of the absorptive villi (215).

Adherence and Colonization

The first suggestion that Stx plays a role in adherence or colonization came from the work of Sjogren and colleagues reported in 1994 (262). They demonstrated that transduction of a diarrheal evoking enteroadherent *E. coli* pathogen of rabbits (called RDEC-1) with an Stx1-converting phage resulted in a strain (named RDEC-H19A) that not only caused a more severe and extensive course of disease in rabbits compared to RDEC-1 alone but also resulted in more wide-spread mucosal colonization/enteroadherence (as assessed by light microscopic analysis of tissue sections) than was seen with the parent strain. Sjogren *et al*. also observed that animals

infected with RDEC-H19A, when compared to those orally inoculated with RDEC-1, were colonized about a day earlier, developed diarrhea sooner and more often, quickly lost more weight, and exhibited greater mortality (262).

While other indirect evidence that Stx can facilitate adherence and colonization of STEC strains accrued over the years [e.g., a signature-tagged mutagenesis study revealed a mutant deficient in its capacity to colonize calves; that mutant had an insert in the gene between stx_1 and the phage lysis genes (69)], direct support for that concept was not reported until 2006. In a study done by Robinson and colleagues, Stx2 was attributed a role in increasing the extent of *E. coli* O157:H7 adherence *in vitro* and colonization *in vivo* (241). Robinson *et al.* used an stx_2 mutant of *E. coli* O157:H7 to facilitate their investigation. These investigators demonstrated that an stx_2 mutant *E. coli* O157:H7 adhered to HEp-2 cells to a lesser extent than did the wild-type *E. coli* O157:H7 strain; furthermore, this adherence deficiency could be overcome by treatment of the HEp-2 cells with purified Stx2. Robinson *et al.* went on to show that enzymatically active Stx2 caused an increase in cell surface-localized nucleolin on HEp-2 cells. They then proposed a mechanism whereby toxin augmented *E. coli* O157:H7 adherence through an increase in cell surface-localized nucleolin; as mentioned earlier, intimin, the major bacterial adhesin of *E. coli* O157:H7, and cell-surface localized nucleolin interact to provide an initial point of attachment for the bacterium (241).

Robinson *et al.* also investigated the role of Stx2 in colonization *in vivo* (241). In single infections of conventional mice, the Stx2-producing wild-type *E. coli* O157:H7 strain demonstrated significantly higher levels of colonization (as assessed by fecal shedding) than did its isogenic stx_2 mutant. However, in a mixed infection (also known

as a competition experiment), the wild-type and stx_2 mutant *E. coli* O157:H7 strains colonized mice to similar levels. These data led Robinson and colleagues to conclude that Stx2 produced by the wild-type was complementing the deficiency of the stx_2 mutant *in vivo* (241).

In addition to the work of both Sjogren *et al.* and Robinson *et al.* (241, 262), others have reported a role for Shiga toxin in the colonization of *E. coli* O157:H7 (38, 64, 126, 180, 317). The same year of Robinson and colleagues' report., Hoffman *et al.* demonstrated that calves infected with a Stx-producing strain of *E. coli* O157:H7 shed higher numbers of bacteria for a more prolonged period than did animals fed a non-toxin producing *E. coli* O157:H7 strain (126). Additional evidence that toxin might facilitate colonization in cattle came after a report that cytotoxins secreted by *E. coli* O157:H7 enhanced adherence of the organism to intestinal tissue of cattle in an IVOC assay (19). Furthermore, the addition of these cytotoxins to low-level colonizing *E. coli* O157:H7 enhanced the adherence capacities of those strains [a similar finding to that reported by Robinson *et al.* in mice (241)] (19). Thus, Baines *et al.* proposed that in cattle coinfected with *E. coli* O157:H7 strains, expression of these cytotoxins (presumed to be Stxs) by one strain facilitated greater overall *E. coli* O157:H7 colonization by all strains as well as an extended duration of *E. coli* O157:H7 shedding (17). This theory was later confirmed by Lowe *et al.* in 2009 who coined the term "piggy-back" colonization. Lowe *et al.* suggested that "piggy-back" colonization was one method by which *E. coli* O157:H7 lineage II strains that exhibit decreased adherence capabilities *in vitro* can be maintained and flourish within feedlot populations (180).

To confirm that Stx was the secreted cytotoxin responsible for the increased colonization of *E. coli* O157:H7 in cattle, Baines *et al.* used *in vitro* organ culture (IVOC) to investigate the effects of Stx2 on adherence to intestinal tissue (17). These investigators found that Stx2 did enhance the adherence of *E. coli* O157:H7 to the intestinal tissues from cattle (colon and jejunum); however, the effects of intoxication on any possible changes in nucleolin expression/localization were not investigated (17). Further *in vivo* evidence that toxin facilitates colonization of cattle was derived from a study by Dean-Nystrom *et al.* in 2008 (64). The report by Dean-Nystrom and colleagues investigated early sites of *E. coli* O157:H7 colonization in a weaned calf model. The authors noted that a wild-type Stx2-producing strain was recovered from the feces of animals at consistently higher levels than was its Stx2-negative counterpart (64).

Shiga toxin was also shown to facilitate colonization of *E. coli* O157:H7 in an additional mouse model (38) as well as in pigs (317). Despite the use of a str-treated mouse model (which in principle should make differences in colonizing capacities of various *E. coli* strains difficult to discern; see above), Calderon Toledo *et al.* reported higher *E. coli* O157:H7 counts in the feces of animals infected with a Stx2-producing strain when compared to a Stx2 non-producing strain [in wild-type C57BL/6 mice and three mutant mouse strains (38)]. Moreover, Yin *et al.* reported a role for Stx2 both in adherence of *E. coli* O157:H7 *in vitro* (HEp-2 cells and the pig jejunal epithelial cells IPEC-J2) and colonization in a pig ligated ileal loop model (317). However, the effect of Stx2 on adherence of *E. coli* O157:H7 to IPEC-J2 cells was not as great as that seen with HEp-2 cells, a finding that likely resulted from the capacity of the organism to bind more efficiently to IPEC-J2 cells. Additionally, the authors described the impact of this toxin-

enhanced augmentation of *E. coli* O157:H7 adherence to IPEC-J2 cells as being

independent of nucleolin and Gb_3, because neither genes nor transcripts for these

products were found during an *in silico* search (317). To resolve the issue of how toxin

might bind to these cells and then impact the cell surface to promote bacterial adherence,

Yin and colleagues suggested that the effect of Stx2 on *E. coli* O157:H7 colonization

might be mediated through Gb_4 (toxin receptor) and β1 integrin (a proposed receptor for

intimin on cell).   Furthermore, the wild-type *E. coli* O157:H7 strain colonized in pig

intestinal loops at statistically higher levels than did an Stx2-negative mutant, although

there was no significant difference in the average volume of fluid that had accumulated in

the loops inoculated with the wild type *E. coli* O157:H7 strain versus its toxin mutant

(317).

Despite these reports that attribute a role for Stx in the augmentation of

colonization by *E. coli* O157:H7, the mechanism by which this phenomenon occurs has

yet to be determined. Robinson *et al.* proposed that the Stx2-mediated enhanced

adherence of *E. coli* O157:H7 to HEp-2 cells was linked to an increase in cell surface-

localized nucleolin following intoxication (241). Hoffman *et al.* attributed the increased

colonization *in vivo* of a toxin-producing *E. coli* O157:H7 strain to the

immunosuppressive effects of toxin; specifically, these investigators suggested that an

Stx2-evoked delay or reduction in the immune response against the bacteria permitted the

Stx2-producing *E. coli* O157:H7 strain to colonize more effectively than did the toxin-

negative strain, 87-23 (126). Yin and colleagues discussed two alternative explanations

for the observed capacity of Stx to facilitate colonization in the pig; they speculated that

an inhibition of activation and proliferation of lymphocytes may have indirectly led to the

facilitation of colonization or that binding of the toxin to crypt Paneth cells may have inhibited antimicrobial peptide secretion (317). Any of these mechanisms, or a combination of them, may indeed be responsible for the promotion of *E. coli* O157:H7 colonization by Shiga toxin. In fact, the mechanism of colonization enhancement by Stx may vary depending on the host that *E. coli* O157:H7 infects.

<u>Therapeutics and Vaccines</u>

Although it has been nearly 30 years since the discovery of *E. coli* O157:H7 as an

enteric pathogen and despite the recent increase in the rate of severe disease associated

with infection by the organism, no treatment yet exists. In general, antibiotic therapy is

contraindicated as it may promote toxin expression from the lysogenized phage.

Moreover, Bellmeyer *et al*. have demonstrated that *E. coli* O157:H7 infection suppresses

the inflammatory response to Stx (22); thus, one can speculate that elimination of the

bacterium might also result in an increased inflammatory response to the toxin.

Additionally, anti-motility agents are not recommended as they can promote the sustained

presence, and consequent toxin expression, of EHEC in the gastrointestinal tract. In

instances where HUS develops, supportive care is provided. Therefore, such strategies as

the use of receptor analogs, passive antibody therapy, or vaccines are under consideration

as ways to protect humans against the systemic effects of toxin; these approaches are

described below.

Receptor Analogs

Since the serious consequences of *E. coli* O157:H7 infection (HC and HUS) are

mediated by the toxin produced by the organism, one potential therapy is to block the

effects of the toxin either by the use of receptor analogs or with anti-Stx antibodies

(discussed below). Receptor analogs are designed to resemble or mimic the Stx receptor,

Gb_3 (a Pk blood group antigen), and bind to toxin. However, one must consider that Stx1

and Stx2 have different affinities for Gb_3 (83) and, ideally, a receptor analog should have

a high binding affinity for both toxins (149). Theoretically, once toxin is bound by the

receptor analog into a complex, the toxin would no longer be free to damage the vasculature of those infected. A reduction (or better yet ablation) in amount of free Stx should, in turn, decrease the likelihood that severe manifestations of *E. coli* O157:H7 infection will ensue.

However, timing of administration of these receptor analog compounds is likely to be of particular importance in prevention or amelioration of *E. coli* O157:H7- (or other STEC-) evoked disease. Karmali suggested that to be maximally effective, these compounds should be given no later than approximately 3 days after the onset of diarrhea and, preferably, even earlier (149). However, given that passive antibody treatment would be optimally therapeutic during this same narrow time period and requires parenteral administration, receptor analogs (specifically those that could be administered orally) are conceptually comparable if not preferable (149).

Several receptor analogs have been developed by various groups for binding to and neutralizing toxin in circulation (Starfish, Daisy, Super Twigs) or adsorbing toxin in the intestines (Synsorb-Pk, CWG308:pJCP-Gb$_3$ bacteria, Gb$_3$ polymer) [reviewed in (149)]. Armstrong *et al.* were the first to suggest the concept of a synthetic oligosaccharide derived from the Pk blood group antigen (Gb$_3$) coupled to an inert matrix as a potential therapeutic for STEC infection (10). This first receptor analog coupled the Pk trisaccharide [Galα(1-4)-Galβ(1-4)-Glcβ1-] to Synsorb, a calcinated, diatomaceous material often used in chromatography (known as chromosorb P). Synsorb-Pk was a toxin affinity-binding agent that was orally administered and indigestible. While Synsorb-Pk was successful in phase I clinical trials [demonstrating safety (13)], phase II trials were inconclusive (11), and phase III clinical trials were abandoned after Synsorb-

Pk seemingly failed to demonstrate any significant benefit in diminishing the severity of HUS in children (291, 296).

The failure of Synsorb-Pk may have been related to its limited access to toxin; Synsorb-Pk was designed to bind to and eliminate toxin in the intestines and thus prevent toxin from reaching the circulation. As a result of the failure of Synsorb, injectable receptor analogs were explored. These products included the decavalent Stx inhibitors, Starfish (157, 209) and Daisy (209). Another set of injectable, receptor analog derivatives known as the Super Twigs were developed by Nishikawa and colleagues (218, 219). The Super Twigs have a carbosilane dendrimeric structure, with multiple trisaccharide molecules attached to the branches (219). Both Daisy and the Super Twigs have been shown to protect against the effects of Stx2 *in vitro* and, more importantly, in mouse models of STEC infection (209, 219).

More effective orally administered receptor analogs have also been evaluated (232, 309). Paton *et al.* developed a "designer probiotic" (CWG308:pJCP-Gb$_3$), an attenuated derivative of *E. coli* R1 that expresses chimeric LPS terminating in the Pk trisaccharide (232). As such, the CWG308:pJCP-Gb$_3$ bacteria express the oligosaccharide receptor analog on their surface. Mice that received twice daily oral administration of this recombinant bacterium (stable colonization was not achieved) were protected from an otherwise lethal infection with Stx2-producing *E. coli* (232). As an alternative to the CWG308:pJCP-Gb$_3$ bacterium, Watanabe *et al.* developed a series of synthetic linear polymers of acrylamide with different numbers of highly clustered trisaccharides (309). The most effective of these Gb$_3$ polymers bound Stx1 and Stx2 *in vitro* and, when given twice daily on days 3-5 of infection, protected *E. coli* O157:H7

strain N-9 (Stx1 and Stx2-producer)-infected mice from morbidity and reduced the extent

of mortality [depending on the Gb_3 polymer used (309)].

Passive Antibody Therapy

In addition to treatment of those infected with *E. coli* O157:H7 of other STEC

with receptor analogs, the administration of neutralizing anti-Stx antibodies to such

infected individuals is under investigation as another means of therapy. Passive antibody

therapy typically involves parenteral injection of antibodies against the desired agent;

such antibodies are usually derived from an immunized animal or immune donor.

Passive transfer of antibodies was first called "serum therapy" and used in late 1800s and

early 1900s; at that time the antibody preparation was obtained from the serum of an

animal or donor (43). Passive administration of such antibody-containing serum was

effective despite some potentially severe consequences, i.e., the onset of immediate

hypersensitivity reactions or even serum sickness. The use of "serum therapy" fell out of

favor in the 1930s following the discovery and development of certain antibiotics. With

the introduction of hybridoma technology in 1975, passive therapy again began to be

exploited for clinical use. The first FDA-approved therapeutic monoclonal antibody

(used in the prevention of organ rejection) became available in the mid-1980s. As of

2004, fifteen monoclonal antibodies were licensed for therapeutic use (43), and more than

70 additional monoclonal antibodies were in various stages of development (296).

Not long after Stx production by *E. coli* O157:H7 was associated with progression

to HUS, monoclonal antibodies for use as a therapeutic against *E. coli* O157:H7 infection

were developed. The first report of an *E. coli* O157:H7 anti-Shiga toxin monoclonal

antibody (STmAb) came from Strockbine *et al.* (268). While these STmAbs (anti-Stx1 antibodies) were originally produced for use in detection assays, in retrospect, mouse lethality studies demonstrated the plausibility of anti-Stx1 as a therapy to prevent Stx1-mediated disease. The first description of passively transferred STmAb to prevent lethal the effects of *E. coli* O157:H7-infection came from work done by Wadolkowski and colleagues in 1990 (307). In this report, they noted that streptomycin-treated mice that were infected with a variant of *E. coli* 933 (produces Stx1 and Stx2) could be protected from mortality by pre-treatment with monoclonal antibody against Stx2 [anti-Stx1 mAb showed no protective effect (307)]. Although the potential applicability of STmAbs to prevent STEC-evoked systemic disease had been noted, it wasn't fully discussed until a book chapter by Edwards *et al.* in 1998 (73). In this chapter by Edwards and colleagues the usefulness of STmAbs as both an immune therapy and a prophylactic agent are noted (73).

Since these initial reports, the development and exploration of anti-Shiga toxin monoclonal antibodies as potential therapeutic agents have flourished. The notion that anti-toxin antibody provided passively could protect against the systemic manifestations of *E. coli* O157:H7 infection has been demonstrated in various animal model systems with both polyclonal and monoclonal antibodies (68, 73, 187, 256, 315). Currently, a number of anti-Stx monoclonal antibodies are under development for use in passive therapy. Several research groups are involved in the development of these neutralizing anti-Stx monoclonal antibodies for therapeutic purposes to prevent or ameliorate HUS. Indeed, both the pre-exposure (73, 307) and post-exposure utility of anti-toxin antibodies have been examined (68, 187, 256). In fact, independent studies in various animal

models of STEC infection indicate that post-infection administration of STmAbs may potentially prevent any ensuing systemic complications (68, 187, 208, 256, 315). For example, an anti-Stx2 mAb given at high enough doses to *E. coli* O157:H7-infected piglets protected the animals from mortality when given at either 24 hours (after the onset of diarrhea in this model) or 48 hours (at the beginning of manifestations of CNS impairment) (296). These findings suggest that STmAbs have the potential to not only protect against the development of HUS when given to children with on-going diarrhea, but additionally may provide some protection when administered to children at or just prior to the onset of HUS (296). As a result of the apparent success of STmAbs in preclinical evaluation and the need for Stx therapeutics, STmAbs are currently in (or moving into) clinical trials. Two STmAbs, including our own, have demonstrated safety and tolerability when passively administered in phase I clinical trials (27, 178). Other STmAbs have also been selected for clinical trials that will begin shortly (296).

Vaccine Candidates

Vaccine strategies for prevention of *E. coli* O157:H7 disease focus on one of two general methodologies: vaccination of people to prevent infection and disease or vaccination of the animal reservoir to prevent transmission. The techniques for achieving vaccination [DNA, toxoid, conjugate, live attenuated, edible] and the antigens used [intimin, toxin, O157, TTSS or effectors] can vary [reviewed in (171)]. Numerous groups are employing various techniques in the development of an *E. coli* O157:H7 vaccine, although most efforts are currently focused on preclinical development for proof-of-concept in animal model systems. However, there are a few *E. coli* O157:H7

vaccines that are moving towards or have entered phase I clinical trials. An O157 LPS

conjugate vaccine has completed phase I trials (163). That vaccine was safe and elicited

an anti-LPS IgG serum response in volunteers (163). Keusch *et al.* prepared large

batches of formalin-inactivated toxin for phase I trials (156), although these trials were

never initiated. While the use of live attenuated vaccines is not as far advanced as a

strategy against *E. coli* O157:H7 infections, a potential advantage of such an approach is

that the vaccine strain may stimulate a local mucosal sIgA response when it colonizes the

intestinal tract [reviewed in (37)].

Toxoid-based vaccines are by far the most popular strategy to prevent STEC

disease because the presence of serum neutralizing antibody against toxin should prevent

systemic manifestations of STEC-infection and prevent development of HUS [as the

latter has been demonstrated in numerous animal models (30, 112, 152, 208, 307, 319)].

In fact, there are several groups, including our own, who are exploring toxoid-based

immunization strategies to ameliorate serious disease due to *E. coli* O157:H7. Indeed, we

have shown that a hybrid Stx1/2 toxoid can elicit protective humoral responses in

vaccinated mice (266). The value of this hybrid toxoid vaccine candidate is that it can

elicit neutralizing antibodies towards both Stx1 and Stx2 (266), toxins that are not cross-

neutralizable (311). While the majority of HUS cases are associated with Stx2-producing

bacteria and the effects of Stx2 have been linked to renal damage in mice (307), the fact

that Stx1-producing strains can also cause disease and HUS makes it important to

consider both toxins in vaccine development (92, 107, 158, 199).

For toxoid vaccines, the issue of immunogenicity is a critical one. Shiga toxins

appear to elicit only low titers (at best) of neutralizing antibodies in patients with HUS

[reviewed in (146)], despite the fact that they are considered to be the major virulence factor of *E. coli* O157:H7. This observation may mean that the toxin is not very immunogenic or, alternatively, it could mean that only a small amount of this potent toxin is required to provoke disease as is the case with botulinum toxin (3). The B subunit of Stx has been shown to induce a humoral response in animal models and that response is protective against the homologous holotoxin (5, 31, 122, 293). Since the B subunit of Stx does not have the associated cytotoxicity of the A subunit or holotoxin, some consider it a prime vaccine candidate (37, 156). However, an Stx1/Stx2 B subunit cocktail would likely be necessary to protect against all Stx types.

An *E. coli* O157:H7 vaccine, aimed at preventing infection and/or disease, while potentially providing the most effective protection, is likely the most controversial and impractical option. Even the determination of the potential target population for immunization would be difficult. While the majority of the disease burden, particularly severe disease, lies with children, Tauxe argues that in the pacific northwestern U.S. disease afflicts both children and adults (279). The finding of a rare but potentially life-threatening disease (HUS) in all age groups makes a case for universal immunization, but universal vaccination is a challenge in and of itself. Furthermore, the social and economical consequences of vaccination against our food supply must be considered, including the impact that licensure of such a vaccine would have on agricultural exports (171, 279).

While an *E. coli* O157:H7 vaccine has not been developed (and licensed) for immunization of humans, there are at least two such vaccines currently in use for cattle. The latter vaccines are designed to reduce or block transmission of *E. coli* O157:H7 from

its zoonotic reservoir to people by reducing carriage of the bacterium in cattle. Bioniche of Canada has developed a vaccine against the *E. coli* O157:H7 TTSS products (207, 263). The first U.S. cattle vaccine to receive a conditional USDA license was Epitopix's O157 Bacterial Extract vaccine (87, 286). Additionally, researchers from our laboratory investigated an "edible plant" vaccination strategy for cattle that was focused on oral vaccination against intimin by means of NT-1 tobacco cells (for proof of concept) and/or corn (140).

Summary of Aims

The goal of the research that formed the basis for this dissertation was to attempt to define the role of toxin in the colonization and pathogenesis of *E. coli* O157:H7. This work was guided by two related hypotheses. First, that Stx2 facilitates colonization in a mouse model in which Stx2-expressing *E. coli* O157:H7 must compete with an intact commensal flora. Second, and as a corollary to that hypothesis, the presence of anti-Stx2 antibody prior to and during *E. coli* O157:H7 infection results in diminished levels of colonization. The specific aims developed to address these theories are as follows:

1. Establish and characterize an oral infection model of *E. coli* O157:H7 colonization and pathogenesis in mice with an intact commensal flora (ICF) and determine the temporal relationship of colonization and Stx production.

2. Evaluate the capacity of Shiga toxin type 2 to promote *E. coli* O157:H7 colonization in the mouse ICF model.

3. Explore the effect of anti-Shiga toxin type 2 antibody on *E. coli* O157:H7 colonization in the mouse ICF model.

4. Verify the capacity of anti-Shiga toxin type 2 antibody to protect against Stx2-mediated disease in the mouse ICF model.

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CHAPTER TWO

PATHOGENESIS OF *ESCHERICHIA COLI* O157:H7 STRAIN 86-24 FOLLOWING ORAL INFECTION OF BALB/C MICE WITH AN INTACT COMMENSAL FLORA

Note: all of the figures and tables shown reflect the work of Krystle Mohawk with the exception of the histopathology, which was done by LTC Erica Carroll. Dr. Melton-Celsa and Tonia Zangari assisted during the experimental procedures. Drs. Melton-Celsa and O'Brien contributed both to the design of the experiments and the interpretation of the data as well as the preparation of the manuscript. The order of this chapter (Abstract, Introduction, Results, Discussion, Conclusion, and Materials and Methods) is in accordance with the submission requirements posed by *Microbial Pathogenesis*. The references have been altered in following with the style of this dissertation.

Abstract

Escherichia coli O157:H7 is a food-borne pathogen that can cause hemorrhagic colitis and, occasionally, hemolytic uremic syndrome, a sequela of infection that can result in renal failure and death. Here we sought to model the pathogenesis of orally-administered *E. coli* O157:H7 in BALB/c mice with an intact intestinal flora. First, we defined the optimal dose that permitted sustained fecal shedding of *E. coli* O157:H7 over 7 days (~10^9 colony forming units). Next, we monitored the load of *E. coli* O157:H7 in intestinal sections over time and observed that the cecum was consistently the tissue with the highest *E. coli* O157:H7 recovery. We then followed the expression of two key *E. coli* O157:H7 virulence factors, the adhesin intimin and Shiga toxin type 2, and detected both proteins early in infection when bacterial burdens were highest. Additionally, we noted that during infection, animals lost weight and ~30% died. Moribund animals also exhibited elevated levels of blood urea nitrogen, and, on necropsy, showed evidence of renal tubular damage. We conclude that conventional mice inoculated orally with high doses of *E. coli* O157:H7 can be used to model both intestinal colonization and subsequent development of certain extraintestinal manifestations of *E. coli* O157:H7 disease.

Introduction

Escherichia coli O157:H7 is a food-borne pathogen that results in an estimated annual incidence of 73,000 cases of diarrheal illness each year in the United States (30). Hemolytic uremic syndrome, or HUS, is the most serious sequela of *E. coli* O157:H7 infection that occurs on average in 4% of those infected (40). However, the recent 2006 spinach-associated *E. coli* O157:H7 outbreak in the United States had a higher rate of HUS (16%) that was perhaps attributable to the emergence of a more virulent clade of *E. coli* O157:H7 (3, 27). HUS consists of a triad of symptoms that include microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (2). Although most people recover from HUS, late complications and even death can result (29).

The sequence of events by which *E. coli* O157:H7 establishes infection and causes disease is imperfectly understood. It is known that *E. coli* O157:H7 can cause illness in a person who has ingested as few as 100 organisms (48). Therefore, at least a portion of that inoculum must be capable of surviving the acidic conditions of the stomach to colonize the intestine [reviewed in Ref. (8)]. Intimate adherence of *E. coli* O157:H7 to the intestinal mucosa is considered to require expression of the bacterial outer membrane protein intimin and other factors secreted through the type III secretion system (TTSS) [reviewed in Ref. (12)].

The development of HUS is associated with the production of Shiga toxin (Stx) by *E. coli* O157:H7 and other Stx-producing *E. coli* (STEC) (19, 20, 35). This toxin is produced by the infecting bacteria in the gut, as evidenced by the presence of Stx in the feces of *E. coli* O157:H7-infected individuals (19, 20, 39). *E. coli* O157:H7 can produce

two types of Stxs, Stx1 and/or Stx2, as well as variants of these toxin types. Stxs are AB_5 toxins that bind to cells through the pentameric B subunits via a glycosphingolipid receptor known as globotriaosylceramide, or Gb_3. Stx is an N-glycosidase that cleaves a purine residue in ribosomal 28S RNA. This catalytic event leads to inhibition of protein synthesis in the target cell and, subsequently, cell death by apoptosis [reviewed in Ref. (36)]. The primary targets for Stx *in vivo* are small vessel vascular endothelial cells that express Gb_3 (37, 38, 41), such as those within the renal glomerulus (7, 41). It is not known how Stx crosses the intestinal barrier to target small vessel endothelial cells in the lamina propria of the gut (thought to be a primary event in the manifestation of hemorrhagic colitis), nor the mechanism by which the toxin enters the blood stream and travels through the circulatory system to the Gb_3-rich small vessel endothelia in the kidney.

Mouse infection models that mirror various aspects of STEC pathogenesis or disease (such as intestinal colonization, renal impairment, central nervous system damage, and death) have been developed [reviewed in Ref. (31)]. While these animal systems are helpful for assessing features of STEC disease, many require that mice undergo artificial manipulations such as prolonged dietary restriction to promote colonization, mitomycin C injection to facilitate Stx expression, or antibiotic treatment to reduce the normal flora that can inhibit the establishment of an exogenous infection (1, 15, 25, 26, 44, 51). Furthermore, in antibiotic-treated or axenic animal model systems the STEC strain is likely to have a pathogenic advantage as it is no longer competing with the entire normal flora microbiome for resources. On the other hand, in a mouse model in which the normal gut flora is suppressed, the infecting *E. coli* O157:H7 strain may not

receive molecular signals made by commensal bacteria that lead, through a quorum-sensing regulatory network, to TTSS expression by *E. coli* O157 (45). Thus, to better reflect the typical gastrointestinal environment to which *E. coli* O157:H7 is exposed following ingestion, we sought to develop an oral model of *E. coli* O157:H7 infection in mice with an intact commensal flora (ICF).

Results

Dose-response studies

As a first step in the development of a mouse model of *E. coli* O157:H7 infection in BALB/c mice with an ICF, we conducted dose-response experiments to determine whether inoculum size correlated with the percent of animals colonized or the level of colonization over time. For this purpose, groups of 5-10 mice were intragastrically administered approximately 10^5, 10^6, 10^7, 10^8, or 10^9 CFU of *E. coli* O157:H7 strain 86-24Nal^R and the degree of bacterial colonization measured as defined by the number of 86-24Nal^R bacteria present per gram [colony forming units (CFU)/g] feces.

The percent of animals colonized on day 1 following infection with strain 86-24Nal^R varied by challenge dose (Fig. 7A). At an inoculum of 10^5 or 10^6 CFU, 40% or 80% of the mice, respectively, had detectable bacterial counts within their feces on day 1 post-infection. All animals challenged with 10^7 CFU or greater shed detectable bacteria on day 1 of infection. The relative proportion of mice that remained colonized after day 1 was also dose-dependent (Fig 7A). Groups of animals given $\leq 10^7$ CFU of *E. coli* O157:H7 strain 86-24Nal^R had fewer infected mice on day 2 than on day 1, with 60% or less of the mice in those groups infected throughout the remainder of the study. Of the animals that received 10^8 CFU, 7 of 8 mice remained colonized for the first 5 days after infection (2 of the original 10 animals died during the study and, thus, were no longer included in this analysis). At the highest inoculum of 10^9 CFU, mice were both consistently (% of mice colonized) and persistently (prolonged, high-level colonization) infected for the 7 days that followed challenge (2 of 5 mice, both highly colonized, died on day 4 post-infection). In summary, as the inoculum dose increased there was a

Figure 7: Colonization of BALB/c mice by *E. coli* O157:H7 strain 86-24Nal^R

(A) Percent of infected mice colonized over time. Animals were intragastrically infected with 86-24Nal^R at inocula of 10^5 (●), 10^6 (■), 10^7 (▲), 10^8 (▼), and 10^9 CFU (♦). Mice were considered colonized if there were recoverable 86-24Nal^R in the feces. (B) Colonization level of 86-24Nal^R overtime at various doses administered intragastrically (symbols as above). For panels C and D, animals were infected with 86-24Nal^R at doses of 10^8 CFU or 10^9 CFU either by intragastric administration (dashed lines with ▼ for 10^8 CFU and ♦ for 10^9 CFU) or pipette feeding (solid lines with ▲ for 10^8 CFU and ■ for 10^9 CFU). (C) Effect of administration route on percent of mice colonized. (D) Impact of administration route on colonization level. For panels A and C, animals that died during the study were excluded after the time of death; these included 2 mice on day 4 from the 10^9 dose (gavage) and one animal each on days 4 and 5 from the 10^8 dose (gavage). For panels B and D, each point represents the GM of the number of 86-24Nal^R shed into the feces from surviving, infected animals, and the limit of detection (indicted by the black dashed line) was 10^2 CFU/g feces.

C

D
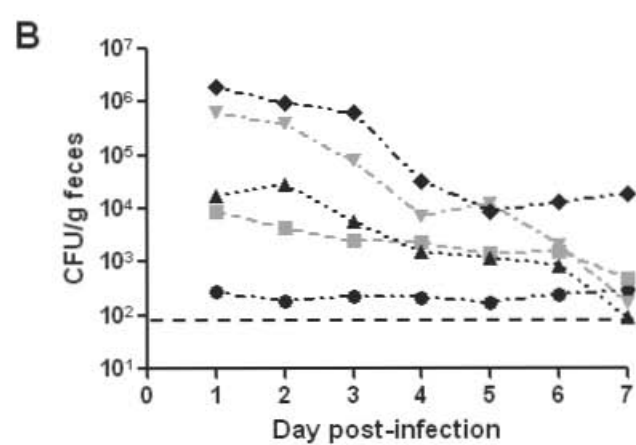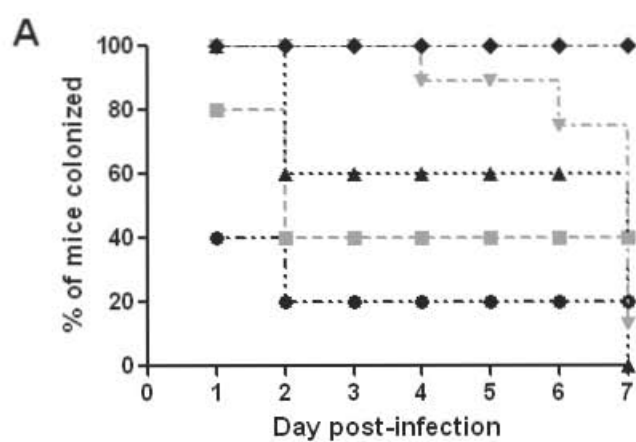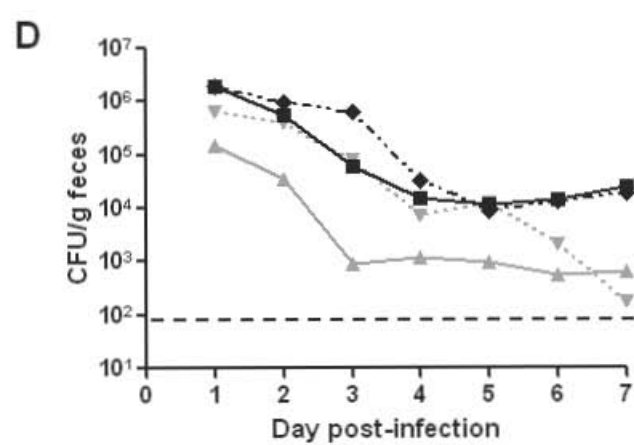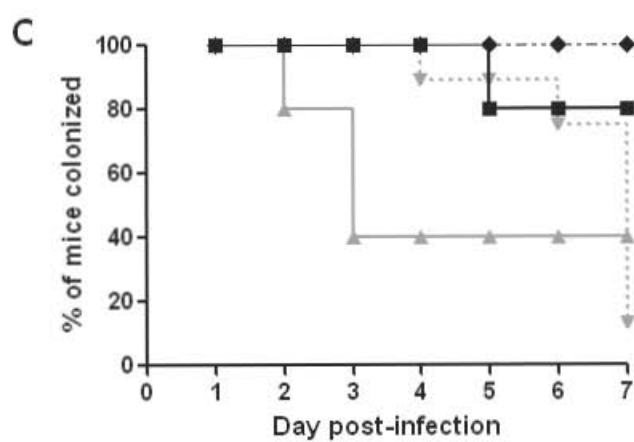
statistically significant increase in the percent of mice that were colonized over time (p=0.005 in a trend analysis).

Next, we assessed the differences in overall levels of colonization among the inocula groups (Fig. 7B). We found that as the challenge dose went up, the number of bacteria shed into the feces increased (p=0.001). At a low inoculum (10^5 CFU), animals shed *E. coli* O157:H7 with a geometric mean (GM) less than 10^3 CFU/g feces over the course of the experiment. The recoverable bacteria from mice infected with 10^6 or 10^7 CFU, while somewhat higher, still remained relatively low (GM at or below 10^4 CFU/g feces). In comparison, animals that received an inoculum of 10^8 or 10^9 CFU had more recoverable bacteria on the day after infection (GMs of 6.5 X 10^5 and 1.9 X 10^6 CFU/g feces, respectively). Mice that received 10^8 CFU had a statistically higher colonization level than animals challenged with 10^5 CFU, irrespective of time (p=0.001). At the largest inoculum of 10^9 CFU, statistically higher levels of colonization were measured compared to doses of 10^5, 10^6, or 10^7 CFU (p≤0.01), again irrespective of time. However, as the overall colonization levels declined during the study these variations by inoculum dose in colonization loads eventually disappeared (except at the highest inoculum when comparing CFU/g feces on day 7 post-infection, Fig. 7B).

We next evaluated the impact on colonization of administration of 10^8 or 10^9 CFU of strain 86-24Nal^R to the mice by intragastric administration versus pipette feeding (orally through a micropipette tip). We observed a more consistent infection after intragastric administration at the highest inoculum of 10^9 CFU (p=0.023, Fig. 7C). When assessing the colonization levels in the various groups (Fig. 7D), significantly higher colonization levels were achieved at higher inocula (p=0.008), as was noted previously

(Fig. 7B), regardless of method used to introduce the bacteria. Additionally, as infection progressed, colonization levels generally declined in all groups (p<0.001). When the data were averaged across all days, there was no overall difference between the procedures used to infect the animals at either dose. However, there was an indication that providing the inoculum by gavage resulted in increased levels of colonization on certain days post-infection, and this was most pronounced on day 3 (p=0.004) (Fig. 7D). Nevertheless, the most consistent and persistent colonization levels in mice were achieved with an inoculum of 10^9 CFU regardless of the route of bacterial challenge used. Therefore, for all subsequent experiments, we orally infected mice either intragastrically by gavage or by pipette feeding with approximately 10^9 CFU of the microbe. The inoculum method applied was largely dictated by the question under study in a particular experiment.

Sites of 86-24Nal^R colonization in the mouse intestinal tract

To determine the site of *E. coli* O157:H7 colonization within the intestines of the ICF mice after challenge with 86-24Nal^R, animals were orally inoculated with about 10^9 CFU of the bacterium by pipette feeding. We elected to use the pipette feeding method for this study because it is more like the natural route of exposure to the organism, ingestion, than is gavage. At various times following infection, groups of animals (n=8-17) were sacrificed, and the numbers of 86-24Nal^R bacteria associated with the tissues of the small intestine, cecum, and large intestine, as well as in the luminal contents of those intestinal segments were determined.

When the 86-24Nal^R counts of the organ tissues were compared, we found that the bacterial load was the highest in the cecum, followed by the large intestine, while the

small intestine had the fewest recoverable *E. coli* O157:H7 (Fig. 8A). The number of *E. coli* O157:H7 bacteria harvested from each of the tissues dropped from 6 h to 24 h, and then leveled off for the remainder of the study. The number of bacteria recovered from the luminal contents showed a similar pattern to that seen for the organs themselves, with the exception that the contents of the large intestines remained high throughout the study (Fig. 8B). We also observed that the large intestine luminal contents had significantly more recoverable *E. coli* O157:H7 than did the large intestinal tissue (compare Fig 8B line with open diamonds to Fig. 8A line with solid diamonds; $p<0.001$). In fact, the large intestine luminal contents had equivalent levels of *E. coli* O157:H7 to those found in the cecum, the cecal contents, or both, depending on the time-point assessed. As was seen for the small intestinal tissue, the small intestine luminal contents had significantly fewer recoverable *E. coli* O157:H7 bacteria throughout the study when compared with the cecal and large intestinal contents ($p<0.001$). Indeed, the small intestinal contents and tissue had the lowest number of 86-24Nal^R throughout the course of infection. Overall, we observed the highest GM CFU/g in the cecal tissue, cecal contents, and large intestinal contents, and these values were similar (compare Fig. 8A and B).

Because the cecal tissue had the highest *E. coli* O157:H7 counts of all the tissues examined, we concluded that the cecum is the site of optimal colonization by 86-24Nal^R. Therefore, we examined cecum-associated *E. coli* O157:H7 via immunofluorescence. Early in infection, we readily found fluorescent bacteria both within the cecal lumen (the majority of fluorescent organisms) and close, perhaps adherent, to the epithelial surface of the cecal tissue (Fig. 9A-F). At later time-points during the course of infection, the

Figure 8: Levels of *E. coli* O157:H7 over time at different sites in the gastrointestinal tract of BALB/c mice orally infected with 6-8 X 10^8 CFU of strain 86-24Nal^R

The GM CFU/g tissue (A) or luminal contents (B) from groups of 8-17 mice each are shown for the tissue of the small intestine (●), cecum (▲), and large intestine (♦) as well as the luminal contents of the small intestine (○), cecum (Δ), and large intestine (◊). Bars indicate the 95% confidence interval.

A

B
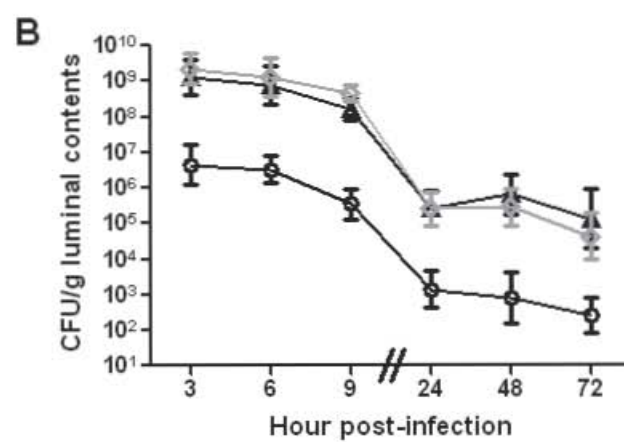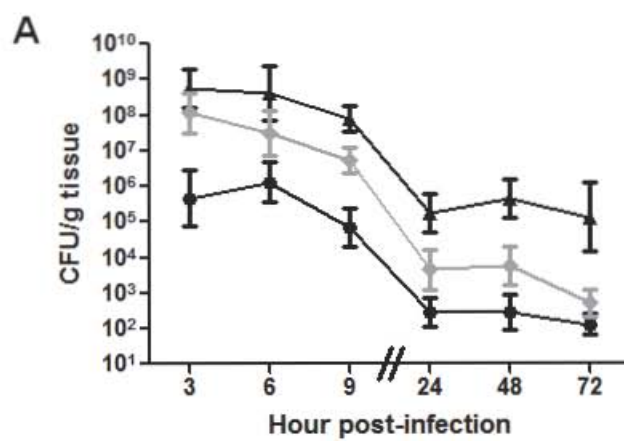
Figure 9: Immunofluorescent *E. coli* O157:H7 in the cecum

Mice were infected with *E. coli* O157:H7 (A-F) or mock-infected (G-I) for 6 h. Images display infected or control ceca stained for O157 (A, D, G), the phase contrast image of the cecal section (B, E, H), and the merged images (C, F, I). Panels D, E, and F are a higher magnification of the boxed area of panel B. For panels A-C and panels G-I the original image was obtained at a magnification of 40X. For panels D-F the original image was obtained at a magnification of 100X.
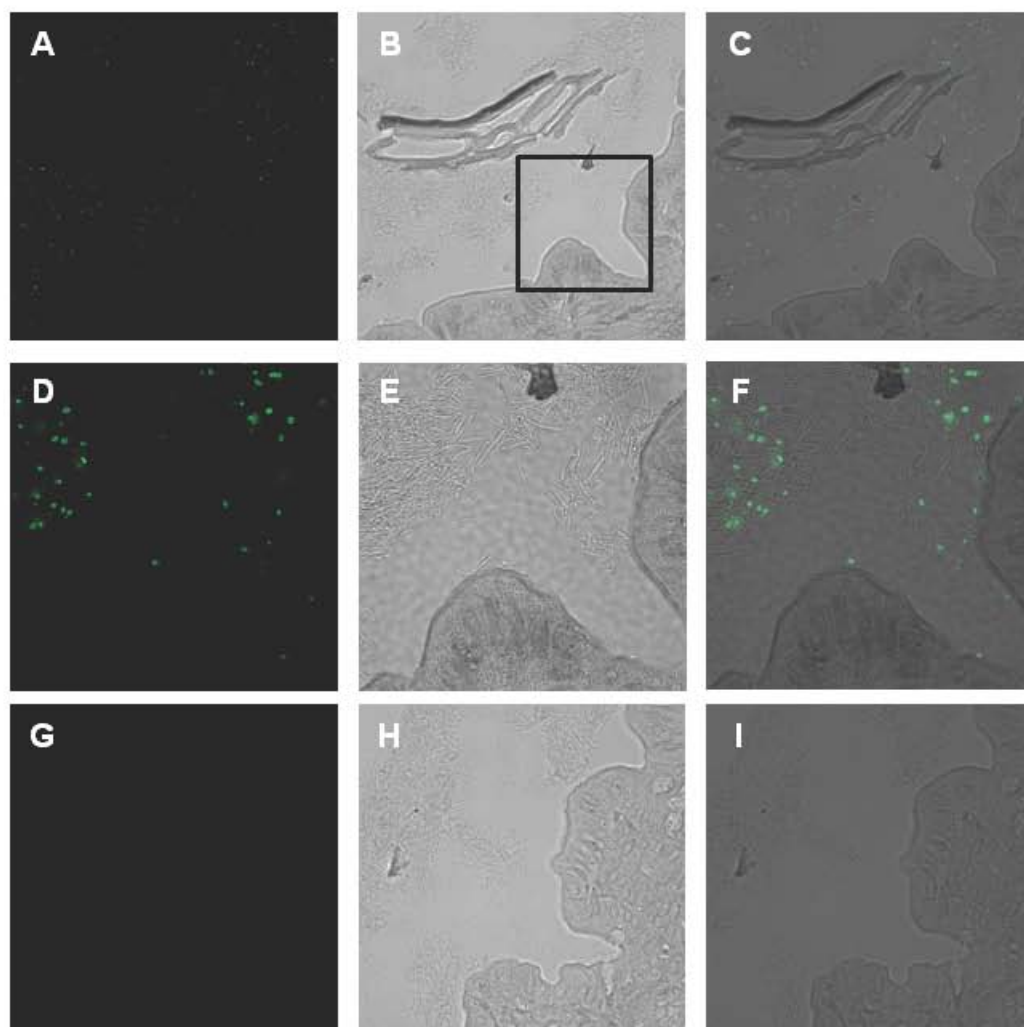
numbers of O157-stainable bacteria decreased (not shown). As expected, *E. coli* O157:H7 was not detected in cecal sections from uninfected control mice (Fig. 9G-I).

Kinetics of intimin and Stx2 expression* in vivo *at intestinal sites

To further evaluate the pathogenesis of *E. coli* O157:H7 in mice with an ICF, we monitored the expression of intimin and Stx2 over time in animals fed 6-8 X 10^8 CFU of strain 86-24Nal^R. Three separate studies were conducted, and the pooled results are shown in Figure 10. For these studies, we did not evaluate small intestine or small intestinal contents because 86-24Nal^R levels were low at that location in the time course study (see Fig. 8).

Intimin was detected at all sites examined (cecum, cecal contents, large intestines, and large intestinal contents) from mice sacrificed at 3, 6, and 9 h post-infection (Fig. 10A). A subset of tissue and tissue content samples were examined for absolute levels of intimin by comparison with values from a standard curve derived from control tissue spiked with different amounts of intimin (Fig. 10B). In this subset of specimens, the highest intimin values were detected within the cecum, cecal contents, and large intestinal contents. These latter findings likely reflect our earlier observations that the largest numbers of recoverable bacteria were found in these locations early in infection (see Fig. 8). Intimin was minimally detected at 24, 48, and 72 h post-infection (Fig. 10A and B), most likely due to the declining numbers of recoverable CFU/g sample after 24 h of infection (see Fig. 8).

The presence of Stx2 in the cecum, cecal contents, large intestines, and intestinal contents was first assessed by enzyme-linked immunosorbent assay (ELISA). Most of

Figure 10: Presence of intimin and Stx2 over time at different sites in the gastrointestinal tracts of BALB/c mice orally infected with *E. coli* O157:H7 strain 86-24Nal^R

(A) Percent of different intestinal samples positive for intimin over time. Each bar represents the mean of 8-17 samples tested. For panels A-D, the tissues are represented by black bars (cecum), white bars (cecal contents), hatched bars (large intestines), and gray bars (large intestinal contents). (B) Levels of intimin in different intestinal sites over time. Each bar represents the mean value of a subset of those samples presented in panel A (n=5-10). The limit of quantifiable intimin was 360 ng/g tissue or tissue contents. For panels B and D, each individual sample is represented by a ○. (C) Percent of different intestinal samples positive for Stx2 over time. Each bar represents the mean of 7-16 samples tested. (D) Levels of Stx2 in different intestinal sites over time. Each bar represents the mean value of a subset of those samples presented in panel C (n=5-10). The limit of quantifiable Stx2 was 6.4 ng/g tissue or tissue contents.
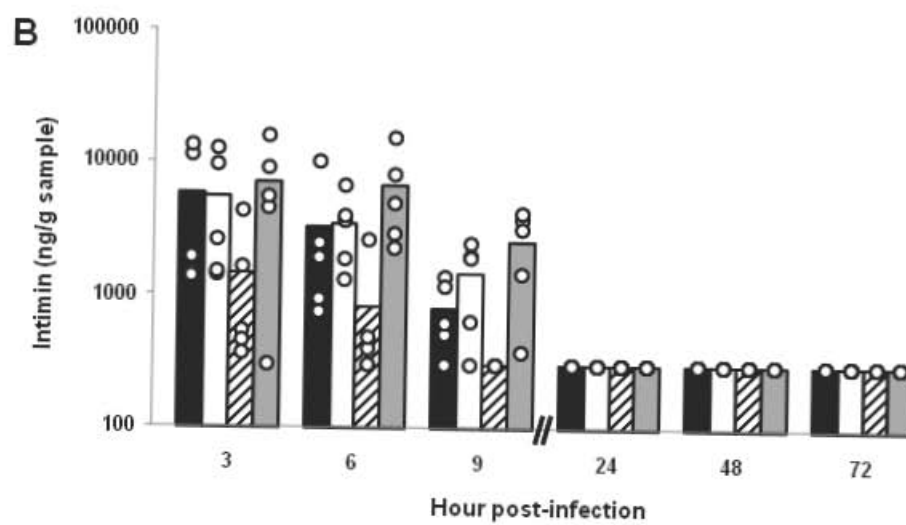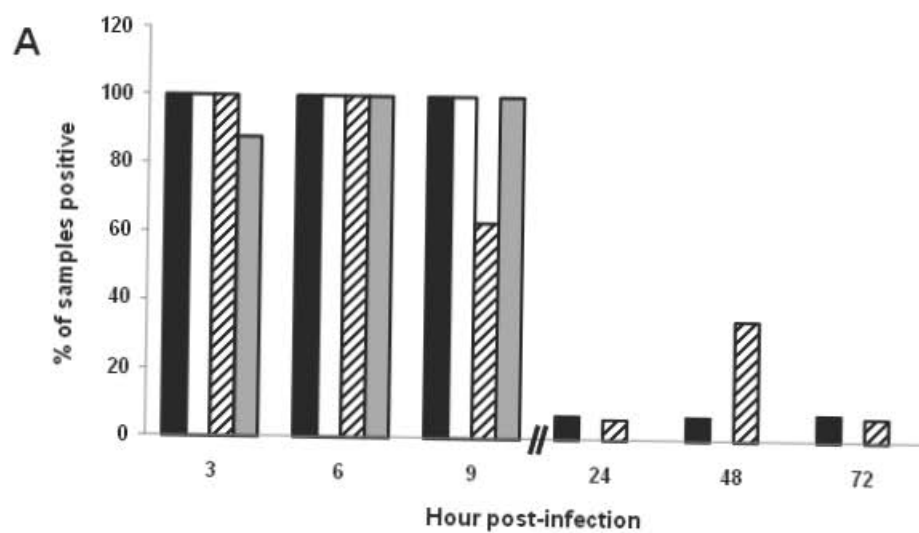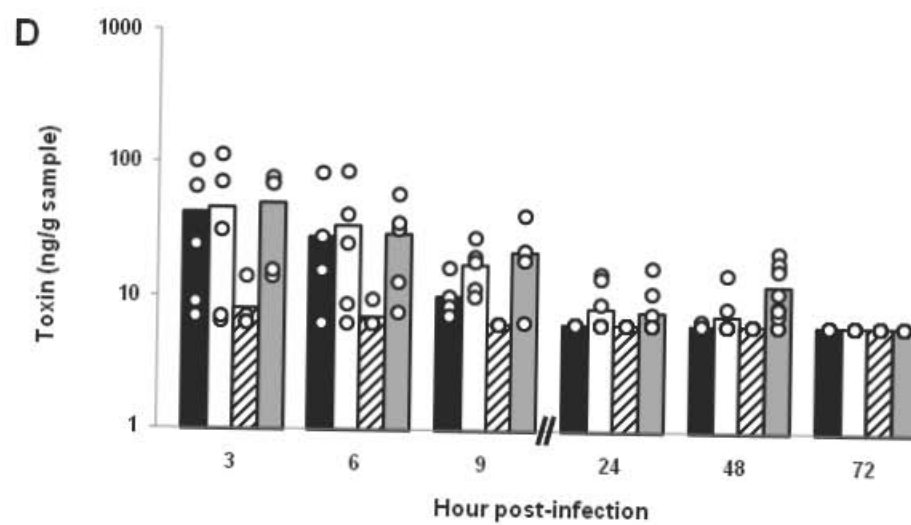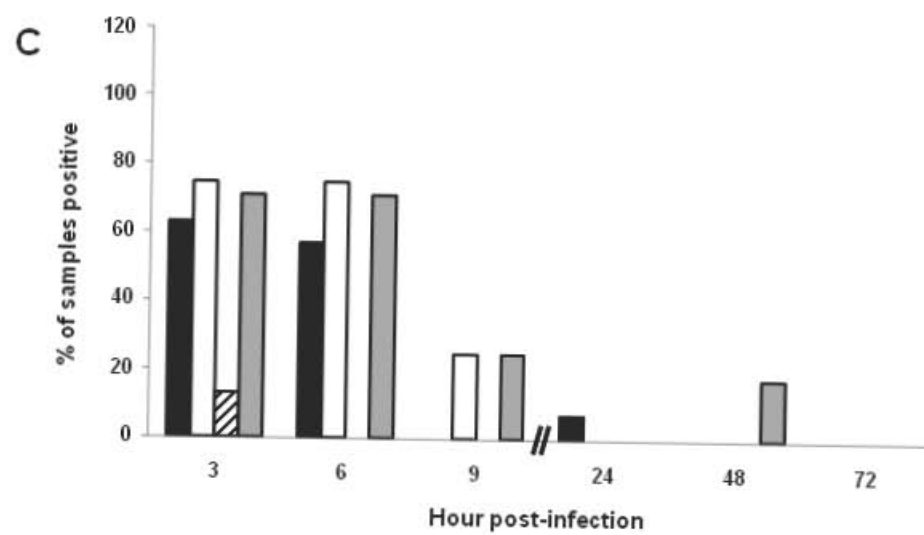
the samples positive for toxin were obtained during the first 9 h of infection (Fig. 10C). We found the luminal contents to have higher detectable toxin levels than the organ tissues when we measured absolute values versus a positive control in a subset of samples ($p \leq 0.002$) (Fig. 10D). Furthermore, we saw the highest detectable levels of toxin in the large intestinal contents ($p \leq 0.006$). In general, the locations where we found higher toxin levels corresponded to intestinal sites that had the highest numbers of recoverable organisms (compare Figs. 8 and 10C). To ensure that the positive results we obtained by ELISA were due to Stx2, we evaluated a subset of the samples for Stx2 by a neutralization assay (not shown). The findings in the neutralization assay and the ELISA were similar; toxin was most readily detectable early in infection and within the contents of the organs tested (the cecum and large intestine).

Systemic effects of infection

Over the course of longer infections, we noticed that a proportion of the animals that were fed 86-24Nal[R] exhibited signs of illness (hunched posture, ruffled fur, lethargy, tremors, etc.) and succumbed to infection. To more closely examine the systemic effects of *E. coli* O157:H7 infection in mice with an ICF, groups of animals were infected by gavage with 10^9 CFU and monitored both for weight loss (indicative of illness) and death. We elected to use gavage for infection because we wanted to increase the likelihood that all animals would be colonized. All animals demonstrated a drop (about 1.5 g) in weight from day -1 to day 0, a phenomenon we ascribed to overnight deprivation of food prior to infection (Fig. 11A). However, the mice returned to near pre-infection weights at day 1 of infection after they were permitted access to food and water

Figure 11: Effect of intragastric infection of BALB/c mice with ~10^9 CFU of *E. coli* O157:H7 strain 86-24Nal^R on body weight and survival

Animals were starved overnight prior to intragastric inoculation with *E. coli* O157:H7 on day 0. (A) Weight was monitored for the control, mock-infected animals (●) and the 86-24Nal^R-infected, experimental group (■). Each point on the graph represents the mean weight of 10 (or less in the case of groups with mortality) animals per group. The bars depict ±one standard error of the mean of weight. Asterisks indicate days that demonstrated a significant difference between weights of infected and control mice ($p \leq 0.001$). (B) Survival of animals infected with 86-24Nal^R or mock-infected. The percent of surviving animals (10 mice per group) on each day post-infection inoculated with 86-24Nal^R (■) or mock-infected (●).
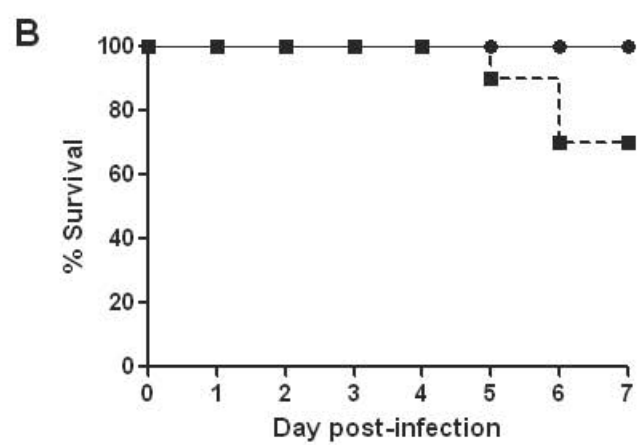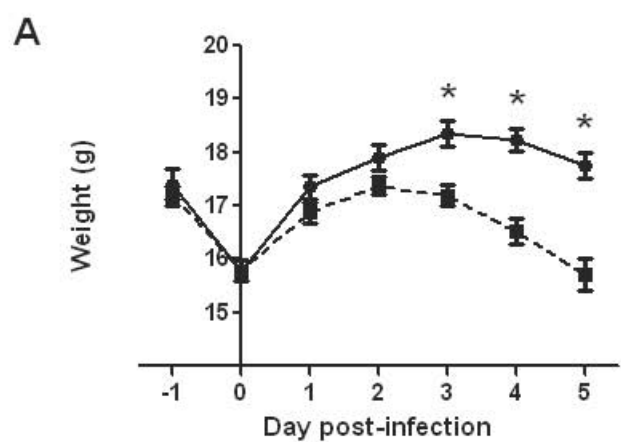
ad libitum. In fact, even after *E. coli* O157:H7 inoculation, all animals gained weight between days 1 and 2. After day 2 of infection, there was a statistically significant divergence in weight of the control and infected animals. Indeed, the control mice continued to gain weight until day 3 when, as a group, their weight plateaued and remained steady at around 18 g. Conversely, the experimental group started to lose weight after day 2 post-infection and weighed significantly less than did the control mice on days 3-5 post-infection ($p \leq 0.001$; Fig. 11A). Moreover, infected mice that demonstrated large amounts of weight loss succumbed to infection on days 5 and 6 post-*E. coli* O157:H7 challenge. In this experiment, 30% of the infected mice died, and, as expected, all control mice survived (Fig. 11B).

We then asked whether the mouse mortality seen after intragastric administration of *E. coli* O157:H7 could have resulted from bacteremia following gavage. To address this question, 20 mice were infected with 10^9 CFU of 86-24Nal^R intragastrically and monitored for mortality and the development of bacteremia. At 6 h post-infection, blood was taken from each mouse and assessed for the presence of circulating bacteria. Five of the animals demonstrated the presence of bacteria in their blood. These same five animals died by 24 h post-infection. The remaining mice were divided into two groups: a group to check for the presence of bacteremia on days 2 and 4 post infection and a mortality group. The animals examined for bacteremia on day 2 post-infection demonstrated no detectable bacteria in their blood. Two animals from the bacteremia-assessment group were sacrificed on day 3 because they were moribund. These latter two animals had symptoms of disease that included neurological manifestations (ataxia, tremor, and convulsions), yet had no detectable bacteria in their blood. The final two

animals from the bacteremia-assessment group were sacrificed on day 4 and were not

bacteremic. In the mortality group, animals lost weight over time; furthermore, deaths

(4/7 mice or 57% mortality) occurred on days 4 and 5 post-infection. All of the animals

in the mortality group that succumbed to infection were free of circulating bacteria in

their blood as determined post-mortem. Thus, bacteremia was not seen, even in

extremely moribund mice, after the first day of infection. Moreover, in a second study

using a lower inoculum (~5 X 10^8 CFU 86-24Nal^R), no bacteremia was detected in any of

the mice at 6 h post-infection. In this latter study, the bacteremia-assessment group also

had no detectable bacteria in the blood at day 2 or 4. The mortality group had a 20%

death rate with no evidence of bacteremia 6 h following gavage or post-mortem. We

concluded that at high challenge doses of 86-24Nal^R given by gavage, animals that

succumb after day 2 post-infection are not dying as a consequence of bacteremia.

Since we observed deaths in mice infected by gavage with high doses of 86-

24Nal^R in the absence of bacteremia, we speculated that this mortality was due to Stx2

produced by *E. coli* O157:H7 in the gut. Indeed, we have shown that streptomycin-

treated mice that would normally die after oral STEC infection remain healthy if

passively treated with neutralizing anti-Stx2 antibodies (21, 26, 49). Here, we sought to

demonstrate the central role of toxin in lethality after *E. coli* O157:H7 infection in an ICF

model by assaying for the presence of Stx2 in the blood of animals gavaged with 10^9

CFU of 86-24Nal^R. However, blood taken from animals that were infected with *E. coli*

O157:H7 showed either no detectable toxin or sub-picogram levels of toxin (found in the

blood of 2/15 mice). We next asked if it was possible to detect intravenously (IV)

administered Stx2 in the blood of uninfected mice. We found that it was necessary to

administer 100-1000 LD_{50}s (100-1000 ng) of Stx2 in order to detect toxin in blood taken from mice 2 h post-intoxication. The amount of Stx2 detected in the blood from these highly intoxicated animals was 0 - 17 pg per 0.1 mL. Therefore, we surmised that the reason we did not detect Stx2 in the blood of infected mice is most likely because the levels of toxin in the blood were below 17 pg/0.1 mL.

To further examine *E. coli* O157:H7 pathogenesis in mice with an ICF, animals were sacrificed on various days post-infection, blood was collected, and kidneys were surgically removed. Sera were analyzed for blood urea nitrogen (BUN) and creatinine levels, and whole blood samples were used to determine complete blood counts (CBCs, Table 2). When compared to the uninfected controls, *E. coli* O157:H7-infected mice showed significantly higher BUN ($p<0.01$) values and higher, but not statistically significant, creatinine levels ($p=0.07$) over the course of infection. The greatest elevations in these kidney function values were seen in animals sacrificed on days 4 and 5 (individual day data not shown). However, none of the key hematological features of HUS were apparent in the infected animals. Specifically, no differences in platelets, hemoglobin, or hematocrit values were noted between infected and control mice. Nevertheless, significant neutrophilia ($p=0.001$) was apparent in the blood samples of the experimental group compared to the controls (Table 2). In fact, as early as day 2 following infection, the blood of infected mice showed an increase in percentage of neutrophils that was evident throughout the experiment (days 2, 3, 4, and 5) with differences of up to 4-fold compared to controls (data not shown).

We then examined the histopathology of the kidneys from the *E. coli* O157:H7-infected and control animals whose blood samples were assayed as above. We found no

Table 2: Average values (and range) of serum chemistry and CBC analyses

of mice infected with *E. coli* O157:H7 strain 86-24 Nal^R

Average values represent data from all days (2, 3, 4, & 5) combined. Blood urea nitrogen is abbreviated BUN. White blood cell is abbreviated WBC. A non-significant *p*-value is indicated by ND or no difference.

Laboratory Test	Control	Infected	*p*-value
BUN (mg/dL)	17.8 (13,28)	32.4 (15,108)	<0.01
Creatinine (mg/dL)	0.26 (0.2,0.5)	0.34 (0.2,0.8)	0.073
% Neutrophils	14.8 (1.8,20.3)	39.0 (22.1,81.4)	0.001
% Lymphocytes	75.9 (61.4,84.8)	53.5 (13.6,74.4)	0.002
Hemoglobin (g/dL)	14.5 (12.4,16.3)	15.5 (12.4,17.2)	ND
% Hematocrit	45.7 (39.4,51.2)	49.0 (39.5,56.6)	ND
Platelets (K/mL)	751 (335,1234)	881 (293,1189)	ND
WBC (K/mL)	4.79 (1.11,8.25)	5.04 (2.43,8.56)	ND

evidence in any of the kidney sections from infected or control mice of lesions in the

renal glomeruli (as assessed by H&E stain), or fibrin deposits in the glomerular

capillaries or interstitial vessels [as evaluated by Mallory's phosphotungstic acid

hematoxylin (PTAH) or a modified Carstair's stain]. Additionally, we probed the kidney

sections for the presence of Stx2. While we detected toxin in the kidney by

immunofluorescence (IF) in one mouse that died following infection (not shown), that

result was not duplicated in a second, moribund animal. That we found only minute

amounts of circulating toxin in the blood also suggests that the quantity of Stx2 present

for detection by IF in the kidneys is quite small. In contrast to the lack of glomerular

changes in infected animals, histological evidence of injury to proximal and distal

convoluted tubules was noted in kidneys from infected animals (Fig. 12A and B) but not

in kidneys from control mice (Fig. 12C). Indeed, kidneys from several infected mice

exhibited acute to subacute, multifocal, minimal to mild tubule degeneration, small

amounts of epithelial necrosis, and varying degrees of regeneration.

Figure 12: Renal lesions in mice infected by gavage with *E. coli* O157:H7 strain 86-24Nal^R

Panels A, B, and C display kidney sections stained with H&E and taken from infected (panels A and B) or control (panel C) mice. The original images were obtained at 40X magnification. (A) The mouse was necropsied on day 5 post-infection. Multiple dilated cortical tubules are lined by epithelial cells that are hypereosinophilic, shrunken, angular, and pyknotic [indicative of necrosis (arrowheads)] or hypertrophic with vacuolated cytoplasm [indicative of degeneration (arrow)]. (B) The mouse was necropsied on day 5 post-infection. Cortical tubule epithelial cells have basophilic cytoplasm, are karyomegalic (arrowheads), and exhibit anisocytosis (presence in the blood of erythrocytes with excessive variation in size) with mitotic figures (arrow), all of which is indicative of regeneration.
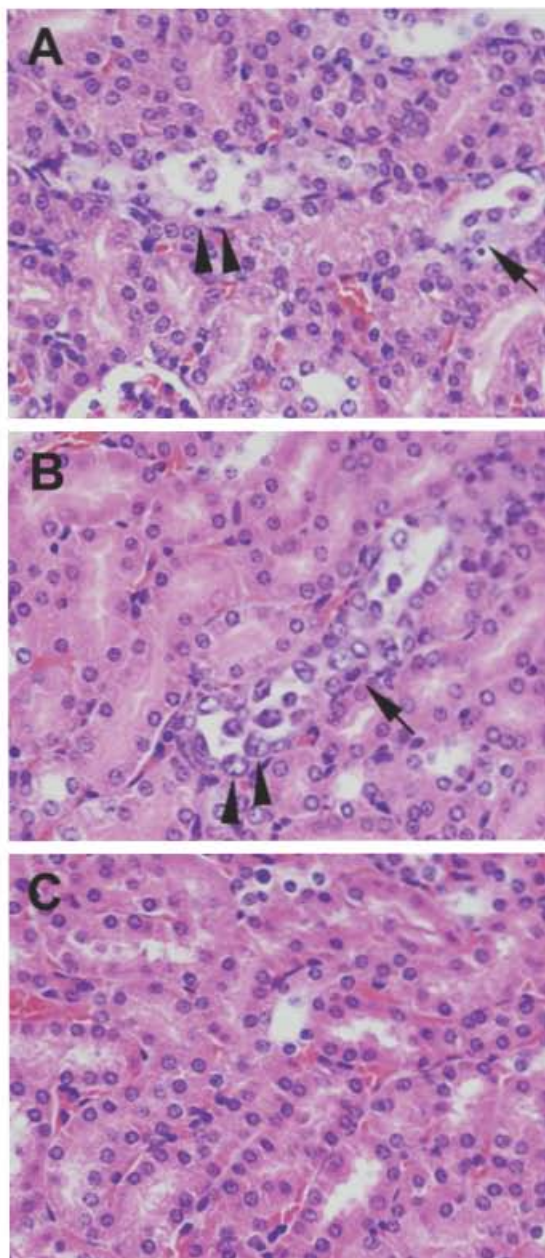
Discussion and conclusions

Discussion

In this investigation, we developed a model of *E. coli* O157:H7 intestinal

colonization and disease in BALB/c mice with an ICF. Although a large dose of *E. coli*

O157:H7 was required in this murine model, high inocula of these organisms are also

needed to achieve colonization and disease in monkeys (18) and rabbits (11). Our data

suggested that the predominate site of *E. coli* O157:H7 colonization was the cecum,

although numerous challenge bacteria were also culturable from the cecal and large

intestinal contents. Furthermore, mice challenged intragastrically with 10^9 *E. coli*

O157:H7 often displayed such manifestations of disease as lethargy and weight loss,

neutrophilia and increased levels of BUN. Moreover, about 30% of animals inoculated

with *E. coli* O157:H7 by the intragastric route died. Finally, we observed evidence of

tubular regeneration and some tubular damage in the kidneys of infected mice. We

interpreted these findings to mean that damaged tubules were in the process of repair. Of

note, tubular damage, in addition to signature glomerular lesions (41, 42), has been seen

in kidney sections from patients with HUS (4, 17, 46). Furthermore, tubular necrosis and

subsequent regeneration were described by Tesh *et al*. in mice that died after toxin

administration (47).

The large numbers of *E. coli* O157:H7 strain 86-24Nal[R] found in the cecum, cecal

contents, and large intestinal contents of infected mice support the hypothesis that these

organisms colonize the cecum and are then shed into the cecal contents before they pass

into the large intestinal contents, where they are ultimately bound in fecal material and

expelled. These results in aggregate indicate that the CFU/g feces formed from the

contents of the large bowel most likely reflect the extent of organism replication in the cecum of the animal. This theory is consistent with the suggestion in the report by Nagano *et al.* that *E. coli* O157:H7 found in the large intestines and feces are shed from bacteria adherent in the cecum (32).

In addition to identifying the site of *E. coli* O157:H7 colonization in the ICF model, we detected intimin in the same areas as the bacteria early during *in vivo* infection but not readily thereafter. This pattern could have reflected down-regulation of intimin expression *in vivo*, as has been documented for the expression of intimin *in vitro* by a related organism, enteropathogenic *E. coli* (EPEC) (23). However, because we also observed a significant decrease in bacterial numbers over this same time period, we could not ascertain whether *E. coli* O157:H7 intimin was down-regulated *in vivo* or if the reduced bacterial load meant that the intimin levels fell below the limit of detection for our ELISA.

As was seen with intimin, we observed the highest levels of Stx2 early in *E. coli* O157:H7 infection, and were only occasionally able to detect toxin in intestinal samples taken later during infection when total recoverable bacterial counts were low. Thus, our inability to detect toxin later in infection probably reflects the reduced bacterial numbers at that time in infection. Cornick *et al.* observed a similar phenomenon when they assessed colonization and toxin production within the small intestines of swine infected with an STEC (6). They found the highest toxin titers when the greatest numbers of STEC were present in the ileum. In our study, the mice manifested systemic effects of disease, such as significant weight loss, despite the lower levels of toxin detected after 9 h of infection. Additionally, mortality in infected animals did not occur until 3-6 days

post-infection. Together these data suggest that a lag period occurs between the time of maximal toxin production in the gastrointestinal tract to the manifestation of clinical disease. Cornick *et al*. also found a lag period between toxin expression and disease manifestations in the pig (6). During this lag period, toxin may transit from the site of infection to the bloodstream and then to the kidneys where it damages the cells within the tubules. That we and others have observed a delay between finding toxin in the intestine and manifestations of systemic disease (6, 44) suggests that therapeutic intervention may be possible during this interval [Melton-Celsa unpublished data, (28, 43, 50)].

Investigators from our laboratory previously reported that attainment of high-level STEC colonization and subsequent mortality in orally infected mice necessitated the use of an antibiotic, i.e. streptomycin, to suppress the normal intestinal flora (26). Subsequently, three other groups of investigators, in addition to ourselves, succeeded in the establishment of an STEC infection (using *E. coli* O157:H7) in mice in the absence of antibiotics (5, 16, 21, 32). However, prior to the study reported here, only Karpman *et al*. successfully administered *E. coli* O157:H7 to untreated mice with morbidity or death as a reported outcome (21). These researchers, who did not monitor colonization, observed neurological and systemic illness in C3H/HeJ and C3H/HeN mice after gavage with strain 86-24 (or the non-toxin-producing *E. coli* O157:H7 strain 87-23) that included gastrointestinal damage, some loose stools, tubular and glomerular damage, and some fragmentation of blood cells (although the red cell fragmentation was not associated with Stx2 production in their study) (21). The disparity in degree of systemic illness between the BALB/c mice used in our study and the C3H/HeN and C3H/HeJ mice used by Karpman *et al*. may reflect genetic differences in the mouse strains; or alternatively, a

difference in the type or extent of their commensal gut flora that could impact the extent

of colonization by the challenge *E. coli* O157:H7 strain. Inherent in the latter explanation

is our presumption that mice colonized to a higher level with *E. coli* O157:H7 are more

likely to display higher morbidity and perhaps mortality. Since both the Karpman and

our studies utilized *E. coli* O157:H7 strain 86-24 (though our strain was Nal[R]), the

differences in the models should not reflect bacterial strain differences. Additionally,

over half of the C3H/HeN and C3H/HeJ mice gavaged with strain 86-24 demonstrated

positive blood cultures just prior to sacrifice. However, bacteremia is a feature not

observed in humans with *E. coli* O157:H7 disease (22).

In our study that examined the impact of *E. coli* O157:H7 strain 86-24Nal[R]

administered by gavage on subsequent bacteremia in mice, we found positive blood

cultures in a small portion of the mice after introducing 10^9 CFU. Those mice with

positive blood cultures for *E. coli* O157:H7 died within 24 h of inoculation. However,

none of the remaining animals in that same study were bacteremic, even when they

appeared extremely moribund or died. Three lines of evidence support the hypothesis

that *E. coli* O157:H7-infected mice that died 2 or more days after gavage with high doses

of *E. coli* O157:H7 did so as a consequence of Stx2 delivered systemically from the gut.

First, we found sub-picogram levels of Stx2 in the blood of some infected animals.

Second, we detected Stx2 in the kidneys of one mouse (not shown). Third, we observed

damage to the Gb_3-rich (9) renal tubules, a pathology also seen after STEC infection in

humans (4, 17).

As noted above, two other groups of investigators reported establishment of *E.*

coli O157:H7 infection in untreated mice but, unlike the original Karpman report or our

study here, morbidity or mortality were not outcomes of infection. In 1998, Conlan and

Perry showed that specific pathogen-free mice (CD-1, BALB/c, and C57BL/6) could be

colonized for an average between 7 and 24 days following intragastric administration of

greater than 10^{10} CFU of *E. coli* O157:H7 (5). In a more recent study, Nagano *et al*.

investigated *E. coli* O157:H7 colonization, in the absence of antibiotic treatment, in a

number of inbred mouse strains that included BALB/c, C3H/HeN, C3H/HeJ, as well as

out-bred ICR (also called CD-1) animals (32). The mice were challenged with as much

as 10^{11} CFU introduced by gavage, and animals were given cimetidine, an H2 blocker,

prior to infection to reduce the acidity of the stomach. Nevertheless, Nagano *et al*. were

unable to detect *E. coli* O157:H7 shed in the feces from the majority of BALB/c mice

with an intact commensal flora one week after infection but did find that ICR mice

remained colonized in the absence of antibiotic treatment 7 days following challenge.

While the reason we observed persistent colonization in ICF BALB/c mice when Nagano

et al. did not remains unclear, two possibilities may account for the varied findings.

First, and likely most importantly, Nagano *et al*. used a different strain of *E. coli*

O157:H7, GPU96MM (an Stx1- and Stx2-producing strain), than we did in this

investigation. The *E. coli* O157:H7 strain used by Nagano *et al.* was associated with an

outbreak in which patients had lower hospitalization and mortality rates than the illness

linked to *E. coli* O157:H7 strain 86-24 used here (13, 33). Furthermore, our laboratory

has previously shown that bacterial strain-specific differences can influence the

colonization pattern seen in mice (26). Second, the level (or type) of commensal flora in

the gastrointestinal tracts of the BALB/c mice bred by the supplier used by Nagano *et al.*

may have been different than the gut flora in the BALB/c mice that we obtained.

Therefore, our current observation that a high initial inoculum of a virulent *E. coli* O157:H7 is necessary to attain elevated levels of the organism and persistent colonization of ICF BALB/c mice is most likely explained by competition for an intestinal niche between the in-coming *E. coli* O157:H7 strain and the already established resident bowel flora.

Conclusions

We established a model of *E. coli* O157:H7 oral infection in BALB/c mice with an intact commensal flora. Our data suggest that the following steps occur in the BALB/c ICF model of 86-24Nal^R infection: 1) the bacteria are ingested; 2) the bacteria colonize within the cecum and to a lesser extent, the large intestine; 3) Stx2 enters the blood stream from the site of *E. coli* O157:H7 colonization within the gastrointestinal tract; and, 4) toxin travels to the kidney, and either directly or indirectly causes renal damage and altered kidney function as seen in HUS. However, the absence of hemolytic anemia or thrombocytopenia indicates that not all the features of HUS are recapitulated in this ICF mouse infection model. Nevertheless, we believe that our small animal model will serve as a useful tool to assess various means to impact colonization levels or persistence of colonization and evaluate treatments that block or ameliorate the systemic, likely Stx2-mediated features of *E. coli* O157:H7 disease. We also speculate that this infection model can be readily applied to examine the relative colonizing capacity and virulence of different strains or clades of *E. coli* O157:H7.

Materials and methods

Bacterial strains and growth conditions

A nalidixic acid-resistant derivative of *E. coli* O157:H7 strain 86-24, referred to as 86-24Nal[R], was used for all experiments described herein (kindly provided by Dr. Arthur Donohue-Rolfe of Tufts University). The parental strain 86-24 produces Stx2 and was isolated in Washington State during a 1986 outbreak associated with contaminated beef products (13). For all mouse infection studies, a sample of 86-24Nal[R] was obtained from a freezer stock of the organism and inoculated onto a Luria Bertani (LB) agar plate supplemented with nalidixic acid (25 µg/mL). The plate was then incubated overnight (O/N) in air at 37°C. A single isolated colony was picked from the agar plate and inoculated into LB broth with nalidixic acid (25 µg/mL). The cultures were then grown O/N with aeration. The bacteria were harvested from the broth by centrifugation, and the pellets resuspended in 20% glucose-phosphate-buffered saline (PBS) to a 40-100X concentration.

Mouse infection studies

Six-week-old female BALB/c mice from Charles River Labs (Wilmington, MA) were used for all animal experiments. The mice were permitted food and water *ad libitum* except prior to *E. coli* O157:H7 infection. Food was taken from the animal cages the night before bacterial challenge. Water bottles were removed from the cages 2 h prior to infection of the mice. Following infection, access to food and water was restored.

Experimental series #1

Dose-response studies were done to determine the optimal infectious dose of 86-24Nal^R required to permit sustained *E .coli* O157:H7 colonization of mice over 7 days. Groups of 5-10 animals were inoculated by intragastric administration, also referred to as gavage, with approximately 10^5, 10^6, 10^7, 10^8, or 10^9 colony-forming-units (CFU) of strain 86-24Nal^R in 100 µL. In a second set of studies, animals were orally infected by pipette feeding 10^8 or 10^9 CFU of bacteria. Pipette feeding (where animals were allowed to ingest the bacterial suspension by lapping the liquid from the end of a micropipette tip) was done in two rounds of 50 µL each with 2 h between feedings to facilitate ingestion of the complete dose. The extent of bacterial colonization was monitored daily for 7 days by quantitation of the *E. coli* O157:H7 shed into fecal pellets. For that purpose, animals were placed individually into clean, empty cages, allowed to defecate, and the feces collected and weighed. The fecal material was diluted 1:10 by weight into PBS then homogenized by mechanical disruption with a sterile wooden stick. Large debris was pelleted by brief centrifugation at 1750 X g with an International Equipment Company clinical centrifuge (IEC Model CL, Damon IEC Division, Needam Hills, MA). The supernatants were diluted and plated onto Sorbitol MacConkey Agar (SMAC) plates supplemented with nalidixic acid (SMAC+Nal, 25 µg/mL) to determine CFU/g feces. Reported values represent the geometric mean (GM) of 5 or more animals per group.

Experimental series #2

Short-term infection studies were conducted to monitor levels of *E. coli* O157:H7

at various intestinal sites after challenge and to obtain organs for immunostaining studies.

Animals were orally infected by pipette feeding of 50 µL containing 6-8 X 10^8 CFU of

strain 86-24Nal^R in three separate experiments. Control animals (generally 1 per time-

point per experiment, with the exception that an additional control animal was required

for the immunostaining experiments) were orally administered 20% glucose-PBS alone

by pipette feeding. Groups of infected mice and control animals were anesthetized in

isoflurane and euthanized by cervical dislocation at 3, 6, 9, 24, 48, and/or 72 h after

infection or buffer inoculation. Whole organs (cecum and kidneys) from at least one

infected animal and one control animal at each time-point were removed and fixed in

Formalde-Fresh (10% buffered formalin, Fisher Scientific, Pittsburgh, PA), then

sectioned, placed on slides, and immunostained for detection of O157 (described in

Histopathology and staining of tissues below). In addition, the cecum, large intestine,

and a terminal section of the small intestines equal in length to that of the large intestine

were surgically removed from the infected and control animals at each time-point. The

luminal contents were then removed from each organ by application of gentle pressure to

the outside of the tissue to expel the internal material. Both the luminal contents and the

organs were weighed and homogenized separately (Omni hand-held homogenizer) in 0.5

mL of PBS. The homogenates were then diluted (final dilution of 1:10 by weight) and

samples were plated on SMAC+Nal plates for enumeration of CFU. An aliquot of each

diluted homogenate was also treated with a protease inhibitor (complete, Mini, EDTA-

free, Roche Applied Science, Indianapolis, IN) and stored at -20°C for subsequent analyses of intimin and Stx2 expression as detailed below in *Detection of intimin by ELISA* and *Stx assay procedures: ELISA, Vero cell cytotoxicity, and neutralization of activity assays*. Values for bacterial counts were reported as the combined GM from all experiments and represent a total of 8 or more (up to 17) animals per time-point analyzed.

Experimental series #3

For this multi-tiered investigation, mice were divided into 4 groups. Group A included infected experimental animals for blood work and tissue harvest (n=25). Group B contained uninfected control mice for blood work and tissue harvest (n=20). Group C consisted of infected experimental mice for morbidity studies (n=10); and, group D was comprised of uninfected controls for the morbidity study (n=10). *E. coli* O157:H7 counts in shed feces were tracked after intragastric administration of a concentrated culture of strain 86-24Nal^R in 20% glucose to groups A and C, or 20% glucose to groups B and D. Animal weights were measured as an indicator of morbidity in all groups. Based on colonization levels and weight loss, five animals were selected on each of days 2, 3, 4, and 5 from the experimental group A, as well as five animals per day from the control group B. Blood was collected from these mice by exsanguination and placed into either microtainer tubes that contained potassium ethylenediaminetetraacetic acid (EDTA, BD diagnostics, Franklin Lakes, NJ) for CBC evaluation or into microcentrifuge tubes to process to obtain sera for kidney function analyses. For the latter purpose, blood in each microcentrifuge tube was first allowed to clot O/N at 4°C. The tubes were then subjected to two consecutive 10 min centrifugation steps at 8000 X g then ≥10,000 X g to separate

the serum from the clotted red cells. Serum samples were stored at 4°C until they could

be analyzed by the Uniformed Services University of the Health Sciences (USUHS)

Diagnostic Services & Comparative Medicine Clinical Pathology laboratory (Bethesda,

MD) for levels of blood urea nitrogen (BUN) and creatinine. Whole blood samples for

determination of complete blood counts (CBC) were similarly stored and evaluated by

the same facility.

The animals from which blood was obtained as above were then sacrificed by

cervical dislocation. Their kidneys were removed at necropsy. These organs were then

placed in the fixative Carnoy (60% ethanol, 10% glacial acetic acid, 30% chloroform) for

2 h or Formalde-Fresh overnight, and then transferred to 70% ethanol for subsequent

histopathological examination (described in *Histopathology and staining of tissues*

below).

Experimental series #4

The possibility that deaths of mice infected with high doses of 86-24Nal^R by

gavage may have occurred as a consequence of bacteremia induced by the procedure was

evaluated. For those assessments two different infecting doses were used and two

evaluation groups (blood evaluation and mortality assessment) were defined with 10

animals in each group. The mice were infected with ~2 X 10^9 CFU (groups A and B) or

~5 X 10^8 CFU (groups C and D) of 86-24Nal^R by gavage. Six hours post-inoculation

blood was collected from the tail vein of all of the mice to test for bacteremia. On days 2

and 4 post-infection, five animals from the blood evaluation groups (B and D) were

anesthetized in isoflurane and sacrificed by cervical dislocation. Blood was collected

directly from the heart by cardiac puncture, and assessed for the presence of bacteria. On a few occasions, no blood was recovered by cardiac puncture and, in those instances, blood was taken from the thoracic cavity or the heart was cut open and the tissue smeared onto SMAC+Nal plates. The mortality groups (A and C) were followed for 10 days and upon death, blood and/or heart tissue was examined for bacteremia. If an animal appeared severely moribund, it was immediately sacrificed. Blood collected from each mouse (1-10 μL) was inoculated into LB broth that contained nalidixic acid. The broth was grown for 48 h statically at 37°C and tested daily for the presence of bacteria by subculture onto SMAC+Nal plates. Additionally, the broth was visually assessed for turbidity. Blood (from the cardiac puncture or directly from the heart) was also plated directly on SMAC+Nal plates and grown overnight at 37°C.

Experimental series #5

Prior to infection, blood was obtained by tail bleed and collected into tubes containing heparin or lithium and heparin. Mice were infected by gavage with 1.9×10^9 CFU of 86-24Nal^R. Colonization and weight were monitored. A group of mice (n=10) were followed for mortality. A separate group of mice (n=15) were sacrificed on days 4 and 5 post-infection with blood taken by retro-orbital eye bleed. The method used to test blood samples for toxin was Meridian's Premier EHEC enzyme-linked immunosorbent assay (ELISA) kit (as detailed in *Stx assay procedures: ELISA, Vero cell cytotoxicity, and neutralization of activity assays* below). Blood cells were gently lysed by 3 rounds of freezing at -80°C and thawing at 37°C prior to use in the ELISA. This procedure was selected because we found in preliminary studies that blood, neat or diluted 1:50,

interfered with interpretation of the Vero cell cytotoxicity assay, another test for Stx activity (described in *Stx assay procedures: ELISA, Vero cell cytotoxicity, and neutralization of activity assays*, below). The amount of Stx2 present in the blood was quantitated by comparison to a standard curve generated by adding purified Stx2 into whole mouse blood and gently lysing the blood/toxin mixture.

To determine the amount of Stx2 in the blood necessary for a positive result in the ELISA, groups of mice ($n = 2$) were administered purified Stx2 intravenously (IV). Prior to infection, blood was obtained by tail bleed and collected into tubes containing heparin or lithium and heparin. The groups then received 1, 10, 100, or 1000 LD_{50}s of Stx2 (corresponding to 1 ng, 10 ng, 100 ng, or 1 µg of Stx2). Two hours post-injection, blood was collected from the mice by tail bleed. Lysed blood was tested for toxin using Meridian's Premier EHEC ELISA kit (as described above).

Detection of intimin by ELISA

A sandwich ELISA was developed to detect intimin in homogenates (see *Experimental series #2*) of the cecum, large intestine, or contents of those organs from 86-24Nal^R-infected mice. The capture antibody in the assay was a goat monospecific anti-*E. coli* O157:H7 intimin antibody that had been purified from polyclonal goat anti-intimin sera (10) by affinity chromatography over a column prepared by coupling full length, purified intimin to Pierce Ultralink Biosupport resin (Thermo Fisher Scientific, Rockford, IL) per the manufacturer's protocol. For the ELISA assay, wells of an Immulon 2HB (Thermo Scientific, Waltham, MA) high-binding microtiter plate were coated overnight at 4°C with 100 µL of a 1:50 dilution of affinity-purified monospecific

goat anti-intimin antibody in a buffered solution of sodium bicarbonate/sodium

carbonate. Next, 200 µL of 5% bovine serum albumin (BSA) in PBS-Tween 0.05%

(PBST) was added to each well to block un-bound sites on the wells, and the plates were

incubated at 4°C for at least 24 h. The wells were washed 4-6 times with PBST and then

homogenate samples (further diluted 1:2 from the original 1:10 dilution for a final

dilution of 1:20) were added to the wells. The plates were incubated for 2 h at 37°C, and

the wells were then washed as above to remove any unbound sample. Intimin present

within the homogenate and bound to the goat anti-intimin capture antibody was detected

by incubation for 1 h at room temperature with 100 µL of each of the following reagents

in sequence with wash steps between each addition: a 1:5000 dilution of rabbit anti-

intimin polyclonal antibody (10) followed by a 1:5000 dilution of horse radish peroxidase

(HRP)-labeled donkey anti-rabbit IgG (H+L) antibody that was reported by the supplier

(Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to display minimal cross

reactivity with sera from other species of animals. Bound antibody was detected with a

3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase enzyme immunoassay (EIA) substrate

kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. The

reaction was stopped with 100 µL of 1N sulfuric acid, and each well was read at an

optical density (OD) of 450 nm on a spectrophotometric plate reader. An infected tissue

or tissue content homogenate sample was considered to be positive for intimin if it had an

optical density reading twice that of the average value for all the uninfected control tissue

and tissue content homogenate samples. Quantitative values for intimin in samples from

one of the three experiments were derived from a standard curve constructed from ELISA

values of dilutions of purified intimin spiked into control tissue homogenates. The limit of detection for the analysis was 360 ng/g tissue.

Stx assay procedures: ELISA, Vero cell cytotoxicity, and neutralization of activity assays

A commercially-available ELISA kit (Premier EHEC ELISA, Meridian diagnostics, Cincinnati, OH) was used to detect Stx2 in homogenates (see *Experimental series #2*) of the ceca, large intestines, or contents of those organs from 86-24Nal^R-infected mice. The ELISA kit was used according to the manufacturer's protocol with the following modifications: homogenates were diluted 1:2 in PBS (for a final dilution of 1:20) and then added directly to the ELISA. A sample was considered to be positive for Stx2 if it had an optical density reading twice the average value of the control tissues in aggregate.   Stx2 levels in samples from one of the three experimental sets were determined based on a standard curve of purified toxin spiked into control tissue homogenates. In our hands, the limit of detection for quantitation with this assay was 6.4 ng toxin/g sample homogenate.

The presence of toxin in a subset of those samples was confirmed with a biological activity assay (Vero cell cytotoxicity and neutralization assay). To measure neutralizable toxin within the samples, a 1:100 final dilution of each homogenate was prepared in PBS and the diluted samples were then clarified at 8000 X g for 10 minutes. The resultant supernatants were filtered first through a 0.8 µm or 0.45 µm syringe filter and then through a 0.2 µm low-protein-binding syringe filter to remove bacteria and debris. Next, the filtrates were incubated in the presence or absence of a 1:5000 (final

concentration of 1:10,000) dilution of polyclonal rabbit anti-Stx2 antibody (24) for ~2 h

(at a 1:1 mixture of filtrate to antibody dilution or PBS) prior to the addition of 100 µL of

the samples onto Vero cells that had been seeded into microtiter plates 24 h previously.

The overlaid Vero cell plates were incubated for 40-48 h at 37°C in 5% CO_2. The

residual cells in the wells were then fixed in Formalde-fresh, stained with crystal violet,

and read at 600 nm on a spectrophotometric plate reader.

Histopathology and staining of tissues

Kidneys and ceca were harvested from infected or control mice at necropsy, fixed

(see *Experimental series #2* and *Experimental series #3*), and subsequently sent to

HistoServ (Germantown, MD) for embedding in paraffin, sectioning, and placement on

charged glass slides. Some sections were stained using hematoxylin and eosin (H&E) or

a modified Carstair's stain at HistoServ or stained using Mallory's phosphotungstic acid

hematoxylin (PTAH) at the USUHS Laboratory of Animal Medicine (Bethesda, MD).

To locate *E. coli* O157:H7 bacteria bound to or near the surface of cecal tissue,

slides with cecal sections were immunostained using an anti-O157 antibody. To reduce

the background autofluorescence normally present in tissue, slides of sectioned tissues

were first placed under fluorescent light for at least 48 h (34). Slides were then

deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated in a

graded ethanol series. To increase antibody recognition, slides were treated with antigen

retrieval buffer [(5X AntigenPlus Buffer, pH10, EMD Biosciences, San Diego, CA)

diluted to 1X, heated in a microwave for ~15 min with replenishment of the buffer as

needed, cooled and dried]. Slides were blocked overnight in fetal bovine serum (FBS)

supplemented with 0.1% Triton-X. Prior to the addition of primary antibody, the anti-

O157 antibody was precleared against a laboratory strain of *E. coli* (DH5α) and a

powdered homogenate of normal mouse cecal tissue (14). The cleared anti-O157

antibody was incubated on the tissue at a dilution of 1:500 in 3% bovine serum albumin

(BSA) in PBS with 0.1% Triton-X for ~1 h. The tissue sections were then washed and

secondary goat anti-rabbit antibody conjugated to Alexa-fluor 488 (also pre-cleared

against the cecal powder) was incubated on the tissue for ~1 h at a dilution of 1:500 in

3% BSA in PBS with 0.1% Triton-X. Prior to microscopic observation, the slides were

rinsed in PBS, mounted using Slowfade reagent, and a coverslip applied to each slide.

Immunofluorescence of stained tissue sections was visualized with an Olympus BX60

microscope with a BX-FLA fluorescence attachment. Digital images of the fluorescent

stains were obtained using a SPOT RT charge-coupled-device digital camera (Diagnostic

Instruments, Inc., Sterling Heights, MI). ImageJ and DeconvolutionJ software (both

developed by the National Institutes of Health) were used for processing the images and

removing out-of-focus fluorescent signals. The images were overlaid and subjected to

green false-coloration using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Statistical analyses

All statistical analyses were done through application of the SPSS v16 software

(SPSS Inc., Chicago, IL). Specific analyses are described below.

Data for the dose response studies (*Experimental series #1*) were analyzed by

both Kaplan-Meier analysis and repeated measures (RM) analysis of variance (ANOVA).

The Kaplan-Meier analysis was performed to ascertain differences in the amount of time

mice were colonized by different doses of inoculum received. The RM ANOVA was used to assess statistical differences in levels of colonization by dose administered. The ANOVA was estimated by a linear mixed models approach that incorporated all available data including information from animals that had died before the end of the study. The model assumes equal correlation among repeated observations on the same animal. For the mixed model analysis, dose was considered the between subjects factor and day was set as the within subjects factor.

A RM ANOVA was also used to test for statistical significance among the numbers of *E. coli* O157:H7 in various segments of the intestine over time (*Experimental series #2*) but, in this case, with time as a between subjects factor and organ as a within subjects factor. To more closely meet the assumptions of ANOVA, the dependent variable (CFU) was assessed on a log scale. After checking for significance by RM ANOVA, we conducted a separate one-way ANOVA to compare bacterial counts at time-points among the organs. We also conducted RM ANOVA for organs at each time-point and made separate pair-wise comparisons.

For the ascertainment of differences in expression of both intimin and toxin, a RM ANOVA was again used. To more closely meet the assumption for a normal distribution, the measurements for quantification of expression (the dependent variable) were assessed on a log scale. Measurements below the limit of detection were given an arbitrary value of 100 to allow the data to be included in the analysis. For the RM ANOVA, time was once again the between subjects factor and organ was again the within subjects factor. Pair-wise comparisons of the main effects for differences both by time and organ were

also conducted using the least significant difference, a procedure that is equivalent to no adjustments for multiple comparisons.

Statistical differences in the mean weight between infected and control mice (*Experimental series #3*) were determined using a RM ANOVA. For the RM ANOVA, day was the between subjects factor and weight was the within subjects factor. Additionally, differences between infected and control mice (*Experimental series #3*) in terms of CBC analyses and serum chemistry results were assessed by performing independent samples t-tests on relevant numbers, such as BUN, creatinine, % neutrophils, % lymphocytes, hemoglobin, % hematocrit, and platelets.

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CHAPTER THREE

NEUTRALIZING ANTIBODIES TO SHIGA TOXIN TYPE 2 (STX2) REDUCE

COLONIZATION OF MICE BY STX2-EXPRESSING

***ESCHERICHIA COLI* O157:H7**

(Manuscript submitted to *Vaccine*)

Submitted as: Krystle L. Mohawk, Angela R. Melton-Celsa, Cory M. Robinson, and Alison D. O'Brien. Neutralizing Antibodies to Shiga Toxin Type 2 (Stx2) Reduce Colonization of Mice by Stx2-Expressing *Escherichia coli* O157:H7.

Note: all of the figures and tables shown reflect the work of Krystle Mohawk with the exception of the lysate feeding work which was done in collaboration with Dr. Cory Robinson. Drs. Melton-Celsa and O'Brien contributed both to the design of the experiments and the interpretation of the data as well as the preparation of the manuscript. The references have been altered in following with the style of this dissertation.

Abstract

Previously, we showed that the Shiga toxin type 2 (Stx2)-expressing *Escherichia coli* O157:H7 strain 86-24 colonized mice better than did its isogenic *stx₂* negative mutant. Here, we confirmed that finding by demonstrating that Stx2 given orally to mice increased the levels of the 86-24 *stx₂* mutant shed in feces. Then we assessed the impact of Stx2-neutralizing antibodies, administered passively or generated by immunization with an Stx2 toxoid, on *E. coli* O157:H7 colonization of mice. We found that such antibodies reduced the *E. coli* O157:H7 burden in infected mice and, as anticipated, also protected them from weight loss and death.

Introduction

Escherichia coli O157:H7 is a food- and water-borne pathogen that can cause diarrhea, bloody diarrhea (known as hemorrhagic colitis), or in a fraction of cases, a life-threatening sequela called hemolytic uremic syndrome (HUS). In a 1999 publication, Mead *et al*. reported that in the United States *E. coli* O157:H7 was associated with an estimated 73,000 cases of intestinal disease each year and that about 3% of infected individuals required hospitalization. The rate of HUS that followed *E. coli* O157:H7 infection was estimated at about 4% and the number of individuals who died of HUS annually was listed as 61 (30). However, the severity of more recent outbreaks of *E. coli* O157:H7 in the United States has increased as indicated by the fact that >50% of ill persons required hospitalization and >10% of infections led to the development of HUS (9, 10, 27).

Shiga toxins (Stxs, also called Vero toxins) made by *E. coli* O157:H7 and other serotypes of *E. coli* (collectively called Shiga toxin-producing *E. coli* or STEC) are considered to be responsible for the development of HUS (21). Stxs are potent AB_5 (one A polypeptide with enzymatic activity and 5 copies of a B or cell-binding polypeptide) cytotoxins. These toxins are N-glycosidases that inhibit protein synthesis by the depurination of a critical ribosomal residue important for protein elongation [reviewed in (34)]. There are two serologically distinct groups of Stx: Stx1 and Stx2 [reviewed in (31)]. The expression of both toxins is associated with human disease, but more recent outbreaks in the United States seem to be associated with STEC that produce Stx2 or a variant of Stx2 (17).

The Stxs are known to act systemically and therefore must transit from the site of STEC colonization in the gastrointestinal tract to the circulatory system [reviewed in (33)]. That Stx may also act locally was suggested by an investigation from our laboratory in which we demonstrated that Stx2-expressing *E. coli* O157:H7 strain 86-24 adhered better to HEp-2 cells in culture and colonized to a greater extent in a mouse model of single organism infection than did its isogenic stx_2 null mutant (38). In that same report, we also demonstrated *in vitro* that Stx2 increases cell-surface expression of nucleolin, a eukaryotic receptor for the *E. coli* O157:H7 adhesin intimin (43, 44). This latter result led to the speculation that Stx2 may augment *E. coli* O157:H7 adherence through its capacity to increase the number of receptors available for intimin-dependent adherence. Intimin is an outer membrane protein of *E. coli* O157:H7 and is the primary mediator of adherence for the bacterium (14, 29). Although the *E. coli* O157:H7 type III secretion system (TTSS) product called Tir (for translocated intimin receptor), is the critical receptor for *E. coli* O157:H7 intimin after its injection into the eukaryotic cell, our previously published *in vitro* and *in vivo* data strongly suggest that nucleolin may play a role in the initial binding of the organism to the target cell surface before Tir is injected (43, 45).

In this study, we first sought to extend our observation that the wild-type *E. coli* O157:H7 strain 86-24 colonizes at higher levels *in vivo* than does its isogenic 86-24 stx_2 mutant by feeding mice Stx2 and then assessing whether the 86-24 stx_2 mutant colonized better than in animals not fed the toxin. We found that pre-treatment with toxin did enhance the capacity of *E. coli* O157:H7 to colonize mice with an intact commensal flora. We then tested the impact of anti-Stx2 neutralizing antibody administered passively or

induced by active immunization on colonization. We found that anti-toxin not only, as expected, protected mice from the morbidity (as reflected by weight loss) and lethality of *E. coli* O157:H7 infection, but also reduced the level of colonization by the *E. coli* O157:H7 challenge strain.

Materials & Methods

Bacterial strains

Wild-type *E. coli* O157:H7 strain 86-24, first isolated in 1986 during an outbreak in Washington State believed to be linked with contaminated beef products (36), was used for all experiments. *E. coli* O157:H7 strain 86-24 produces Shiga toxin type 2 (Stx2) only. We made use of a toxin null mutant isogenic to strain 86-24, TUV 86-2. Both the wild-type and the isogenic mutant TUV 86-2 were generously provided by Dr. Arthur Donohue-Rolfe of Tufts University (19). To aid in recovery and differentiation of the bacterium from normal flora *in vivo*, strains of both the wild-type and mutant resistant to nalidixic acid (Nal^R, 25 µg/mL) were used in this investigation. Bacteria were taken directly from a freezer stock and grown on Luria Bertani (LB) plates with nalidixic acid. Subsequently, a single colony was inoculated into LB broth supplemented with nalidixic acid for use as a starter culture. For infection of mice, bacterial starter cultures were diluted 1:100 into larger broth cultures and were grown overnight with aeration prior to concentration by centrifugation and resuspension to 40X in 20% glucose-phosphate-buffered saline (PBS).

Anti-Stx2, normal rabbit serum, and immunoblot procedure

Polyclonal rabbit anti-Stx2 serum (24) was used in passive immunization studies and as a primary probe for the detection of Stx2 toxin or toxoid in immunoblot assays. Normal rabbit serum (NRS, purchased from Rockland Immunochemicals, Inc., Gilbertsville, PA) was used as a control in passive immunization experiments and immunoblot analyses.

Prior to use in animals, samples of the anti-Stx2 antiserum and NRS were pre-cleared against a laboratory strain of *E. coli* (to remove non-specific anti-*E. coli* antibodies) as follows. Two volumes of an overnight culture of DH5α were harvested by centrifugation (10 minutes at 5,000 rpm), washed three times in PBS, and resuspended in one volume of the serum sample. This mixture was allowed to incubate end-over end at 37°C for ~2 hours and then the bacteria were pelleted by centrifugation. The resulting supernatant that contained the pre-cleared serum was filtered through a 0.22 μm syringe filter. The filtrate was tested for sterility by spotting 100 μL of the material onto LB agar followed by overnight incubation of the plate at 37°C.

For immunoblot analyses, samples were either spotted directly onto nitrocellulose with the use of a dot blot manifold or electrophoretically mobilized into a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Samples run by SDS-PAGE were transferred to nitrocellulose with a semi-dry transfer apparatus. Once samples were applied to nitrocellulose, the blots were rinsed in PBS-Tween (PBST) then blocked overnight with 5% dry milk in PBST. Blots were washed in PBST prior to incubation in primary antibody (anti-Stx2 or NRS at a dilution of 1:5,000) for ~2 hours. Blots were washed again and incubated for an additional hour in secondary antibody conjugated to horse radish peroxidase (goat anti-rabbit-HRP, Bio-Rad, Hercules, CA). After a final wash, blots were incubated with Amersham Bioscience's enhanced chemiluminescence (ECL) Plus Western blotting detection reagents (Amersham Bioscience, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Blots were then developed onto Kodak XAR-5 film (purchased from VWR International, West Chester, PA) using a Series XXXV A Rapid Processor (Ti-Ba Enterprises, Inc., Rochester, NY).

Mouse infection model

For all experiments, we used an intact commensal flora (ICF) mouse model to study *E. coli* O157:H7 colonization and pathogenesis (32). For that purpose, six week-old female BALB/c mice were purchased from Charles River Labs (Wilmington, MA). The mice were housed in filter-top cages, in a temperature-, light-, and humidity-controlled room. Animals were provided access to food and water *ad libitum*. To ensure colonization with *E. coli* O157:H7 upon infection, animals were fasted overnight prior to infection; additionally, water access was restricted 2 hours before infection and returned thereafter.

Mice were infected orally either by pipette feeding or intragastric administration (IG, gavage) with ~10^9 CFU of strain *E. coli* O157:H7 strain 86-24Nal^R (wild-type) or TUV 86-2 Nal^R (*stx$_2$* mutant) [at a feeding dose of 10^9 CFU, oral infection by pipette feeding or IG results in essentially equivalent colonization levels (32)]. Animals were monitored for *E. coli* O157:H7 colonization by enumeration of the number of challenge organisms in their feces. Fecal pellets were suspended in PBS (1:10 dilution by weight), homogenized with a wooden stick, and debris pelleted by slow speed centrifugation. The resultant fecal supernatants were diluted and plated on sorbitol MacConkey Agar (SMAC) supplemented with nalidixic acid to determine the colony-forming-units (CFU) shed/g feces. In many experiments animals were housed individually after infection to prevent secondary infection from hyper-shedding animals.

Stx2 toxin and toxoid

Toxin feeding experiments were done with a histidine-tagged Stx2 protein (called Stx2-6H) that was generated and purified as previously described (38). The resulting toxin was tested for activity on Vero cells and found to be about 10-fold less toxic than purified Stx2 without the tag. Nevertheless, the specific activity of Stx2-6H was still high [8 X 10^7 50% cytotoxic doses (CD_{50})/mg protein].

The clone for expression of a fully inactive Stx2 toxoid with a similar C-terminal 6-histidine tag (Stx2 Y77S E167D-6H) was created as follows. Splicing by overlap extension (SOE) PCR was used to introduce a mutation into the clone that expressed Stx2 E167D-6H (38) so as to ultimately generate a second amino acid change in that toxoid (Y77S). The DNA segments to be linked together with nucleotide changes were amplified by PCR from the Stx2 E167D-6H clone template [forward primer 2Y77S (TCAGTGGCCGGGTTCGTTAATACGG); reverse primer pTrcR (CCAGGCAAATTCTGTTTTATCAGACCGC); forward primer pTrcF (GACAATCTGTGTGGGCACTCGACCGG); reverse primer 2Y77SR (CCGTATTAACGAACCCGGCCACTGATAAATTATTTTGCTCAATAATCAGACG AAGATGGT)]. These upstream and downstream fragments were then connected by SOE PCR with pTrcF and pTrcR as primers. The resulting PCR product was subjected to a double restriction enzyme digest and ligated into pTrcHis2C. Transformants were screened by PCR for the presence of the mutated DNA. That the plasmid DNA inserts from those transformants that were positive by PCR were indeed those of the mutant genotype was confirmed by sequencing. The toxoid was expressed and purified in a similar manner to that of Stx2-6H protein described previously (38).

Toxin feeding and O157:H7 challenge studies

A crude preparation of Stx2 was made by sonically-disrupting a concentrated overnight culture of 86-24. The lysate was clarified by centrifugation and then filter sterilized. The concentration of Stx2 in the lysate was estimated by comparison with purified Stx2 on Western blot. The lysate was then adjusted to contain 0.1 µg, 1 µg, or 10 µg of Stx2 in 20% glucose-PBS. We selected those toxin amounts based on our knowledge of the load of bacteria present within the cecum of an infected, ICF mouse, 10^5-10^6 CFU of *E. coli* O157:H7 86-24Nal^R (data not shown); that level of bacteria produces approximately 0.1 µg of Stx2 *in vitro*. As we thought there might be loss of toxin during transit to the site of infection, we used 1-, 10-, and 100-fold doses (0.1 µg, 1 µg, or 10 µg) of Stx2 to maximize the chance that sufficient toxin was available to affect colonization. Prior to infection and daily thereafter, groups of three mice were fed 20% glucose-PBS alone or one of the lysates that contained Stx2. Mice were infected by pipette feeding with either the wild-type or the *stx₂* mutant bacteria. Colonization was monitored over the first 9 days post-infection. Colonization levels are reported as the geometric mean (GM) of CFU/g feces after normalization by inoculum load. Specifically, daily CFU counts were normalized to wild-type inocula by division of the CFU of the strain inoculum over the CFU of the inoculum of wild-type.

For the studies in which mice were given purified Stx2, five µg of pure Stx2-6H in Non-Fat Dry Milk (NFDM) or NFDM alone was administered orally, once daily by pipette to groups of 5 mice on days -1 through 7 and again on days 11 through 21. Mice were than challenged with a concentrated culture of *E. coli* O157:H7 strain 86-24Nal^R or

TUV 86-2 Nal^R given orally by pipette feeding. Colonization was monitored by fecal

shedding (see *Mouse infection model*) and reported as geometric mean (GM) of CFU/g

feces, normalized by inoculum level (see above).

Assessment for presence of anti-Stx2 serum and Stx2 in the feces of mice

Animals monitored for antibody shed into the feces after two IP injections of the

precleared, neat polyclonal rabbit anti-Stx2 antiserum (described above in *Anti-Stx2,*

normal rabbit serum, and immunoblot procedure). Fecal pellets were collected from

infected (n=8) and uninfected (n=8) animals at various times post-infection (1, 2, 3, 4, 5

days) and analyzed for the appearance of the Stx2 antibody by an anti-Stx2 ELISA (see

Determination of fecal and serum anti-Stx2 ELISA titers). Fecal material was also

assessed for the presence of toxin using a commercially-available ELISA kit (Premier

EHEC ELISA, Meridian diagnostics, Cincinnati, OH) as per the manufacturer's protocol.

Passive immunization with anti-Stx2 antiserum or normal rabbit serum

Mice were injected IP with 200 µL of the polyclonal rabbit anti-Stx2 antiserum

(described above in *Anti-Stx2, normal rabbit serum, and immunoblot procedure*) or 200

µL of NRS (pre-cleared and filter-sterilized as for the polyclonal anti-Stx2). Mice were

then intragastrically infected with wild-type strain 86-24Nal^R. Animals received two

doses of anti-serum (anti-Stx2 or NRS), 24 hours apart prior to infection (at either -24

and -1 hour or at -48 and -24 hours, depending on the study). Following infection,

colonization was monitored by fecal shedding (see *Mouse infection model*).

Active immunization with an Stx2 toxoid

Prior to immunization, serum and fecal material were collected from the various groups to serve as a pre-immunization control. Groups of 11 animals were then immunized IP with either 5 µg of the toxoid Stx2 Y77S E167D-6H or PBS mixed 1:1 with the adjuvant TiterMax Gold (TiterMax USA, Inc., Norcross, GA). Animals received an initial injection followed by five boosts at three week intervals.

Three weeks after the fifth boost animals received a final dose of concentrated antigen (200 µg of toxoid in a total volume of ~100 µL) by intragastric (IG) administration. To generate this antigen, purified toxoid was further concentrated by use of a Centriplus centrifugal filtration device with a molecular weight cut-off of 10 kDa (Amicon Bioseperations, Millipore, Billerica, MA). The final toxoid concentration was determined by bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, Rockford, IL) in conjunction with an immunoblot analysis that included toxin standards.

After each IP boost and the single IG immunization, fecal pellets and blood from tail vein bleeds were collected from each animal to determine serum and fecal anti-Stx2 titers by a neutralization assay and an ELISA (see *Assessment of Stx2-neutralizing antibody capacity in fecal supernatants* and *Determination of fecal and serum anti-Stx2 ELISA titers*). Animals were then infected by gavage with greater than 10^9 CFU of *E. coli* O157:H7 strain 86-24Nal^R and colonization was monitored (see *Mouse infection model*).

Assessment of Stx2-neutralizing antibody capacity in fecal supernatants

Fecal samples were diluted 1:10 by weight into PBS, homogenized, and large debris pelleted. Fecal supernatants were removed, further diluted 1:10 into PBS, and filtered through a 0.80 µm or 0.45 µm syringe filter followed by a 0.22 µm syringe filter. The filtered fecal supernatant material was stored at -20°C prior to use.

The fecal supernatants were analyzed by a Vero cell neutralization of cytotoxicity assay for the presence of anti-toxin antibody. Each fecal sample was pre-incubated (at the 1:100 dilution) with 8 pg of Stx2 diluted into sample at a 1:1 ratio for a final concentration of 4 pg/100 µL for 2 hours at 37°C. Vero cells were then overlaid with 100 µL of the sample/toxin mixture and incubated 40-48 hours at 37°C 5% CO_2 prior to fixation and staining in crystal violet. The wells in the Vero plate were read at 630 nm on a spectrophotometer.

Determination of fecal and serum anti-Stx2 ELISA titers

Serum was obtained from the blood collected prior to O157:H7 infection. Fecal samples were obtained and processed as described in *Assessment of Stx2-neutralizing antibody capacity in fecal supernatants*. Microtiter plates (96-well U-bottom) were coated with purified Stx2 (100 ng/well) in PBS overnight. Plates were blocked with 3% bovine serum albumin (BSA) for at least 16 hours prior to use. Before addition of samples, plates were washed in PBST. Separately, serum samples were diluted 1:50 in PBS then further serially diluted at 1:5 increments. Fecal samples were serially diluted at 1:2 in intervals from a filtered, 1:100 stock dilution (see *Assessment of Stx2-neutralizing antibody capacity in fecal supernatants*) or from a fecal supernatant obtained from feces

diluted 1:10 by weight (see *Assessment for presence of anti-Stx2 serum in the feces of mice*). The sample dilution series was incubated on the pre-blocked, washed plates at 37°C for 2 hours (serum) or at 4°C overnight (fecal). Next, plates were washed in PBST and a 1:3,000 dilution of secondary antibody, either goat anti-mouse IgG or goat anti-mouse IgA both conjugated to HRP, was added to each appropriate well. The plates were then incubated at room temperature for 1 hour and subsequently washed in PBST. Substrate solution [3,3',5,5'-tetramethylbenzidine (TMB) peroxidase enzyme immunoassay (EIA) substrate kit, Bio-Rad Laboratories, Hercules, CA] was added to the wells of the washed plates and incubated for 15 minutes before the reaction was stopped with 1N sulfuric acid. The plates were then read on a spectrophotometric plate reader at a wavelength of 450 nm. For this assay, polyclonal mouse anti-Stx2 serum was used as a positive control.

Statistical analyses

Statistical analyses were calculated through application of the statistical software program SPSS v16 (SPSS Inc., Chicago, IL). Specific analyses were performed as described below.

For evaluation of the colonization levels of *E. coli* O157:H7 wild-type or toxin mutant in the lysate feeding experiment, all data were transformed to a log base 10 scale to meet the assumption for normality. A two-way analysis of variance (ANOVA) was done with the log base 10 CFU/g feces as the dependent variable and both group and day as fixed factors. Main effects of both group and day were investigated.

For most other statistical assessments of colonization levels, individual animals were monitored over time and repeated measures (RM) ANOVA was used to appraise differences in colonization levels among the groups. The RM ANOVA was estimated by a linear mixed model approach to incorporate all available data (to include results obtained from animals that died prior to the conclusion of the study). In these analyses, group was the between subjects factor and day was the within subjects factor. Differences on various days post-infection were then analyzed by means of a one-way ANOVA, with the data split by day post-infection. For the latter analysis, group became the independent variable.

For evaluation of the effect of vaccination of serum and fecal response by ELISA, we made use of a nonparametric statistical test on related samples (the Wilcoxon signed-rank test) as our samples failed to meet the assumption for normality even after transforming the data to log base 10.

Results

Role of Stx2 in **E. coli** *O157:H7 colonization*

In our previous study on the impact of Stx2 on colonization of mice by *E. coli* O157:H7, we found that when a mixture of wild-type 86-24Nal^R and the toxin null mutant TUV86-2Nal^R were fed to the animals, toxin produced by the wild-type bacteria complemented the defect in the colonizing capacity of the mutant (38). Based on that observation, we hypothesized that Stx2 given orally to mice would increase intestinal colonization of a toxin null mutant [Note: US Recombinant DNA guidelines forbid the complementation of such a mutant by transformation with a plasmid that expresses Stx2 holotoxin (1)]. To test this theory, we did a preliminary study to determine whether crude toxin preparations supplied exogenously would complement the colonization defect of the toxin null mutant. For that purpose, we fed groups of 3 mice varying dilutions of an Stx2-containing lysate of 86-24Nal^R such that animals received no toxin (20% glucose-PBS), 0.1 µg, 1 µg, or 10 µg of crude Stx2 prior to infection. The following day, we challenged lysate-treated or 20% glucose-PBS-treated mice orally with TUV86-2Nal^R and 20% glucose-PBS-treated mice with wild-type 86-24Nal^R as a control. We continued to administer the Stx2-containing lysates or 20% glucose-PBS alone to the animals daily thereafter while we monitored levels of O157:H7 shed in the feces as a surrogate for intestinal colonization (Fig. 13A). Although in this experiment we did not get good "take" of either wild-type or the toxin null mutant at day 1, we were still able to confirm our previous finding that the mutant alone colonized to a lesser extent than did the wild-type alone (p=0.001). In addition, each lysate that contained toxin increased colonization by the mutant strain (p≤0.001).

Figure 13: Colonization levels of *E. coli* O157:H7 *stx$_2$* mutant in mice following oral infection in the presence or absence of Stx2 provided *in trans*

(A) Mice infected with the *stx$_2$* mutant of strain 86-24 were orally administered sterile toxin-containing lysate of wild-type strain 86-24 adjusted to contain 0.1 µg (▲), 1.0 µg (♦), or 10 µg (▼) of Stx2. Lysate was given prior to and daily following infection. Control groups included mice that received 20% glucose-PBS alone and were infected with the wild-type (●) or the *stx$_2$* mutant (■). Colonization was monitored over a period of 9 days by the CFU shed/g feces. (B) Mice orally infected with the *stx$_2$*mutant of strain 86-24 were fed 5 µg of purified toxin in NFDM (▲). Control groups of both wild-type (●) and *stx$_2$* mutant- (■) infected mice were given NFDM alone. Mice were provided toxin or NFDM alone prior to and following infection on days 0-7 and days 11-21. Colonization was monitored over a period of 23 days by the CFU shed/g feces. All reported values are the GM for the group and the limit of detection is 10^2 CFU/g feces (marked by an arrow).
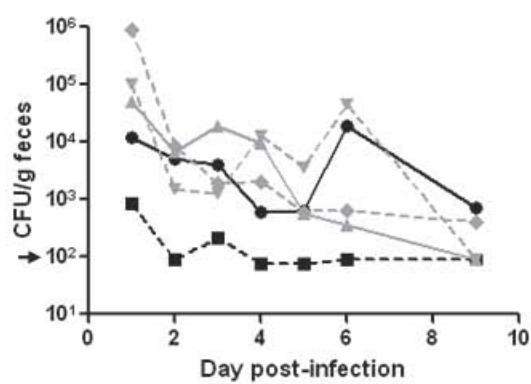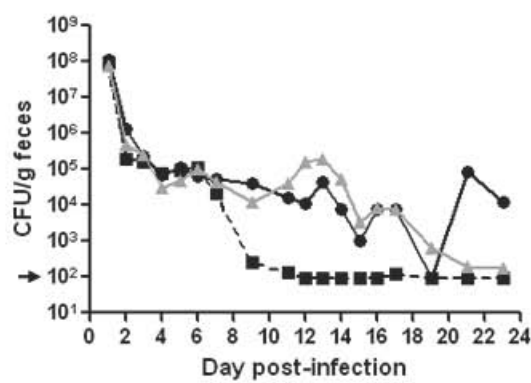
To ensure that Stx2 alone was responsible for the increased colonization of TUV86-2Nal^R when animals were given the 86-24 lysate, animals were fed 5 μg of purified Stx2-6H in NFDM (mutant treated group) or NFDM alone (mutant or wild-type control groups). As with the lysate-treatment experiments toxin was given 1 day before infection to prime the gastrointestinal tract. The mice were then orally infected by pipette with greater than 10^9 CFU of either wild-type 86-24Nal^R or the isogenic toxin-null mutant, TUV 86-2Nal^R. In addition, animals continued to receive toxin in NFDM or NFDM alone (depending on the infection group).

One day after infection, the groups appeared comparably colonized with *E. coli* O157:H7 organisms, i.e., they shed between 10^7 - 10^8 CFU/g feces (Fig. 13 B). In fact, during the first week post-infection, colonization levels were similar [except at day 2 where the wild-type GM CFU/g feces was greater than the mutant (p=0.034)]. This was likely due to the high rate of initial colonization by both strains in this experiment. However, the bacterial load in the mutant-infected, NFDM-alone-treated group began dropping at day 9 and continued to decline, so that by day 12 post-infection this group had colonization levels below the detectable limit. As expected, the wild-type toxin-producing strain colonized to higher levels than did the toxin null mutant throughout the remainder of the experiment. Moreover, when toxin was supplied exogenously with the mutant, significantly higher levels of colonization, comparable to that of wild-type, were achieved than with the mutant alone from days 9-17 of infection. Specifically, on days 11-14 CFU levels were higher in the animals fed purified toxin when compared to the mutant alone by nearly 2 logs or greater (p≤0.025 for days 11-14). Thus, while there was not an obvious difference in colonization pattern early in infection (except at day 2; see

above), at later time-points toxin administration resulted in increased colonization levels

for the stx_2 mutant. We speculate that the large number of bacteria present during the

first week of infection obscured the beneficial effects of toxin on colonization.

Impact of passively administered anti-Stx2 serum on O157:H7 colonization

Since exogenously supplied Stx2 increased colonization of TUV86-2, we next

tested the impact of passively administered polyclonal rabbit anti-Stx2 serum on

colonization by wild-type *E. coli* O157:H7 strain 86-24Nal[R]. First we confirmed that this

antiserum (24) both recognized Stx2 in a Western blot (Fig. 14A) and neutralized the

cytotoxic activity of Stx2 toward Vero cells (Fig. 14B). Additionally, when animals

received two doses of the antiserum, the anti-Stx2 was still detectable up to 5 days post-

immunization in 3/10 mice [day 5 was the latest time-point assessed (Fig. 15)]. The

detection of the anti-Stx2 antiserum in the feces of animals after IP administration

indicated that at least a portion of antibody provided by this route transited to the

gastrointestinal tract. Thus, we felt confident that we could assess the role of passively

administered toxin-neutralizing antibody on colonization by wild-type *E. coli* O157:H7.

In a first experiment to examine the effect on 86-24 colonization by the anti-Stx2 serum,

groups of 5 animals were inoculated IP with two doses of either NRS or anti-Stx2

antibody and, later, infected by IG administration with about 2×10^9 CFU of *E. coli*

O157:H7. Colonization levels of the animals were monitored by fecal shedding twice

daily through day 4 and then once daily from days 5-7 (Fig. 16). The groups

demonstrated a comparable level of initial colonization as reflected by the shedding of

approximately 10^7 CFU/g feces on day 1 post-challenge. However, on days 3-5 post-

Figure 14: Evaluation of polyclonal rabbit anti-Stx2 serum for capacity to recognize

and neutralize Stx2

(A) Dot blot of 200 ng of purified Stx2 probed with polyclonal rabbit anti-Stx2 antibody or NRS. (B) Vero cell cytotoxicity neutralization assay in which polyclonal rabbit anti-Stx2 or NRS were incubated with 1:5 dilutions of Stx2 starting at 700 ng and then overlaid onto Vero cells. Dark wells indicate that the monolayer remained intact. For comparison, Vero cells overlaid without antibody and cells overlaid in the absence of toxin are also shown.

A

B
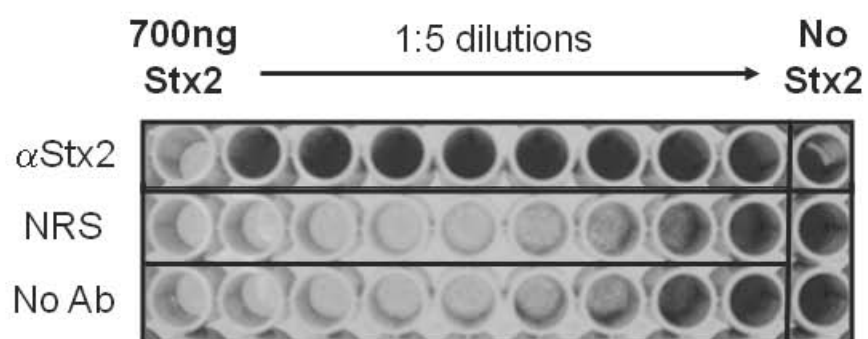
Figure 15: Detection of rabbit anti-Stx2 antibodies in feces of mice following IP

administration of antiserum

The percentage of animals (n=10) that had evidence of rabbit anti-Stx2 antibodies (at least 1.5 OD values above background in an ELISA) in their fecal samples on various days post-administration is depicted.

225
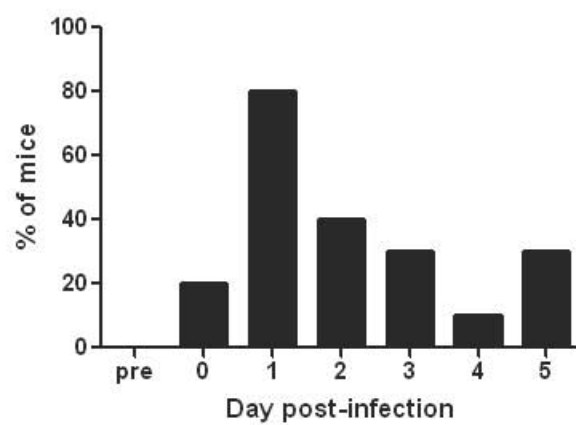
Figure 16: Impact of passively administered anti-Stx2 neutralizing antibodies on *E. coli* O157:H7 strain 86-24 colonization level

Mice received polyclonal rabbit anti-Stx2 serum (▲) or NRS (■) twice by IP administration prior to infection. Colonization was monitored following infection, twice daily for the first 4 days, and then once daily through day 7. Colonization was determined by enumerating the CFU shed/g feces and is reported for each animal (n=5). The dashed lines represent the GM for the anti-Stx2 (gray) and the NRS (black) treatment groups. Asterisks indicate specific days when statistically different colonization levels were observed. The limit of detection was 10^2 CFU/g feces (marked by an arrow).
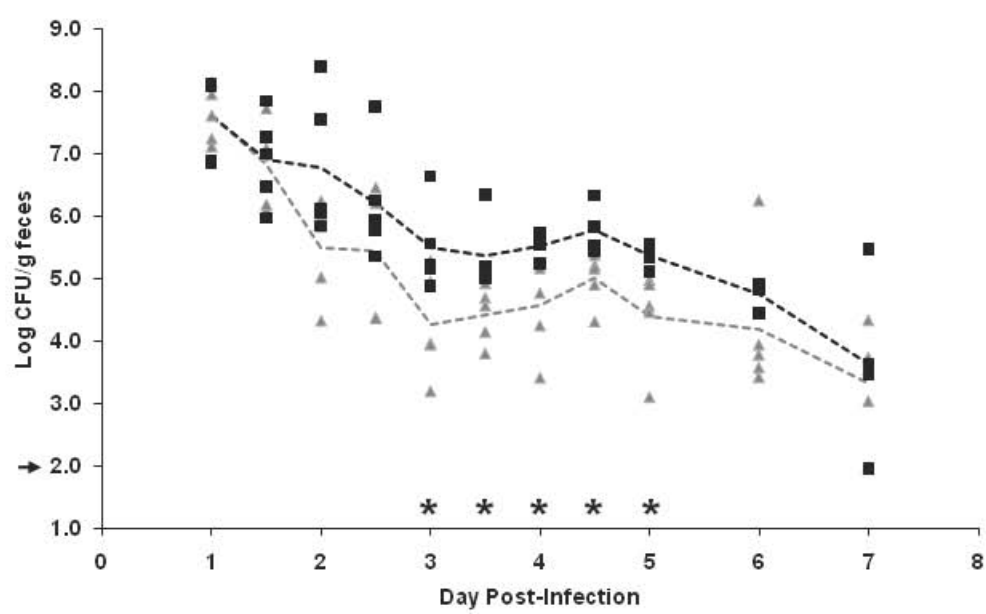
infection, animals that received the anti-Stx2 antiserum had lower geometric mean

colonization levels than did control mice (p<0.05). By day 7 post-infection, the

colonization levels of both groups had dropped to just over 10^3 CFU/ g feces. We

repeated these studies on two occasions and found a similar trend (data not shown).

Local and systemic impact of passively administered anti-Stx2 serum on toxicity of Stx2

delivered by **E. coli** *O157*

In the above study, a severely moribund infected animal within the NRS group

was sacrificed to prevent undue suffering. We suspected that this mouse died due to the

production of Stx2 by the infecting bacteria. Therefore, we asked whether we could

demonstrate neutralization of toxin made *in vivo* in the gut by the passively administered

anti-Stx2 antibody. To address that question, groups of 8 mice were administered anti-

Stx2 serum or NRS (as a control), challenged IG with 86-24Nal^R, and feces from the

animals collected for 5 days following infection. Supernatants of these fecal samples

were evaluated by ELISA for rabbit anti-Stx2 antiserum or Stx2. Anti-Stx2 antibodies

were detected in the feces of infected animals that received anti-Stx2 serum but, as

expected, not in the feces of animals that received NRS (Fig. 17A). There was also a

difference in detection of Stx2 in the feces of animals from both groups (Fig. 17B).

Fewer mice given anti-Stx2 antibody had detectable Stx2 within their feces on day 1

post-infection than did mice given NRS. Moreover, by day 2 post-infection, mice given

anti-Stx2 antibody had no detectable Stx2 in their feces while a portion of mice

administered NRS still had measurable levels of Stx2 through day 3 of infection (Fig.

17B). These findings suggest that the anti-Stx2 antibody at least partially bound the toxin

Figure 17: Post-infection detection of passively administered anti-Stx2 antibody or

Stx2 produced by the infecting wild-type *E. coli* O157:H7 strain 86-24

The group of mice given NRS is depicted by black bars and the animals administered anti-Stx2 are represented by gray bars. (A) Percentage of mice (n=8) from the NRS and anti-Stx2 administration group with detectable anti-Stx2 antibody shed into the feces after *E. coli* O157:H7 infection. (B) Percentage of mice (n=8) from the NRS and anti-Stx2 administration group that shed detectable levels of Stx2 into the feces post-infection.

A
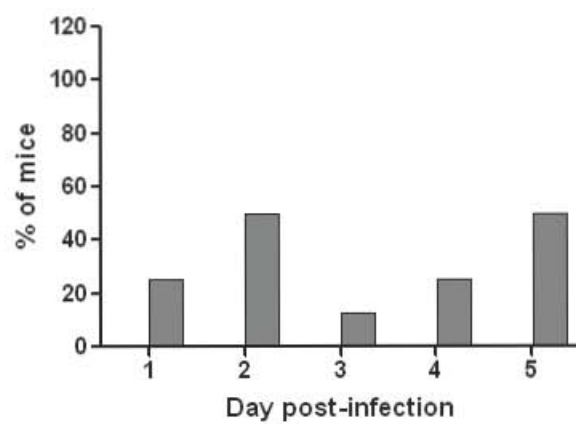
B
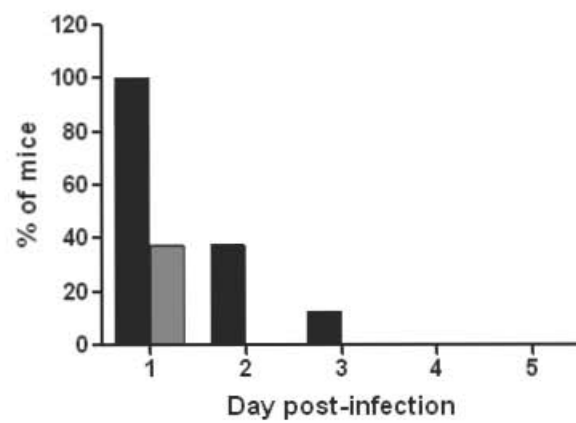
produced at the site of infection within the gastrointestinal tract.

In the experiment described above, animals that received NRS lost weight [in comparison to mice given anti-Stx2 serum (Fig. 18A)] and 7 of 8 mice succumbed to infection between days 4 and 5 (Fig. 18B). Overall, the cumulative lethality data from all passive transfer experiments were as follows: 12/38 mice given NRS and then infected with 86-24Nal^R died versus 0/43 mice treated with anti-Stx2 serum and then challenged with the bacterium. Therefore, passively administered anti-toxin antibody not only reduced the bacterial burden of *E. coli* O157:H7 in the gut but also protected the infected animals from Stx2-mediated death.

*** Effect of active immunization with Stx2 toxoid on serum and fecal anti-Stx2 responses and on colonization ***

Next, we assessed the impact of active immunization with an Stx2 toxoid on *E. coli* O157:H7 colonization. From a pilot study, we knew that a lengthy immunization protocol would be required to detect an Stx2-neutralizing antibody response in the feces and that such a response was necessary to reduce colonization by the challenge strain (data not shown). Therefore, mice were given Stx2 Y77S E167D-6H over a series of inoculations (see Materials and Methods for details of procedure). Fecal and serum samples obtained after boosts number 5 (last IP immunization) and 6 (IG administration) were then analyzed for the presence of anti-Stx2 antibodies by ELISA and by the capacity to neutralize Stx2 on Vero cells (Fig. 19). High titers of anti-Stx2 IgG antibodies were present in the sera from all vaccinated mice by boost 5 with lower level titers seen in the

Figure 18: Effect of passive administration of rabbit anti-Stx2 serum or NRS on

weight and survival of mice following *E. coli* O157:H7 infection

Groups of 8 animals received either two IP injections of anti-Stx2 antibody (▲)

or NRS (■) prior to oral infection with wild-type *E. coli* O157:H7 strain 86-24. (A)

Weight was monitored following infection and is displayed as the average of the group in

grams. Animals that succumbed to infection were subsequently listed at their last

recorded weight. Bars indicate the standard error of the mean. (B) Percentage of animals

surviving by day post-*E. coli* O157:H7 infection.

A
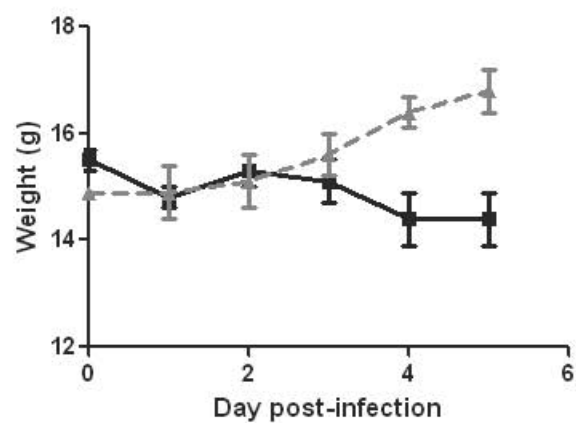
B
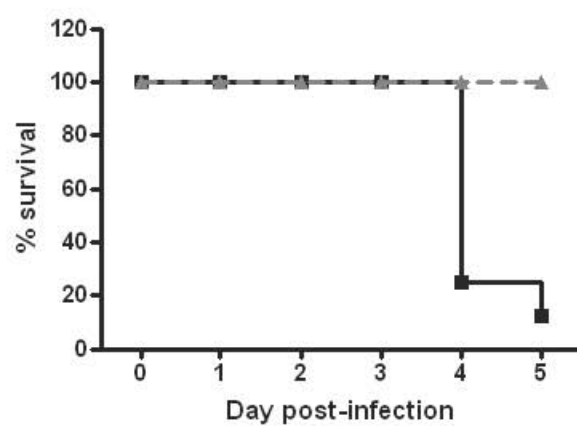
Figure 19: Evaluation of anti-Stx2 antibody response in serum or fecal filtrates by

ELISA and in fecal filtrates by the capacity to neutralize Stx2 on Vero cells

Samples were obtained from mice following the last IP-immunization (boost 5, B5) and again after IG administration (boost 6, B6) of toxoid (▲) or PBS (■). (A) Serum IgG titers of Stx2 antibodies in vaccinated or mock-vaccinated mice. (B) Fecal IgG titers of Stx2 antibodies in vaccinated or mock-vaccinated mice. (C) Fecal IgA titers of Stx2 antibodies in vaccinated or mock-vaccinated mice. (D) Percent neutralization of 4 pg of Stx2 ($\sim$ 1-2 50% cytotoxic doses or CD_{50}s) by fecal filtrates from vaccinated or mock-vaccinated mice.

234
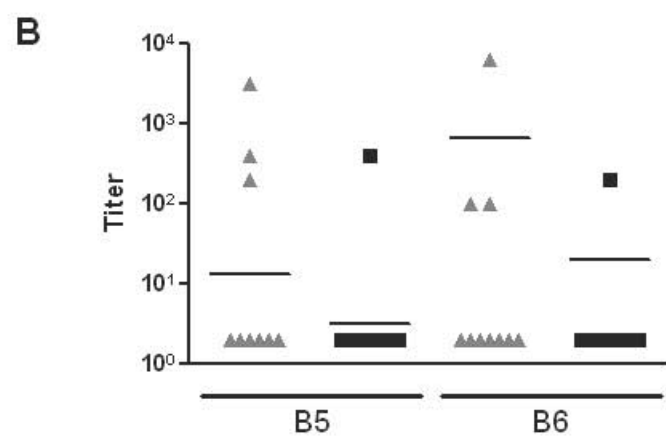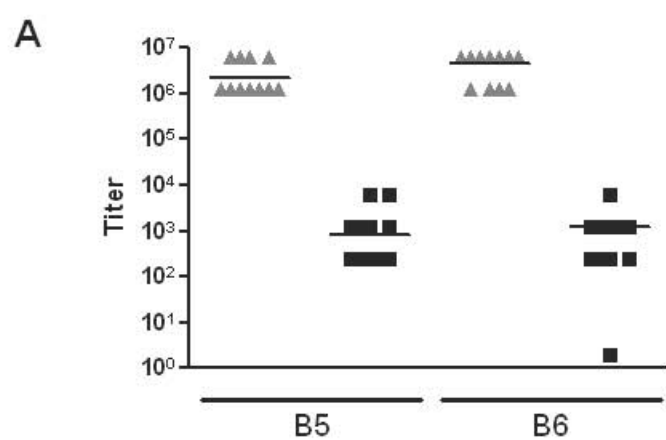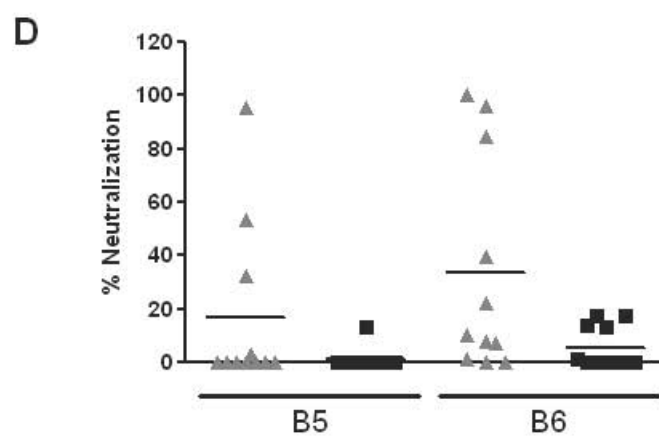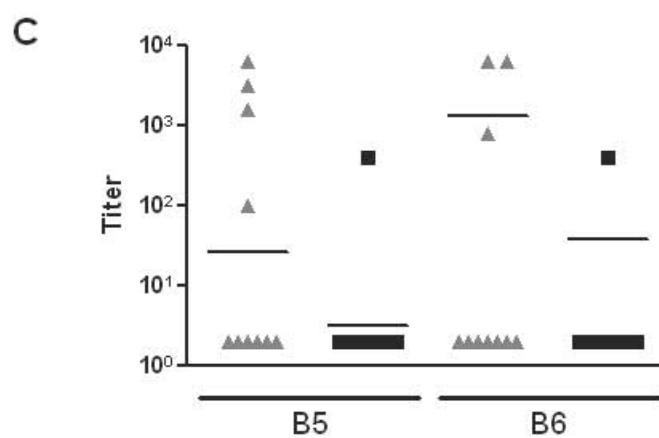
unvaccinated animals (Fig. 19A). Much lower ELISA titers of anti-Stx2 IgG (Fig. 19B) and IgA (Fig. 19C) antibodies were detected in fecal homogenates. In addition, antibodies were only detected from the feces of a proportion of the animals (Fig. 19B and C). Furthermore, IG administration of a concentrated amount of toxoid antigen (boost 6) did not result in significantly increased ELISA titers (Fig. 19B and C). Nevertheless, the fecal homogenates from some of the vaccinated animals after boost 5 and more of the mice after boost 6 had the capacity to ablate the cytotoxicity of Stx2 on Vero cells (Fig. 19D), an indication of Stx2-neutralizing antibody in those samples.

Following the detection of toxin-neutralizing antibodies in the feces, mice were infected with 2.8×10^9 CFU of wild-type strain 86-24Nal^R and colonization was monitored by fecal shedding (Fig. 20). Fecal shedding of 86-24Nal^R on day 1 post-infection was comparable between groups. Thereafter, the vaccinated group showed a steady decline in colonization levels out as far as two weeks post-infection, and the bacterial burden of the group remained below 10^3 CFU/g feces for the remainder of the experiment. The unvaccinated group displayed an overall decline in colonization levels past day 3 post-infection but also seemed to undergo cyclical rebounds in colonization levels (seen on days 3, 7, 10, 16, and 17) before the GM CFU/g feces in that group finally dropped below 10^3 on day 22 and remained there for the duration of the experiment. Thus, as was seen with passive immunization, active immunization against Stx2 resulted in decreased colonization levels (p=0.048 by mixed model, RM ANOVA) and a shorter duration of colonization (p=0.047) as compared to the controls. However, unlike the passive experiments, no systemic disease (emaciation, fur ruffling, unsteady gait, or death) was apparent in any of the mice. This absence of an obvious toxic effect from

Figure 20: Effect of active immunization with Stx2 toxoid on *E. coli* O157:H7 colonization levels

Mice were vaccinated with Stx2 toxoid (▲) or mock-vaccinated with PBS (■). Colonization was monitored daily following infection for the first 21 days, then every other day through day 35, and then weekly through day 63 post-infection. Colonization was determined by enumeration of the CFU shed/g feces and is reported for each animal (n=10-11/group). The lines represent the GM for the vaccinated (gray) and the mock-vaccinated (black) group. The limit of detection is 10^2 CFU/g feces (marked by an arrow).
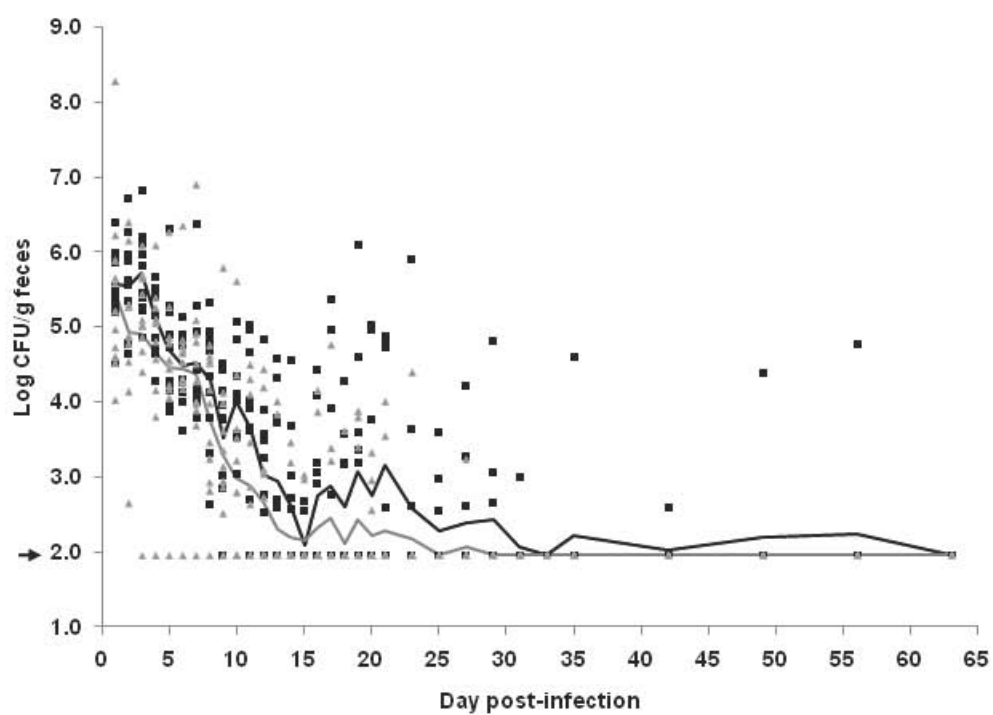
Stx2 may reflect the fact that these mice were considerably older and, thus, heavier (presumably with a lower toxin to body weight ratio) than the animals used in the passive immunization experiments.

Discussion

Three major findings were derived from this investigation. First, we demonstrated that Stx2 provided exogenously to mice challenged with an *E. coli* O157:H7 *stx₂* mutant enhanced the intestinal colonizing capacity of that mutant. Second, we showed that Stx2-neutralizing antibodies administered passively to mice reduced the burden of wild-type, Stx2-expressing *E. coli* O157:H7 and protected animals from death. Third, we provided evidence that active immunization with an Stx2 toxoid resulted in toxin-neutralizing antibodies in the gut and reduced the level of wild-type, Stx2-expressing *E. coli* O157:H7 shed in the feces over time compared to unvaccinated, infected controls.

The notion that Stx plays a role in colonization came from work that suggested a rabbit diarrheagenic *E. coli* strain transfected with a phage that encodes Stx1 not only caused more serious disease than the control strain, but was also more enteroadherent (46). In 2006, our laboratory presented evidence that Stx2 plays a role in *E. coli* O157:H7 adherence *in vitro* and colonization *in vivo* (38). Subsequently, other investigators reported that Stxs can augment colonization of *E. coli* O157:H7 (3, 7, 13, 20, 25, 50). Conversely, several groups of researchers found no such role for toxin (5, 11, 16, 37, 41). However, in those latter studies, a variety of factors may have affected the results. For example, introduction of the organism by a route that bypassed most of the gut (41) or the use of animals with no or reduced flora (16) could preclude detection of a role for toxin. Additionally, the conclusions in some of the reports are based on either a single time-point post-infection (37) or a limited temporal analysis (11, 16). Finally, in one instance the authors excluded a role for Stx in gut adherence yet only

measured colonization levels of an Stx-negative *E. coli* O157:H7 strain without inclusion

of a wild-type toxigenic strain for comparison (5).

The findings described here and elsewhere suggest that Stxs can promote bacterial

colonization by *E. coli* O157:H7 (3, 7, 13, 20, 25, 38, 50). The discovery that toxin plays

a role in colonization by bacteria appears to be generalizable to a broader group of toxin-

producing enteric microbes. In fact, there are reports of a role in gastrointestinal

colonization for heat-labile toxin of enterotoxigenic *E. coli* (ETEC) (2, 4), the accessory

toxins of *Vibrio cholerae* (35), VacA toxin of *Helicobacter pylori* (12, 39), and, most

recently, *Clostridium difficile* transferase (CDT) toxin of *C. difficile* (40). The

mechanisms by which toxin directly or indirectly promote colonization in the

gastrointestinal tract likely differ among these pathogens and are not fully understood.

Some possible explanations include: a toxin-mediated increase in the level of a bacterial

receptor on the surface of eukaryotic cells (as we have suggested for the effect of Stx2 on

a eukaryotic intimin receptor, nucleolin), the capacity of toxin to directly function as a

bacterial adhesion molecule, an indirect increase of/exposure to receptor as a result of

toxin-mediated cell death, a toxin-promoted host-cell cytoskeletal change required for

bacterial adherence, and/or a dampening of the host response in favor of initial

colonization (2, 35). Regardless of the mechanism, toxin production may either be

required to augment colonization or simply provide a competitive advantage to the toxin-

producing bacterium [see review by (12)].

Because Stx plays a role in both colonization and systemic disease, we propose

the use of either toxoid vaccination or passive administration of anti-Stx antibodies to

reduce or prevent STEC-mediated disease in people. The transfer of specific antibodies

or anti-serum to patients is a well-established strategy to prevent or treat certain

infectious disease agents or toxins [reviewed in (8, 23)]. That anti-Stx2 antibody might

be of use in the treatment of the serious consequences of *E. coli* O157:H7 infection was

first demonstrated by Wadolkowski *et al.* in a mouse model (47); in that and other early

studies, such antibody was given before infection (15, 22, 47). In subsequent evaluations

of the efficacy of such passive therapy, anti-Stx antibodies were shown to provide

protection against STEC-mediated illness and death even when given up to 4 days after

bacterial challenge (15, 28, 42, 49). In this study, we found that anti-Stx2 antiserum

administered IP prior to *E. coli* O157:H7 challenge prevented both weight loss and death

of the infected mice. We also found that such treatment decreased the likelihood that

mice would become highly colonized with *E. coli* O157:H7. To the best of our

knowledge, the latter observation is a novel finding about the impact of passively

administered anti-Stx antibodies in an animal model.

Several groups of investigators, including our own, have reported that active

immunization with an Stx toxoid by parenteral (6, 18, 26) or oral routes (48) protects

animals from the systemic manifestations of STEC disease. In a more recent paper by

Zhu *et al.*, StxB1 was used to immunize rabbits through a transcutaneous procedure. The

animals were then challenged with an Stx1-producing rabbit diarrheagenic *E. coli* strain

(51). Immunized animals demonstrated systemic protection in the form of enhanced

weight gain and a reduction in the histopathological effects of intoxication and also

showed a statistically significant decrease in enteroadherent bacteria on the surface of

their cecal tissues when compared to both naïve and adjuvant- alone-treated controls (51).

The latter finding is consistent with our observation that active immunization against

Stx2 resulted in decreased levels of *E. coli* O157:H7 colonization of mice after infection when compared to mock-vaccinated control.

In sum, our results suggest that a toxoid-based vaccine against STEC would be beneficial for two reasons. First, such a vaccine would prevent the systemic toxicity due to the Stxs that can lead to such serious consequences as HUS. Second, a toxoid-based vaccine would reduce the bacterial burden of *E. coli* O157:H7 in the gastrointestinal tract if infection should occur and would thus decrease the level of toxin made *in vivo* as well as the likelihood of person-to-person transmission of this serious food-borne pathogen.

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CHAPTER FOUR

DISCUSSION

Summary of results in the context of the dissertation objective, specific aims, and

proposed model

The overall objective of this dissertation was to investigate the role of Stx2 in the

colonization and pathogenesis of an Stx2-expressing strain of *E. coli* O157:H7. In an

effort to meet this objective, we developed two related hypotheses that guided the work

for this dissertation. Our first hypothesis was that Stx2 facilitates intestinal colonization

of *E. coli* O157:H7 in mice with an intact commensal flora (ICF). To investigate this

theory, we first developed and described a murine model of *E. coli* O157:H7 intestinal

colonization in which the infecting organism was administered orally (by pipette feeding

or gavage). In this model, high doses of *E. coli* O157:H7 were required to achieve

consistent, persistent colonization in the face of the ICF of the animal. We determined

that within the mouse gastrointestinal tract, the primary site of *E. coli* O157:H7 was the

cecum. We surmised that the bacteria were shed into the cecal content where they

transiently colonized or directly passed through the large intestine in the luminal contents

prior to becoming encased in feces pellets that were subsequently expelled. Thus, we

deduced that the *E. coli* O157:H7 colony counts in the feces primarily reflected the level

of the colonization within the cecum. Investigation of the expression of intimin and Stx2

revealed that both virulence factors were readily detected early in infection when *E. coli*

O157:H7 numbers were high. With the mouse ICF model, we not only demonstrated that

E. coli O157:H7 colonized the animals but also that it caused systemic disease as

reflected by weight loss, morbidity, and death of some of the infected mice. In addition,

ICF mice infected with *E. coli* O157:H7 displayed evidence of renal tubular damage with

increased blood urea nitrogen (BUN) and slightly increased levels of creatinine, both of

which can indicate renal impairment. These blood chemistry findings are seen in patients with HUS that sometimes follows *E. coli* O157:H7 infection. Therefore, through these data and others, we inferred that Stx2 produced by *E. coli* O157:H7 at the site of infection within the gastrointestinal tract could enter the blood stream and harm the kidneys of the mice in a manner parallel to that presumed to happen in some *E. coli* O157:H7-infected humans.

After the development of the ICF mouse model of *E. coli* O157:H7 infection, we sought to demonstrate the capacity of Shiga toxin type 2 to promote colonization by the microbe. For this purpose, we exogenously supplied Stx2 *in trans* to an *stx₂* mutant *E. coli* O157:H7. This addition of Stx2 *in trans* to the *stx₂* mutant restored the capacity of the mutant to colonize in the ICF model. We concluded that our first hypothesis was correct: toxin does in fact promote *E. coli* O157:H7 colonization in the ICF mouse model.

Our second hypothesis, which was a correlate of our first theory, stated that the presence of anti-Shiga toxin type 2 antibody prior to and during *E. coli* O157:H7 infection would result in diminished levels of colonization by the microbe. To address this premise, we sought to explore the effect of passively administered anti-Stx2 on *E. coli* O157:H7 colonization in the mouse ICF model. We discovered that when administered prior to infection, anti-Stx2 neutralizing antibodies significantly decreased the likelihood that a mouse would become highly colonized with *E. coli* O157:H7. In addition to this novel observation, we confirmed that these Stx2-neutralizing antibodies could protect *E. coli* O157:H7-infected mice against such manifestations of Stx2-mediated disease such as weight loss and death. Moreover, we found that mice repeatedly immunized with an Stx2 toxoid developed an Stx2-neutralizing fecal response

and that when challenged with *E. coli* O157:H7 shed fewer organisms in their feces for a shorter duration than sham-vaccinated control mice.

From our evaluation of *E. coli* O157:H7 strain 86-24 colonization of the BALB/c ICF mouse, we propose the following model to explain the role that Stx2 plays in the initial steps of *E. coli* O157:H7 infection (depicted in Figure 21). We theorize that the bacteria enter the gastrointestinal tract and then transit to the primary site of colonization [the cecum in mice (49, 54)] where they then elaborate Stx2. Data generated *in vitro* with HEp-2 cells indicate that the action of the toxin causes an increase in cell surface-localized nucleolin (62). Nucleolin is a eukaryotic binding partner for intimin and thus a eukaryotic receptor for intimin-expressing *E. coli* O157:H7 (75, 76). Intimin from the *E. coli* O157:H7 and cell-surface nucleolin interact to provide an initial point of attachment for the bacterium. Once the organism associates with the host cell through the intimin/nucleolin interaction, intimin binds to Tir, the bacterially-encoded intimin receptor that gets translocated by the type-III secretion system (TTSS) into the host cell. The intimin/Tir interaction, coupled with the effects of other TTSS effector proteins, culminates in the formation of the characteristic attaching and effacing lesion/pedestal (Figure 21A).

Stx2 was detected *in vivo* as early as 3 hours after *E. coli* O157:H7infection of ICF mice. This finding suggests that toxin production may occur during transit of the organism through the gastrointestinal site. Certainly Stx2 was expressed early in infection in the cecum and at other sites where *E. coli* O157:H7 numbers were high. Furthermore, the presence of large amounts of toxin within the large intestinal luminal contents implies that toxin may be absorbed into the circulation from this location as well

Figure 21: Model of the role of Stx2 in *E. coli* O157:H7 adherence and

colonization

Panel A was derived from *in vitro* data while panels B and C were derived from *in vivo* data. The symbol legend for panels A, B, and C is listed in association with panel A. (A) *E. coli* O157:H7 elaborates Shiga toxin early during the colonization/adherence process. Stx2 exerts an effect upon the host cell epithelium leading to increased levels of cell-surface-localized nucleolin. Nucleolin acts as an initial receptor for intimin allowing *E. coli* O157:H7 to bind to the host epithelium and inject various TTSS effectors into the host cell. One such effector, Tir, is a bacterially-encoded receptor. The intimin-Tir interaction, coupled with the cellular effects of other TTSS effectors, lead to host cell cytoskeletal rearrangement and formation of the characteristic A/E lesion. (B) Stx2, produced by the WT organism or provided to an *stx$_2$* mutant, facilitates colonization of the gastrointestinal tract. (C) Neutralizing anti-Stx2 antibody present prior to and during *E. coli* O157:H7 infection results in reduced levels of *E. coli* O157:H7 colonization of the gastrointestinal tract.
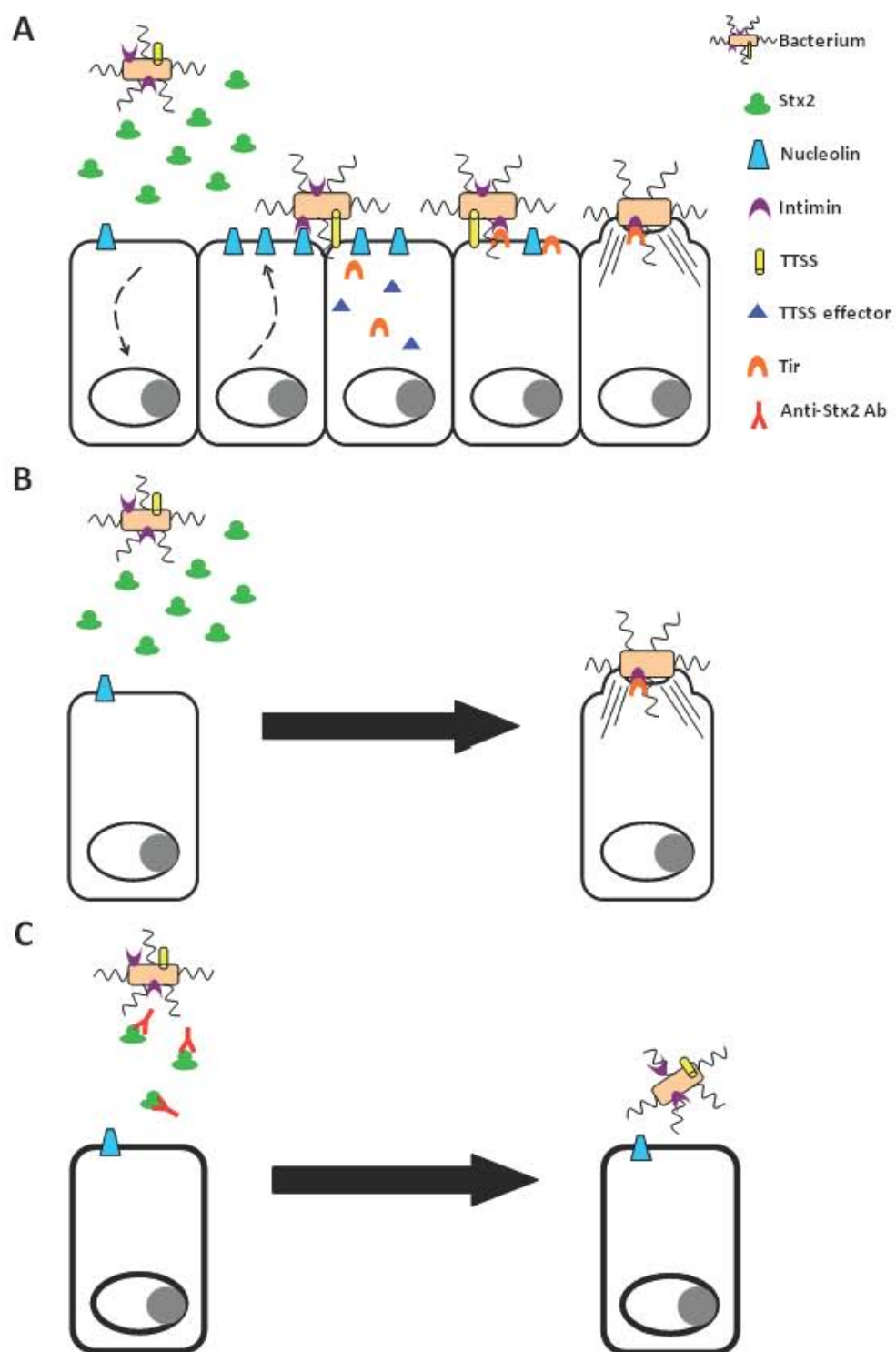
as from the cecum. Our failure to detect Stx2 in the bloodstream of most *E. coli*

O157:H7-infected animals may suggest that only a small percentage of the toxin

produced in the gastrointestinal tract enters the circulation. Alternatively, the observation

that only a fraction of the Stx2 injected intravenously was detectable in the circulation

may suggest that Stx2 is readily bound to the microvascular endothelium. Nevertheless,

we presume that at least a portion of the toxin produced within the gastrointestinal tract

enters the blood stream because: 1.) renal tubular damage was evident in infected mice

and toxin was detected in the kidney of a least one infected animal, and; 2.) parenteral

inoculation of anti-toxin antibody protected mice from the systemic manifestations of

disease.

Given that Shiga toxin not only acts systemically but also facilitates *E. coli*

O157:H7 colonization [chapter 3 and (62)], we propose that a therapeutic strategy

directed against toxin would be quite beneficial. To this end, we first showed that toxin

provided exogenously to a toxin-null mutant resulted in increased *E. coli* O157:H7

colonization (chapter 3). Thus, toxin, produced by the WT or provided exogenously to

the mutant, had the capacity to increase the colonization levels of *E. coli* O157:H7 in

mice (Figure 21B). We went on to demonstrate that anti-toxin antibody, either pre-made

and administered to animals or actively generated following vaccination, reduced the

overall levels of *E. coli* O157:H7 shed by mice and protected animals from systemic

manifestations of disease to include weight loss and death (Figure 21C).

Mouse Model of *E. coli* O157:H7 infection and pathogenesis

We developed a mouse model in which *E. coli* O157:H7 introduced orally by either pipette feeding or intragastric administration (gavage) was forced to compete with an intact commensal flora to become established within the gastrointestinal tract. We chose such a model because we deemed that intestinal colonization by *E. coli* O157:H7 in the face of normal gut flora more accurately reflected the nature of exposure of humans to the pathogen than did establishment of the organism in the gastrointestinal tract of streptomycin-treated, axenic, or protein calorie-restricted animals. While high doses ($\sim 10^9$ CFU) of *E. coli* O157:H7 were required to establish colonization and manifest disease in the ICF model [as opposed to humans in which the ID_{50} is less than or equal to 50 organisms (83)], we contend that the benefits of this model for the evaluation of colonization and the impact of toxin delivered from the gut outweigh the detriment of this requisite for an abnormally high challenge inoculum.

Indigenous gastrointestinal microflora

The overall composition of the microbiota found within the gastrointestinal tract is stable but does vary by location (Figure 6) in terms of both the number and type of species present [reviewed in (9)]. While the oral cavity is rich in microbes, the stomach and proximal small intestines contain only low numbers of organisms. The distal small intestines is considered the "transition zone" that separates the relatively sparsely colonized upper bowel from the more densely colonized lower bowel that includes the ileocecal junction/cecum, and the large intestines. The large intestine, with its slower rate

of motility and low oxidation-reduction potential, is the primary site of indigenous microbial colonization in humans.

Influence of the microbiota on the host

The presence of an indigenous microbiota influences the physiological, biochemical, morphological, and immunological development of the host [reviewed in (9)]. The importance of the effects that an indigenous microbiota has on its host has been demonstrated in studies that compare conventional and germfree animals [reviewed in (9)]. Thus, germfree animals have enlarged ceca, decreased rates of villus epithelial cell turnover, and underdeveloped gut-associated lymphoid tissues and secondary lymphatic organs compared to animals with normal flora (9). Other more specific effects of the normal microflora (or microbiota) include the following. Members of the gastrointestinal microflora are responsible for the production of vitamins (B_{12} and K) used by the host (9) and for priming the immune system to respond to invading bacterial pathogens. This latter phenomenon is thought to occur by general antigenic stimulation [through host pattern recognition receptors (TLRs and NLRs) (70)] and perhaps by bacterial translocation to the lamina propria and mesenteric lymph nodes (9). Furthermore, the indigenous microflora helps to maintain peristalsis and mucosal integrity within the intestines (9, 32). Recently, indole, secreted by commensal *E. coli* and demonstrated to be present in human feces, was shown to positively impact intestinal epithelial cells *in vitro*; the mucosal barrier was strengthened, mucin production increased, and the expression of proinflammatory molecules was inhibited (6). As a result of the considerable influence that the microbiota has on the host, Berg has suggested that

germfree animals, even once colonized with microflora, can never be truly considered representative of conventional animal (9).

Colonization Resistance

Arguably, the most important role of the normal microbiota is in colonization resistance (also known as bacterial antagonism, bacterial interference, or microbial interference). As discussed previously, colonization resistance is the phenomenon whereby the normal microflora interferes with the capacity of exogenous pathogens to colonize within the gastrointestinal tract and cause disease. Several different explanations for this normal flora-mediated impediment to pathogen colonization have been proposed and these include: 1.) competition for both available nutrients and receptors/adherence sites along the intestinal epithelium; 2.) the production of inhibitory substances (antimicrobial or toxic by-products); 3.) stimulation of mucin secretion or antimicrobial peptide production by the host, and; 4.) gut mucosal barrier stabilization and improved peristalsis (80, 81). However, the actual mechanism of colonization resistance is not fully understood (80) and may represent a combination of these or other factors (as discussed in *Benefits of ICF Model*).

Effects on Pathogen Colonization and Disease

The precise role of the gastrointestinal microbiota in the establishment of *E. coli* O157:H7 infection or in the likelihood of ensuing disease has not been fully delineated. Several studies have demonstrated the susceptibility of *E. coli* O157:H7 to colonization resistance in conventional and gnotobiotic (colonized with a defined microflora) mice in

comparison to str-treated and germ-free animals (3, 29, 43, 73, 74, 82). While the exact mechanism/s have not been completely elucidated, various reports have attempted to define potential mechanisms by which resistance to *E. coli* O157:H7 colonization occurs. Several possibilities have been suggested such as competition for such nutrients as proline between the established normal flora and the in-coming *E. coli* O157:H7 (51), production of bacteriocins by certain members of the normal flora that kill susceptible *E. coli* O157:H7 (55, 65), production of organic acids (lactate and acetate) by species within the microbiota that inhibit *E. coli* O157:H7 motility (51), or production of other substances, such as indole, that may attenuate *E. coli* O157:H7 infection (7).

While the indigenous microbiota can exert colonization resistance against *E. coli* O157:H7 as described above, Durso *et al.* showed that *E. coli* O157:H7 can also act *in vitro* as a competitive antagonist against both commensal *E. coli* strains and other strains of *E. coli* O157:H7 (23). As an explanation for these findings, Durso and colleagues proposed that the *E. coli* O157:H7 strains used in their study made bacteriocins (colicins) that inhibited the growth of other organisms (23). Indeed, Scotland *et al*. (69) had previously shown that certain *E. coli* O157:H7 strains produce colicins (to which they are naturally resistant) even though other *E. coli* O157:H7 strains were subsequently demonstrated to be sensitive to colicins made by different *E. coli* strains (52). Another advantage that *E. coli* O157:H7 may have in the face of normal microbial flora was suggested by the work of Fabich *et al*. who demonstrated that *E. coli* O157:H7 can metabolize different carbon sources than those used by commensal bacteria (26). Thus, *E. coli* O157:H7 may have the capacity to consume sugars in the GI tract that are not available as energy sources for indigenous microbiota (26).

Furthermore, several studies have shown that the particular constituents of the indigenous microbiota may influence *E. coli* O157:H7 colonization and subsequent pathogenesis. In an example of the former, Momose and colleagues demonstrated that the microbiota of gnotobiotic mice reconstituted from human infant feces can influence *E. coli* O157:H7 persistence; in one instance *E. coli* O157:H7 was eliminated and in another carriage of the microbe was achieved (50). Investigation of the microbiota shed in the feces of these animals revealed that the mice that eliminated *E. coli* O157:H7 were colonized with the species of commensals seen in the feces of healthy infants (50), whereas the mice that exhibited sustained carriage of *E coli* O157:H7 shed species not typically found in the stools of healthy infants. These results lend support to the contention that the severe disease seen in some *E. coli* O157:H7-infected individuals may reflect differences in the composition of that individual's intestinal microbiota. Indeed, differences have been reported in the gut microbiota between infants and the elderly as compared to adults (31) and, perhaps, these variations in the normal flora composition in aggregate or at particular sites in the intestines may underlie the susceptibility of the former groups to *E. coli* O157:H7 infection and subsequent disease. Additionally, children and adults appear to differ with respect to the production of colicins by their respective indigenous flora; specifically, *E. coli* O157:H7 inhibitors (presumably colicins) are found more frequently in the feces of middle-aged individuals (40-59 years) compared with the stools of children 6 years and under (84).

Commensal gut organisms may also alter the amount of Stx produced by *E. coli* O157:H7 in the gastrointestinal tract. For example, a reduced level of *E. coli* O157:H7 in the gut as a consequence of microbiota-induced colonization resistance would indirectly

limit toxin production (and the subsequent likelihood of systemic disease) (20).

Conversely, Gamage *et al*. showed that certain normal flora *E. coli* strains can be

transduced *in vitro* with Stx-converting phage acquired from *E. coli* O157:H7 leading to a

lysogenic or lytic infection of the transductant (28). Furthermore, Gamage and

colleagues demonstrated transduction of an introduced *E. coli* strain inoculated into mice

concurrently with an *E. coli* strain lysogenized with the Stx2-converting phage 933W

(30). In a more extensive analysis, Gamage *et al*. demonstrated that coinfection of *E. coli*

O157:H7 with a phage-susceptible strain led to an increase in the overall level of toxin

production in the gastrointestinal tract compared to that seen on coinfection with a phage-

resistant strain; presumably the *E. coli* O157:H7 have undergone lysis and the phage-

susceptible commensal *E. coli* have become transduced and can themselves be induced to

produce toxin (29). In addition, the production of secreted molecules from certain

commensals can either enhance or inhibit transcription of *stx₂* (20). Moreover,

Reissbrodt *et al*. reported that cultures of *E. coli* (obtained from human fecal material)

can inhibit Stx production *in vitro* (59), although a mechanism for this decrease in toxin

synthesis remains to be elucidated. Additionally, *in vitro* cultures of a variety of

probiotics or media conditioned with human fecal microbiota reduced the level of

transcription of *stx₂* (14, 20). Furthermore, the presence of *Bifidobacteria* in str-treated

animals did not affect the initial colonization by STEC (although subsequent effects on

persistence were seen), but did inhibit mitomycin C-induced Stx production in the first

few days of *in vivo* infection (3). Despite the inhibition of *E. coli* O157:H7

growth/pathogenesis in the presence of inhibitors suspected of being colicins (84),

Toshima *et al*. reported that DNase colicins increased Stx2 production *in vitro* by

induction of the SOS response (85). Conversely, this same group found that RNase

colicins inhibited Stx production (85). Finally, Gamage and colleagues showed that an

isolate of commensal *E. coli* recovered from a healthy child could neutralize the cytotoxic

effects of Stx2 (but not Stx1) *in vitro*; however, this finding has not been confirmed *in

vivo* (27, 30).

Benefits of an ICF Model

To reiterate: the use of an animal model with an intact commensal flora for

studies of *E. coli* O157:H7 infection and pathogenesis has the advantage of reflecting a

complex microbiological environment in the gut such as that typically found in an

individual who ingests *E. coli* O157:H7 in contaminated food or water or through person-

to-person contact. Streptomycin treatment to reduce the normal microbiota or the use of

axenic animals without a normal microbiota not only have the disadvantage of lacking

(all or some of) the potentially beneficial influences of this flora but also have disrupted

(or non-existent in the case of axenic animals) colonization resistance mechanisms.

While the exact reason(s) these animals are susceptible to low-dose enteric infection is

unclear, Strecher *et al*. have suggested that the elimination of a specific microbial species

or genera that would normally confer colonization resistance may be responsible (80).

As alternative explanations, these investigators posed the possibilities that animals with

reduced or non-existent gut microbiota can be readily infected because of a simple

overall reduction in microbial numbers or because of the absence of the effects on the

physiology of the gut mucosa that are typically mediated by commensal flora (80).

Despite the incomplete understanding of the basis for colonization resistance, the capacity of a pathogen to overcome this microflora hindrance by whatever mechanism is critical for that organism to become established at a mucosal site. Since *E. coli* O157:H7 can colonize the gastrointestinal tract of people (as was described in the introduction, chapter 1) and conventional mice (as was described in chapter 2), it is safe to assume that *E. coli* O157:H7 must have developed strategies to overcome colonization resistance. Clearly, the mechanisms by which *E. coli* O157:H7 can surmount colonization resistance can only be dissected in an animal model with an established flora. Finally, one might speculate that if an infecting microbe does not need to employ pathogenic strategies to overcome colonization resistance because the host animal has no or a reduced microflora, then the overall expression of virulence factors by that agent as well as the subsequent pathogenesis of disease mediated by it could be altered.

Quorum Sensing

E. coli O157:H7 has the capacity to exploit quorum sensing, or bacterial cell-to-cell signaling, mechanisms to modulate its virulence (37). Through this system, *E. coli* O157:H7 can respond to autoinducers (hormone-like signaling molecules) produced by itself, other *E. coli*, or even other bacterial species. Furthermore, *E. coli* O157:H7 can also make use of host-derived neuroendocrine molecules in an analogous manner to that of a particular autoinducer (AI-3). Thus, the pathogenicity of *E. coli* O157:H7 can be modulated by self, intraspecies (commensal *E. coli*), interspecies, or interkingdom (host) communication. Therefore, the presence of an indigenous microflora within the gastrointestinal tract is likely to play a role in *E. coli* O157:H7 pathogenesis.

Both pathogenic and commensal bacteria are known to encode the *luxS* gene, the product of which is an enzyme responsible for the universal quorum sensing system (67, 90). The LuxS system in *E. coli* O157:H7 is responsible for the production of at least two autoinducers, AI-2 and AI-3 (79). Both of these autoinducers have been detected in the cultures of stools from healthy volunteers (16, 79), findings that suggest that the indigenous microbiota has the capacity to produce AI-2 and AI-3. Further investigation revealed that the activity of both autoinducers can be detected in such commensal bacteria as *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (16). Thus, these autoinducer molecules may serve as signals for intra- and inter-species communication among the indigenous microflora components and may be further manipulated to modulate the virulence of *E. coli* O157:H7 and other enteropathogens.

The AI-3 autoinducer that can be detected in culture supernatants of both *E. coli* O157:H7 and commensal *E. coli* cultures can activate transcription of the LEE operons and the flagella regulon as well as stimulate production of toxin (16, 78, 79). Speculation is that the quorum sensing via AI-3 (and neuroendocrines like norepinephrine and epinephrine) may facilitate *E. coli* O157:H7 colonization by signaling the bacteria when they have reached the lower bowel (where large numbers of commensals are present and likely producing AI-3) and inducing the expression of virulence genes needed for both colonization and systemic disease (16). If this scenario is correct, then the diminishment/abrogation of signaling in animal models that have reduced/lack indigenous microflora likely influences the location at which *E. coli* O157:H7 colonizes. Additionally, in the absence of the production of excess AI-3 made by indigenous microflora, a delay in virulence expression in *E. coli* O157:H7 could result and thereby

influence the pathogenic process of *E. coli* O157:H7. Finally, the application of an animal model with an ICF that permits the full signaling cascade (intraspecies, interspecies, and interkingdom) to occur will likely be required to fully investigate the role that quorum sensing may play in the pathogenesis of *E. coli* O157:H7.

Competition for Nutrients – Metabolic Switching

One proposed mechanism for colonization resistance is that the incoming organism must compete with normal flora microbes for nutrients like carbon sources. Commensal *E. coli* in particular have been demonstrated to simultaneously use a number of scarce nutrient sources for growth in the intestines. While *E. coli* O157:H7 is capable of the co-metabolization of different sugars to maintain growth *in vitro* (and likely *in vivo*), *E. coli* O157:H7 lacks the capacity to perform metabolic switching. This is a rapid process by which an increase in a metabolic intermediate triggers a switch to the utilization of a second, unrelated nutrient (i.e. fucose to ribose utilization) (4). That *E. coli* O157:H7 cannot undergo such metabolic switching was demonstrated *in vivo* in a series of competition studies in which the step-wise introduction of several metabolic defects in *E. coli* O157:H7 resulted in additive colonization deficiencies compared to the wild-type parent strain (26). In comparison, commensal *E. coli* organisms have the capacity to undergo metabolic switching (26): Autieri *et al*. have proposed that metabolic switching allows commensal *E. coli*, and presumably other commensal organisms, to efficiently grow within the nutrient-limited environment of the gastrointestinal tract without being eliminated (4).

The failure of *E. coli* O157:H7 to perform metabolic switches, and the organism's dependence on multiple carbon sources for efficient growth, hinders its capacity to effectively compete in models with an indigenous microbiota. Thus, the use of streptomycin treatment to eliminate the majority of the facultative anaerobes, like the commensal enterobacterium *E. coli*, or the use of germ-free animals provides an advantage for *E. coli* O157:H7. To overcome this apparent deficiency, *E. coli* O157:H7 has developed the capacity to utilize sugars that are not preferentially used by commensal *E. coli*, namely galactose, mannose, and ribose (26). This metabolic mechanism would likely be favored in situations where *E. coli* O157:H7 competes with an intact commensal flora. Thus, to completely explore and characterize the mechanisms by which *E. coli* O157:H7 overcomes nutrient limitation necessitates the use of an animal model in which these metabolic strategies would likely be employed.

Finally, Fabich *et al*. have hypothesized that metabolic switching is an adaptation mechanism that allows members of the diverse commensal population to co-inhabit in particular niches of the gastrointestinal tract in the face of "host-strain-nutrient variations" (26). Given that the nutrient availability is likely dictated by the particular commensals present, it is intriguing to speculate that the varying levels of resistance to *E. coli* O157:H7 infection may be dictated in part by the nutrient sources available for use within a given individual's intestinal microbiota.

A Triad of Interaction – Host, Commensal, Pathogen

Our knowledge that interplay exists between the host, the microbiota, and the invading pathogen makes the use of germfree or streptomycin-treated models less

desirable. Obvious morphological, physiological, biochemical, and immunological

differences exist between conventional and germfree mice. Furthermore, the use of

antibiotics, streptomycin in particular, has been shown to disturb "the mouse microbiota

composition and numbers, potentially affecting the downstream host-microbiota, host-

pathogen and microbiota-pathogen interactions" (70). The development and utilization

of models to study the pathogenesis of *E. coli* O157:H7 in the presence of this multitude

of interactions and signals is vital if we are to contemplate and combat the full battery of

pathological mechanisms employed by *E. coli* O157:H7.

E. coli *O157:H7 Infection in the ICF Model*

The ICF mouse model was developed to explore the role of Stx2 in colonization

of E. coli O157:H7.   With the model, we were also able to investigate several parameters

of the colonization process such as dose required to establish infection, duration of

persistent shedding of the microbe, ways to intervene with colonization through antibody

therapy, development of disease following infection, as well as the prevention of the

systemic manifestations of infection. Certain findings from those investigations that were

not described in detail in the preceding chapters are discussed below.

Colonization Levels

While we observed colonization in a portion of ICF BALB/c mice at every dose

of *E. coli* O157:H7 tested, a large initial *E. coli* O157:H7 inoculum was required to

achieve high level, persistent colonization of mice. Thus, we propose that this high

challenge dose of *E. coli* O157:H7 is needed to overcome colonization resistance by the

normal microbiota. In support of this hypothesis, our dose- response infection studies in ICF mice revealed three types of responses to infection among animals. The first group included the "nontakers" (resistant to colonization); these non-takers comprised a subset of those animals challenged with the lowest doses of *E. coli* O157:H7 tested. We surmised that the "nontakers" represented animals that failed to become infected with *E. coli* O157:H7 perhaps because the organism at lower doses could not establish a foothold in the presence of the indigenous microbiota. The second group consisted of animals that received a higher inoculum of 10^8 CFU. While these animals exhibited high initial colonization levels, as a group they were only transiently infected with *E. coli* O157:H7. We labeled these animals "takers-not-keepers" (transient colonization). Finally, animals given the highest dose of bacteria, 10^9 CFU, had a more persistent infection and were considered "takers and keepers" (persistent colonization). The "takers and keepers" included those animals that demonstrated the greatest morbidity and mortality.

A link between STEC colonization level, toxin titer, and clinical disease was also reported in an edema disease model of swine (18). We surmise that the reason for this linkage, noted by Cornick and colleagues and by us, is that animals in either the "nontakers" group or "takers-not-keepers" group never achieve the colonization and Stx expression level to advance the course of pathogenesis to that of systemic intoxication. In contrast, animals in the "takers and keepers" group are likely those animals in which colonization and subsequent toxin production levels are sufficiently high for the systemic effects of disease (i.e. intoxication) to become manifest. Furthermore, the infection courses in the groups (resistant, transient, and persistent) may reflect the broad spectrum of disease seen with *E. coli* O157:H7. Some people appear like the "nontakers"; they are

exposed to the bacteria, become infected, and clear the organism with little or no disease manifestations. Others become infected, show some signs of disease, but eventually clear infection, likely without ever seeking treatment; this group of infected individuals may be similar to what we call "takers-not keepers". Finally, still other individuals become infected with *E. coli* O157:H7 and then manifest symptoms that are severe enough that they (or their caretakers) seek treatment. These *E. coli* O157:H7-infected persons are often hospitalized and some develop HUS. The increased risk of severe outcome of infection in this latter category of individuals is comparable to the highly and persistently infected "takers and keepers" group of *E. coli* O157:H7-challenged ICF mice.

In our studies, not every member of the "takers and keepers" succumbed to infection even though the mice were persistently colonized with *E. coli* O157:H7. Perhaps, as is seen in people, there are those mice in this group that act as shedders; they are colonized for prolonged periods and represent a source of re-infection for others in the group. Furthermore, even among animals that receive a similar inoculum, we often observed a considerable spread in colonization levels after initial *E. coli* O157:H7 infection (the "take"). In fact, there were instances where some animals never really became colonized by the challenge organism (i.e. the inoculum did not "take") and cleared infection in a day or two. Variable "takes" seem to be a part of all experiments in these and other studies in mice with a normal commensal flora (and probably in humans as well). The explanation for disparities in individual animal up-take of *E. coli* O157:H7 is not clear. The mice that we used were genetically homogeneous (i.e. inbred), exposed to the same environment, and given the same feed. Nevertheless, there may still have been differences in the intestinal microbiota of the animals (as discussed in *Effects on*

Pathogen Colonization and Disease and *Competition for Nutrients*), the amount of food

they ingested (11, 13), or their hormone levels (7) that may have altered the efficiency

with which *E. coli* O157:H7 survived, multiplied, and/or competed for establishment in

their gastrointestinal tracts. Furthermore, these same factors may play a role in

determining who, when infected with *E. coli* O157:H7, becomes asymptomatically

colonized, develops mild diarrheal disease, or presents with HC or even HUS

Methodologies for Inoculation

The studies described in this dissertation applied either of two techniques to

introduce *E. coli* O157:H7 orally into mice: pipette feeding or intragastric administration

(gavage).   We evaluated the effect of inoculum delivery method on *E. coli* O157:H7

colonization and found that similar levels of colonization were achieved by oral pipette

feeding or intragastric administration of the bacterium at the highest inoculum (10^9 CFU)

tested. We concluded that either approach could be used for infection studies with

similar results. While the method we selected for *E. coli* O157:H7 challenge in a

particular experiment was largely dictated by the question under study, overall we

dispensed the organism by gavage more often than by pipette feeding. Each method of

inoculation has its advantages and disadvantages as are discussed below.

Intragastric administration, or gavage, is a quick and easy method of oral infection

that dispenses the desired dose of the microbe to the intestines more directly than does

feeding the suspension of bacteria by pipette tip. Additionally, this technique permits the

oral delivery of a larger bulk of inoculum. However, if done improperly, gavage can

result in death of the mouse by asphyxiation or introduction of the bacteria into the

bloodstream. As this methodology results in the administration of the bacteria into the stomach, it circumvents the requirement for the bacterium to transit through the esophagus. For that reason, we elected to use the pipette feeding method of oral infection for the evaluation of the passage of the organism through the gastrointestinal tract as well as for the assessment of intimin and Stx2 expression by *E. coli* O157:H7 over time. This inoculum delivery approach provides a more natural route of infection than does gavage in that the microbe must transit through the esophagus prior to entry into the stomach. Moreover, oral infection by pipette feeding is of little risk to the animals. One major drawback of this pipette feeding technique is that only a small volume of material can be administered to a mouse at a time, a restriction that in some instances necessitated the administration of half-doses to the mice two hours apart. Likely as a result of this need to dose the animals twice and our inability to ensure that the entire inoculum was orally delivered (as opposed to spit out by the mouse), this technique sometimes resulted in a less consistent inoculum among animals within a group compared to mice within a group given the organism by gavage.

Virulence Factor Expression

Shiga toxin and the LEE-encoded factors are considered to be the major virulence factors of *E. coli* O157:H7, with Stx responsible for the development disease and the LEE vital to colonization. Therefore, much research has focused on the expression and regulation of these factors/genes [(47, 63), reviewed in (48)]. However, most of the data in the literature that describe expression of these STEC virulence factors have been derived from experiments done *in vitro* (cell culture, IVOC). That these *E. coli* O157:H7

proteins are expressed *in vivo* is indicated by their reactivity in immunoblots when probed with human convalescent sera (35, 38). Little information has been published on the temporal course of STEC pathogenesis and even fewer studies have addressed whether current paradigms about the pathogenic process of *E. coli* O157:H7 and other STEC infections that are based on experiments done *in vitro* are actually valid *in vivo*. Thus, one goal of the published report presented in chapter 2 was to expand the body of knowledge on *E. coli* O157:H7 pathogenesis *in vivo* with a specific focus on the site of colonization and the timing of expression of toxin and intimin by the organism.

A number of studies have described the temporal expression of Shiga toxin in various animal models (18, 34, 42, 72, 87, 92), although a few have focused on the detection of Stx at a single time-point *in vivo* (1). Moreover, there are inherent difficulties in the applicability of the findings from these investigations to STEC infections in humans because many of them used axenic or antibiotic- treated animals or applied such harsh conditions as prolonged protein restriction to promote colonization or mitomycin C injection to facilitate Stx expression (1, 34, 42, 72, 87, 92). While the expression, temporal or otherwise, of Stx has been investigated to some degree *in vivo*, data on the expression of LEE-encoded factors are lacking. Immunohistological studies done by Sinclair *et al*. revealed the presence of Tir beneath adherent *E. coli* O157:H7 in the intestines of a mouse, calf, and pig on day 2 or 3 post-infection (75); however, intimin was not detected in these tissues. Therefore, our study (49) was not only the first to describe the measurement of toxin expression over time at specific sites in an animal early after *E. coli* O157:H7 infection but also the first to report detection of the expression of intimin *in vivo*.

Relationship to Disease

Our detection of Stx2 production early during the course of *E. coli* O157:H7 infection when intimin was also measurable lends support to a model of colonization that involves the role of toxin in the initial steps of *E. coli* O157:H7 colonization of the gastrointestinal mucosa (Figure 21). Sinclair *et al.* used a conventional mouse model to demonstrate the necessity for intimin in colonization by *E. coli* O157:H7 (75). Additionally, Sinclair *et al.* demonstrated by immunostaining that *E. coli* O157:H7 and nucleolin (as well as β1 integrin) localized in close proximity to each other in tissue sections from infected mice (str-treated to attain sufficient levels of bacteria for detection), pigs, and calves. Furthermore, Robison *et al.* demonstrated that *in vitro* exposure of HEp-2 cells to Stx2 increased nucleolin expression and subsequently the amount of *E. coli* O157:H7 that adhered to the cells (62). That both intimin and Stx2 are detected early in the pathogenic process also suggests that toxin production may precede *E. coli* O157:H7 colonization because once the bacteria are adherent to cells intimin cannot be detected either *in vitro* or *in vivo* (75, 76).

Site of Colonization and Production of Stx2

In our ICF mouse model of *E. coli* O157:H7 infection, as well as in the model reported by Nagano *et al.*, the cecum was the primary site of colonization by the microbe. Furthermore, in our study, Stx2 was most readily detected in the contents of the cecum and the large intestines with the highest levels found in the material contained in the large intestines. In people, the majority of the gastrointestinal damage that occurs during *E.*

coli O157:H7 infection is observed in the lower bowel, specifically the right side [cecum, ascending colon, and transverse colon (33, 39, 60)]. However, Chong *et al.* and Phillips *et al.* proposed that this colonic damage associated with *E. coli* O157:H7 infection results from the presence of Stx in the colon in the absence of attached bacteria (15, 58). This theory of toxin-mediated damage to the colon without evidence of *E. coli* O157:H7 mucosal colonization is supported by the histopathological findings of Kelly *et al.* (39). Our data would also favor this notion. In fact, we hypothesize that *E. coli* O157:H7 that are adherent to the epithelium in the region of the ileocecal junction (the cecum in the case of mice) release toxin into the luminal contents. Stx then travels to the large intestines where, in humans, it can cause destruction of the colonic epithelium (hemorrhage, ulceration, edema).

Toxin-Mediated Systemic Disease

The ICF mouse model permitted us to evaluate both colonization of *E. coli* O157:H7 following oral infection and any ensuing intoxication. Because the mice had an ICF that generally restricted growth and colonization by challenge *E. coli* O157:H7 organisms, we were surprised to find that some of the mice exhibited signs of disease, i.e. lethargy and weight loss. Indeed, a decrease in body weight, compared with the controls, was indicative of a poor disease outcome. Specifically, *E. coli* O157:H7-infected animals generally lost weight and demonstrated other systemic changes such as increased BUN and creatinine levels and immune cell modulation (change in % neutrophils and % lymphocytes). Moreover, a portion of the animals that lost weight ultimately succumbed

to infection. Certainly a drop in body mass would result in an increase in the overall concentration of toxin (amount per g of animal weight).

In chapter 2 we described our ability to detect: 1.) toxin production at the site of *E. coli* O157:H7 colonization; 2.) minute amounts of toxin in blood of a small number of infected mice; 3.) toxin in the kidneys of a moribund animal in one instance, and; 4.) renal tubular damage in infected animals but not controls. When taken together, these findings suggest that the death of infected animals resulted from toxin produced by bacteria at the site of infection in the gastrointestinal tract and then trafficked to the kidneys. In chapter 3, we provided further proof that the systemic manifestations of *E. coli* O157:H7 infection, including mortality, occurred as a consequence of the effects of toxin by the demonstration that antibody against Stx2 reduced or eliminated the symptoms of disease. Specifically, in the passive transfer studies described in chapter 3, animals that received anti-Stx2 antibody had significantly higher weights post-infection than did animals given NRS. Furthermore, infected animals that received NRS exhibited severe morbidity, tended to have the highest colonization levels, and were much more likely to succumb to infection than those mice administered anti-Stx2 serum. We propose that the high levels of *E. coli* O157:H7 colonization in mice that received NRS likely resulted in the robust production of Stx2 that, without antibody to neutralize its effects, probably contributed to the morbidity and mortality seen in this group (further discussed in *Colonization Levels*).

The Role of Shiga Toxin in Colonization

Our laboratory was among the first of several groups to ascribe a role for Shiga toxin in the adherence and/or colonization of *E. coli* O157:H7 (62). However, there are other investigators who have reported that Stx has no effect on the colonization of *E. coli* O157:H7. While the findings of these researchers are mentioned in the discussion of chapter 3, we extend our analyses of their reports below.

Conflicting Evidence

The number of reports that attribute a role to toxin in the colonization process of *E. coli* O157:H7 has increased in the last two years (5, 12, 22, 45, 91), nevertheless, other publications have refuted such an effect of toxin (10, 17, 25, 61, 71). However, in many of the studies that found no evidence for a role of Stx in colonization, the authors' made use of manipulated model systems (25, 71) or based their conclusions on either a single time-point post-infection (61) or a limited temporal analysis (17, 25). Furthermore, some investigations utilized animal model systems that do not reflect the normal environment in which the challenge STEC interacts with the gastrointestinal tract (25, 71). A study done by Ritchie and colleagues in 2003 explored the contribution of Stx2 to *E. coli* O157:H7 colonization and disease in infant rabbits (61). While Stx2 played a role in disease development and inflammation, the authors found no effect of toxin on intestinal colonization. However, their comparative colonization analyses of Stx2-expressing and non-expressing isogenic *E. coli* O157:H7 strains were limited to day 7 post-infection; thus, these investigators may have missed a point in infection where Stx2 facilitated colonization (61).

In another study, done by Best *et al.*, insufficient data were presented to support

the authors' generalizations about the role of Stx in intestinal adherence of *E. coli*

O157:H7 (10). Best and colleagues investigated the capacity of a toxin-negative *E. coli*

O157:H7 strain to colonize the ceca of chickens.   Because the animals were persistently

colonized by the toxin-negative *E. coli* O157:H7 strain, the authors concluded that Shiga

toxin does not have a significant role in mediating gastrointestinal colonization of

chicken. Furthermore, they postulated that "LEE encoded mechanisms of colonisation

may not play a significant role in colonisation in this model" (10). Although the

investigators' may have been correct, they only infected the chickens with an Stx-

negative O157 strain. Their exclusive evaluation of a toxin-negative strain did not permit

a direct comparison of colonization levels with an Stx^{+} strain that may well have

colonized to a higher level than was seen with the toxin mutant. Thus, their contention

that Stxs produced by the organism must not play a significant role in colonization of the

gastrointestinal tract in this model seems overstated. Additionally, without directly

testing for a role of LEE-encoded factors, such as intimin and Tir, in colonization the

authors' conclusions regarding the role of LEE products in colonization is unfounded.

Sheng *et al.* conducted a study exploring the colonization of *E. coli* O157:H7 or a

toxin mutant in cattle. Sheng *et al.* found that in a model involving the direct application

of *E. coli* O157:H7 or the toxin mutant to the recto-anal junction, Stx did not promote

colonization of *E. coli* O157:H7 (71). However, that infection portal does not take into

account any role toxin may play in survival, passage through, and persistence in the

gastrointestinal tract of cattle before reaching the recto-anal junction. Furthermore,

sampling was done at limited time intervals (days 1, 4, 7, 11, 18, 25, and 32). We have

seen that effects of toxin may occur in a brief period of time, which may have been missed in the analysis done by Sheng and colleagues. Before any positive impact of Stx on colonization of *E. coli* O157:H7 in this model can be ruled out, we contend that samples should be obtained daily for colony counts from days 1 through 11 of infection.

In a study performed by Cornick *et al.* they concluded that Stx does not facilitate colonization of sheep by *E. coli* O157:H7; however, like Sheng e*t al.* (71), Cornick and colleagues did not collect daily samples for analyses (17). Instead they look only early (combining data from days 2, 3, and 4), 2 weeks (days 13, 14, and 15), 1 month (days 28, 29, and 30), and 2 months (days 58, 59, and 60) post-infection. By combining results from 3 separate days and taking so few samples, they likely missed periods when Stx may have provided a benefit in *E. coli* O157:H7 colonization. In fact, early following infection both the Stx-negative and phage-negative strain numbers were reduced compared to the WT, although these differences were apparently not statistically significant. Perhaps if daily colony counts had been obtained and analyzed, the subtle effect toxin can have in augmenting *E. coli* O157:H7 colonization may have been revealed. Furthermore, the use of animals not tested for resident *E. coli* O157:H7 precludes a definitive conclusion about the impact of Stx on colonization because resident *E. coli* O157:H7 could provide the benefit of toxin *in trans*. Certainly sheep have been documented to carry *E. coli* O157:H7 and other STEC strains (40, 41).

Finally, most recently, Eaton *et al.* explored the role of toxin in colonization of germ-free mice by *E. coli* O157:H7. They found no difference in the levels of colonization between 86-24 and 86-24Δstx_2 and between EDL933 and EDL933Δstx_2 at 1 week post-infection; in all instances the colonization achieved in germ-free mice was

greater than 10^{10} CFU/g feces. Thus, germ-free mice appear to be exquisitely susceptible to *E. coli* O157:H7 infection, as well as infection with bacteria in general (81). The application of this germ-free model to analysis of the impact of Stx on colonization of *E. coli* O157:H7 fails to address the possibility that Stx provides a competitive advantage in the presence of a normal intestinal microbiota.

Accessory Role for Stx2

Intimin is the major adhesin molecule of *E. coli* O157:H7 (see introduction for detail on this protein and its role in virulence of *E. coli* O157:H7). Indeed, the presence of antibody to this outer membrane protein in the gastrointestinal tract reduces the duration of *E. coli* O157:H7 shedding in conventional mice and significantly diminishes the level of colonization and ensuing disease in piglets that suckle from intimin-immunized dams (21, 36). While the administration of antibody to Stx prevents the toxin-mediated systemic manifestations of *E. coli* O157:H7 disease, anti-Stx antibody is less effective than anti-intimin antibody at reducing colonization by the organism. This observation is likely a result of the more subtle effect of toxin in *E. coli* O157:H7 colonization. Unlike intimin-negative strains of *E. coli* O157:H7, toxin-negative strains can colonize the gastrointestinal tract in different animal models, albeit at lower levels than for wild-type toxigenic strains. Therefore, the role of toxin in colonization is ancillary to that of intimin. Nevertheless, based on our findings in the ICF murine model, we postulate that in conventional animals (or people) infected orally with wild-type *E. coli* O157:H7, Stx acts in concert with intimin to facilitate higher level, more persistent

colonization of the gastrointestinal tract than would be attained by a toxin-negative strain of the microbe.

Our work also showed that the impact of toxin on *E. coli* O157:H7 colonization in mice was often only apparent for a limited period of time and the timing of that window correlated with the overall load of challenge organisms shed by the animals. Thus, we found that the higher the initial colonization levels of *E. coli* O157:H7 (as reflected by the CFU/g feces), the more delayed the appearance of the effect of toxin. We speculate that the accessory role of toxin is precluded or masked in these instances. Thus, we contend that it is necessary to monitor fecal shedding of an Stx-producing strain and its Stx mutant daily or at least frequently to ensure that the relatively subtle influence of toxin on levels of colonization (compared to the impact of intimin on bacterial load and persistence) is apparent.

Toxin and Colonization in other Enteropathogens

In a review by Cover and Blanke, the authors suggested that most bacterial protein toxins likely contribute to the capacity of the microbe to colonize the host (19). However, few reports that assign this function to bacterial toxins (other than Stx) can be found in the literature. The findings from these relatively recent publications are described in detail below.

Labile Toxin of Enterotoxigenic E. coli

The current paradigm for the pathogenesis of enterotoxigenic *E. coli* (ETEC) is that ETEC colonize the small intestine by way of fimbrial adhesins known as

colonization factors (or CFs). The ETEC then elaborate enterotoxins, including heat-

labile toxin (LT) or heat-stable toxin (ST), that are considered to be responsible for the

profuse watery diarrhea associated with ETEC infection. However, Berberov and

colleagues demonstrated that LT not only promoted disease but colonization as well

(similar to Stx2) in a gnotobiotic piglet model (8). These investigators reported that

animals infected with the WT ETEC strain, when compared to animals inoculated with its

LT-negative derivative, showed increased weight loss, higher rates of development of

dehydration, and more frequent occurrences of intestinal ischemia, bacterial

translocation, and secondary septicemia. Contrary to what we observed with an Stx$^-$

strain of *E. coli* O157:H7, animals infected with the LT$^-$ strain still manifested diarrhea,

severe weight loss, and became moribund. Most importantly to the discussion here, LT

activity appeared to enhance bacterial colonization of the small intestine by ETEC;

animals infected with the LT$^-$ strain demonstrated decreased levels of colonization, as

assessed by both ileal tissue plate counts and histological scoring of bacteria adherent in

the ileum (and jejunum).

In a more recent study, Allen *et al.* developed an immunocompetent, outbred

mouse (CD-1) model of ETEC colonization (absence of disease symptoms) and reported

that LT production and secretion facilitated ETEC colonization (2). Not only did a toxin

mutant (which contained a deletion in the A subunit) colonize the mice at a level 10-fold

lower than WT, but this toxin mutant was also outcompeted by WT in a competition

experiment. In addition, a toxin competent, secretion incompetent mutant also displayed

reduced fitness for colonization when in competition with WT. The finding that WT

outcompeted the toxin mutant in a competitive infection suggests that, contrary to what

Robinson *et al*. found with Stx2 (62), the colonization deficiency in the LT mutant of

ETEC cannot be complemented *in trans*.

While the mechanism for the capacity of LT to increase colonization of ETEC has

not been investigated in publications to date, several theories to explain the finding have

been posited. One such explanation is that LT may evoke host cell surface changes that

facilitate colonization, perhaps by increasing the expression of a receptor or by resulting

in beneficial actin cytoskeletal changes (2, 8). While LT may serve directly as an

adhesin, this possibility is considered unlikely (8). An alternative suggestion is that LT

can alter the host's response to the challenge microorganism so as to be more beneficial

for ETEC colonization (2). A final hypothesis as to why LT increases ETEC colonization

is that LT may function to alter intestinal function (i.e. motility, secretion of various

factors) in favor of bacterial colonization and growth (8).

Accessory Toxins of Vibrio cholerae

Vibrio cholerae El Tor (hemolytic O1 biotype), in comparison to classical cholera

strains, makes three additional secreted toxins: multifunctional autoprocessing RTX toxin

(MARTX), HA/protease, and hemolysin (57). Oliver *et al*. showed that an El Tor strain

that did not express these toxins was defective in its capacity to initially colonize the

small intestines of adult, anesthesia- and str-treated mice and to persistently colonize

(past 24 hours) the distal small intestines when compared to the wild-type El-Tor strain

(57). This colonization deficient phenotype was apparent in the absence of cholera toxin

(CT) production, the latter of which was shown to have minimal, if any, role in

colonization of these mice. The capacity to complement the accessory toxin mutant's

defect by coinfecting with a strain capable of accessory toxin production implied that

rather than directly affecting the bacterium, the secreted toxins modulate the local host

environment to promote persistent colonization by the bacterium (57). To extend this

finding that a multitoxin mutant strain of El Tor showed a defect in persistent

colonization of mice, Olivier *et al.* investigated the impact of single deletions of the

accessory toxin genes in the absence of CT production on colonization of adult,

anesthesia-treated (ketamine-xylazine) mice (56). These researchers reported that

hemolysin and MARTX toxin single mutants displayed reductions in colonizing

frequency and that a double mutant defective for production of both toxins exhibited a

drastic colonization deficiency. However, none of the toxins were found to be essential

for *Vibrio* colonization (56). That hemolysin and MARTX are capable of increasing the

colonization capacity of *V. cholerae* but are not required for colonization is similar to our

findings with Stx2.

While the exact mechanism by which the accessory toxins function to prolong

colonization remains unclear, several possibilities were proposed. First, Olivier *et al.*

suggested that the accessory toxins might function directly as adhesin molecules;

however, the authors considered this explanation unlikely given the capacity to

complement the deficiency in colonization by providing the accessory toxins *in trans*

(57). The second possibility posited by Olivier and colleagues was that the accessory

toxins might play an indirect role in adhesion of *V. cholerae* El Tor by mediating

localized intestinal cell death and thereby facilitating the exposure of a eukaryotic cell

receptor for adherence (57). In support of this possibility, the authors found sloughed

cells (albeit of unidentified origin) in the intestinal lumen of mice infected with strains

that expressed the accessory toxins (57). The third and final explanation put forth by these investigators for the increased colonization and persistence of *V. cholerae* evoked by accessory toxin expression was that the toxins may target immune cells at the site of infection that would normally function to diminish bacterial numbers (56, 57).

VacA Toxin of Helicobacter pylori

All strains of *Helicobacter pylori* isolated from humans that have been examined contain the gene for the vacuolating cytotoxin (VacA). This correlation suggests a role for VacA in the pathogenesis of *H. pylori* infections, although early studies failed to uncover precise mechanisms by which VacA might contribute to infection and disease (66). However, in 2001 Salama *et al.* demonstrated that in a mixed infection of mice, the WT *H. pylori* outcompeted the *vacA*-null mutant and, additionally, the mutant had an increased ID_{50} (66). The two strains demonstrated no differences in terms of localization and neither strain resulted in inflammation. Furthermore, the *vacA*-null mutant was outcompeted in the first few days following infection, an observation that suggests that the mutant was truly deficient in its capacity to initially colonize.

The mechanism by which VacA can facilitate the initial colonization of *H. pylori* in a mouse model is unknown. VacA has been ascribed immunosuppressive capabilities, attributes that could support the persistence of *H. pylori* at the site of infection (19). However, the lack of inflammation noted by Salama and colleagues indicates that it is unlikely that VacA is facilitating immunomodulation, at least in this mouse model (66). Another proposed mechanism by which VacA may support *H. pylori* colonization involves changes in the local environment in response to the cytotoxic activity of VacA

on the gastric epithelium. For example, such VacA-mediated destructive activity could result in host cell turnover that might present a new cell type to which *H. pylori* could adhere or, alternatively, the tissue damage might permit the survival of *H. pylori* by releasing important nutrients or inhibiting acid secretion. Finally, the demonstration that *vacA*-null mutants are capable of colonizing in various models of *Helicobacter pylori* infection (24, 89) suggests that VacA is not absolutely required for colonization; this subtle role for VacA in colonization is similar to that described herein for Stx2. That differences in colonization between wild-type *H. pylori* and a *vacA*-negative mutant were only evident in competition studies suggests that, unlike what we observe for Stx2, very little if any complementation *in trans* occurs.

CDT Toxin of Clostridium difficile

   Schwan *et al.* explored the early effects of CDT from *Clostridium difficile* (as well as other pathogenic *Clostridium*) on gastrointestinal cells *in vitro* (68). CDT is a member of the actin-modifying ADP-ribosylating toxins that act on G-actin to inhibit the polymerization of actin. On prolonged exposure of Caco-2 cells to CDT in the study by Schwann et al, the cells shrank and became rounded. In a morphological analysis of cells exposed to CDT for shorter periods of time, the appearance of cellular protrusions was noted. These protrusions proved to be dynamic (growing/changing) microtubules. The induction of the cellular protrusions by CDT resulted in increased adherence of *C. difficile* to the Caco-2 cells.

   Most importantly, in a gnotobiotic mouse model there were differences in colonization of *C. difficile* in the presence and absence of antibodies that could neutralize

CDT. Specifically, animals colonized by a hypervirulent strain of *C. difficile* (and treated with control serum) had significantly higher levels of colonization and more bacteria appeared to be adhered to the cecal and colonic mucosa than in mice that received anti-toxin (CDT-neutralizing anti-iota toxin serum, a related ADP-ribosylating toxin) antibody. Furthermore, administration of anti-iota toxin to *C. difficile* infected animals resulted in a reduction of the manifestation of disease symptoms. However, in the case of *C. difficile*-infected animals treated with anti-iota toxin antibody, these symptoms included diarrhea; macroscopic examination of the intestines on necropsy revealed colitis and inflammation of the cecum. The authors concluded that at least one major function of CDT was to promote adherence to epithelial cells and thus increase colonization of the pathogen *in vivo*. That the *in vitro* effects mediated by CDT of *C. difficile* are shared by other members of the *Clostridium* toxin family suggests that the toxin-induced formation of cellular protrusions may be a common mechanism by which these toxins facilitate adherence (68).

The Effectiveness of Passively Administered Antibody

As anticipated, anti-Stx2 neutralizing antibody provided protection against the systemic manifestations of *E. coli* O157:H7 disease in our ICF murine model. This observation is consistent with reports from various animal modeling systems of the effectiveness of both pre-infection and post-infection administration of anti-Stx antibody (discussed in chapter 1). In addition, we discovered that when anti-Stx2 antibody was administered intraperitoneally prior to *E. coli* O157:H7 infection, the likelihood that a mouse would become highly colonized with *E. coli* O157:H7 decreased. To the best of

our knowledge, this latter observation about the impact on *E. coli* O157:H7 colonization of passive administered anti-Stx antibodies is a novel finding.

Antibody Detection in the GI Tract

We observed that when anti-Stx2 antibodies were administered parenterally to mice, we could detect the antibodies shed in the gastrointestinal tract. Similarly, we found anti-Stx2 neutralizing activity in the feces of mice following their development of high-titered serum responses against toxin elicited by repeated parenteral toxoid immunization. We are uncertain about the immunoglobulin type that was responsible for the reduction in *E. coli* O157:H7 colonization seen and, furthermore, we only monitored for the shedding of anti-Stx2 IgG in our passive experiments. Although IgA and IgM can be directly transported across the epithelium into the lumen of the bowel by the polymeric immunoglobulin receptor (pIgR) [reviewed in (64)], supposedly IgG cannot. So then how do we explain the appearance of anti-Stx2 IgG in the feces of mice following parenteral administration of antibody?

We believe that in our passive studies, parenteral administration of high-titer, neutralizing polyclonal antisera resulted in spillover from the circulatory system into the intestinal lumen. While this may have been aided by some heretofore unobserved *E. coli* O157:H7-induced damage to the intestinal epithelium of mice, our ability to detect parenterally administered antibody in the feces of uninfected animals suggests that this transit into the lumen can occur even in the presence of an intact intestinal epithelium. One explanation for this "spillover" of serum antibody into the lumen of the bowel relates to the rate and possible outcome of IgG turnover. The rate of IgG turnover is considered

to increase as the levels of serum IgG rise (64). The consequences of this turnover that

occurs in endothelial cells are that the IgG can be recycled back into the circulation,

degraded by lysosomal action within the cells, or released into luminal secretions (such

as in the intestine). We theorize that in our passive transfer of anti-Stx2 antibody studies,

the presence of an excess amount of circulating antibody likely increased the rate of IgG

turnover and subsequently led to the transport of anti-Stx2 IgG into the intestinal lumen

for clearance. Furthermore, we speculate that in the animals challenged with *E. coli*

O157:H7, the anti-Stx2 antibody that was transported into the gut lumen bound Stx2 (and

precluded the effect of the toxin on colonization and disease) before clearance of the IgG

could occur. This observation that parentally administered IgG antibodies can be

detected in the gut is not a novel finding in that such results were reported in a passive

immunization study designed to block rotavirus (88) infection in the gastrointestinal tract

and in an investigation intended to neutralize orally-administered, Stx-related, ribosome-

inactivating toxin, ricin (46).

Toxoid Vaccine Candidate

The dissertation herein describes the effectiveness of anti-Shiga toxin antibodies

(both passively administered and actively generated via toxoid immunization) in the gut

on reducing *E. coli* O157:H7 colonization and in the blood stream on protecting against

systemic disease manifestations. Animals immunized with an Stx2 toxoid displayed both

lower *E. coli* O157:H7 colonization levels and a less prolonged infection. These findings

are similar to those reported by Zhu *et al*. (93). These investigators demonstrated a

reduction in the numbers of enteroadherent Stx1-producing *E. coli* in an RDEC rabbit

model of infection following transcutaneous immunization against Stx1B (93). The

potential utility of anti-Shiga toxin antibodies in reducing the STEC burden and duration

of infection in conjunction with the recognized capacity of such antibodies to prevent

toxin-mediated systemic disease makes the inclusion of an Stx toxoid component in an *E.*

coli O157:H7 vaccine doubly beneficial.

Suggested Future Directions

Expanding the ICF Mouse Model

The ICF mouse model of *E. coli* O157:H7 infection has thus far been used to evaluate *E. coli* O157:H7 colonization levels (as assessed by fecal shedding) and the systemic manifestations of *E. coli* O157:H7-mediated disease (weight loss, morbidity, and mortality). While bloody diarrhea was not an obvious outcome in this model, we have yet to investigate the histopathology of the gastrointestinal tract to ascertain whether intestinal damage, as is reported in people, is evident in infected animals. In our initial description of *E. coli* O157:H7 pathogenesis in the ICF model, we reported renal tubular damage in infected animals. We did not look for histopathological evidence of CNS involvement in the *E. coli* O157:H7-infected mice. Nevertheless, neurological manifestations were evident in some of the infected animals; these observations suggest that CNS damage did in fact occur. Thus, I would propose a more complete necropsy of the infected animals to investigate the potential for intestinal and CNS damage.

The kidney damage we reported after *E. coli* O157:H7 infection is only one facet of the triad of clinical findings associated with HUS, i.e. microangiopathic hemolytic anemia, thrombocytopenia, and renal damage. While we found histopathological evidence of renal damage in *E. coli* O157:H7-infected animals and observed that the mice also had increased levels of BUN and creatinine that further indicated renal dysfunction, the infected animals did not display either hemolytic anemia or thrombocytopenia. Moreover, our hematological analyses of blood from the infected animals, revealed some evidence of hemoconcentration (i.e.an increase in hematocrit values), a feature of STEC infection of mice that has been previously reported (25, 44).

The finding of hemoconcentration is likely a consequence of dehydration resulting in a decreased plasma volume; however, hemoconcentration can obscure the assessment for anemia in the infected animals. Additionally, while the increases in BUN and creatinine are likely attributable to the renal damage seen in the kidneys of infected animals, I would propose further studies to confirm the observation of renal damage and to determine its physiological basis.

To determine the cause of the observed dehydration, which may or may not be related to the renal damage, I would suggest studies designed to: 1.) monitor the animals' fluid intake, and; 2.) seek evidence of vascular leakage by gross macroscopic examination of the lungs. Although we have previously been unsuccessful at rehydration therapy as a way to ameliorate hemonconcentration that may mask anemia, another attempt at such therapy might be worthwhile. Peripheral blood smears to assess for the presence of schistocytes is another methodology to provide evidence of microangiopathic hemolysis; additionally, analyses of bilirubin levels and haptoglobin would provide information regarding red blood cell lysis. As a means of monitoring for intravascular clot formation and breakdown, D-dimers, fibrin degradation products, and PTT could be evaluated. Finally, while we have already shown evidence of renal failure (as described above), the use of urine test strips to assess for the presence of protein and red blood cells in the urine would be indicative of glomerulonephritis.

Function of Commensals in E. coli *O157:H7 Colonization*

One important difference between the str-treated model of STEC infection previously adapted in our laboratory (86) from a model developed by Myhal *et al*. (53)

and the ICF model described in Chapter 2 is the relative virulence associated with *E. coli* O157:H7 infection. Thus, str-treated animals administered a high dose of Stx2-expressing *E. coli* O157:H7 shed very high levels (~10^7CFU/g feces usually) of the challenge strain for weeks yet few animals ever became moribund or died (86). While the reason for this apparently contradictory result (higher levels of colonization, lower systemic illness) remains unclear, the enhanced virulence of *E. coli* O157:H7in ICF mice compared to str-treated animals may imply a role for commensals in the pathogenesis of the microbe.   To further explore differences between the str-treated and ICF models, I suggest as a first step that the numbers of *E. coli* O157:H7 at different intestinal sites over time after infection in str-treated mice be monitored. Next, I suggest that the expression patterns of primary *E. coli* O157:H7 virulence factors, specifically toxin, in those animals be determined. A comparison between the kinetics of virulence factor expression by *E coli* O157:H7 may shed light onto the reason/s for the enhanced virulence of *E. coli* O157:H7 seen in the ICF model when compared to the str-treated model of infection.

To further investigate the role of commensals in the pathogenesis of *E. coli* O157:H7 in the ICF model, I would propose studies to investigate the composition of the intestinal microbiota of BALB/c ICF mice. Specifically, an analysis of the any differences among the commensal population of animals that seem resistant to infection and those that are highly infected may reveal the difference between a "taker" and a "non-taker". Also, one could evaluate the changes (if any) that occur in the commensal flora population in response to *E. coli* O157:H7 infection.

Mechanism Linking Stx2 and Colonization

Sinclair *et al*. showed that nucleolin localized with *E. coli* O157:H7 and Tir in immunostained intestinal sections from *E. coli* O157:H7-infected animals (75). These investigators provided the first data from an *in vivo* study in support of a role for nucleolin in *E. coli* O157:H7 attachment to the gut. However, in that report, the authors were unable to see any co-localization of intimin with nucleolin. This failure to demonstrate such an intimin:nucleolin association may be more reflective of the technical difficulties inherent in visualizing intimin in tissues [or even in *E. coli* O157:H7-infected cell cultures (76)] rather than of the absence of an interaction between the bacterial and eukaryotic cell proteins. Also, tissue sections provide just one snap shot of an infectious process. As Sinclair and colleagues have proposed that intimin of *E. coli* O157:H7 only binds nucleolin as an initial step in infection and then rapidly engages Tir (76), the window when one might detect the co-localization of intimin and nucleolin *in vivo* is likely quite narrow. Currently, we are attempting to demonstrate a link between toxin expression and increased cell surface levels of nucleolin *in vivo*. For that purpose, I had previously immunostained cecal tissue sections from *E. coli* O157:H7-infected ICF mice for nucleolin. I had also subjected cecal homogenates from conventional mice fed large, but sublethal, doses of toxin to Western blot analysis to detect nucleolin. I was unsuccessful at both of these attempts to assess for nucleolin in tissues.

In addition to attempting to reveal a connection between Stx2, colonization, and nucleolin *in vivo*, we are working to describe the mechanism responsible for the increase in cell surface-associated nucleolin that occurs after Stx treatment of cells *in vitro*. In initial efforts to dissect this mechanism, Dr. Cory Robinson demonstrated a reduction in

the toxin-evoked, cell surface adherence of the *stx2* mutant strain by treatment of

intoxicated cells with a small molecule inhibitor of ERK [extracellular signal related

kinase (C. Robinson, unpublished data)], a member of the MAP kinase family that is

involved in signaling for initiation of an apoptotic response. As a next step, I would

suggest that those data be confirmed and then extended to determine the effect of this

inhibitor, as well as others, on nucleolin expression and/or localization. Additionally, and

perhaps more directly relevant, I would deem it important to measure the surface

expression level of nucleolin and to determine the kinetics of nucleolin surface

expression in response to toxin. While initial studies focused on determining the

response of HEp-2 cells to intoxication, I would suggest that this investigation be

expanded to the more relevant HCT-8 cell line. In fact, preliminary data generated by Dr.

Cory Robinson indicated that not only did toxin increase the adherence of *E. coli*

O157:H7 to HCT-8 cells, but, additionally, toxin treatment resulted in increases in

nucleolin expression albeit with a delayed kinetic profile (C. Robinson, unpublished

data).

While we believe that the mechanism by which Stx2 enhances colonization *in*

vivo requires fully enzymatically active toxin based on the findings of Robinson *et al*. *in*

vitro (62), this presumption needs to be confirmed. Additionally, we currently do not

know whether Gb_3 is necessary for the effects of toxin on colonization either *in vitro* or *in*

vivo. Dr. Steve Zumbrun in our laboratory is working to create stable Gb_3- and Gb_4-

knockdown cell lines. I would propose that the Gb_3-knockdown cells be used to

investigate whether Gb_3 expression by the host cell is required for the Stx2-promoted

enhancement of *E. coli* O157:H7 adherence.

Role of Stx1 in Colonization and Pathogenesis

My efforts and those of Robinson and colleagues in our group focused on evaluation of the impact of Stx2 on *E. coli* O157:H7 colonization *in vitro* and *in vivo*. This serogroup of Stx was selected to address the role of toxin in *E. coli* O157:H7 colonization because it is considered the more relevant toxin in terms of serious disease. However, a current idea in our laboratory is that while Stx2 is more frequently linked to systemic manifestations of *E. coli* O157:H7 infection in humans than is Stx1, Stx1 may be responsible for the more localized effects of disease and may in fact have a more pronounced impact on colonization than does Stx2. I conducted some preliminary experiments to test this concept. I used an *stx₂* mutant of *E. coli* O157:H7 strain 86-24 (note that the WT strain only makes Stx2) in a flow cytometeric-based adherence assay. When I provided either Stx1 or Stx2 *in trans*, I found that Stx1 exerted a much larger cytotoxic effect on HEp-2 cells than did Stx2; this observation is consistent with the higher specific cytotoxic activity against Vero cells (CD50 per mg toxin) reported for purified Stx1 compared to Stx2 (77). Although Stx1 treatment resulted in a decrease in the total numbers of viable cells and, as a result, there were fewer HEp-2 cells to which bacteria were attached, the remaining viable population of HEp-2 cells with associated bacteria showed more adherent *E. coli* O157:H7 bacteria per cell than was seen after Stx2 treatment. I speculated that Stx1 may indeed have more efficiently facilitated adherence than Stx2 but that these same Stx1-treated cells with numerous adherent bacteria were more highly intoxicated and thus more likely to undergo apoptosis. To confirm this interpretation of my findings, I would suggest that these analyses be repeated, preferably

with an stx_1 and an stx_2 mutant of an *E. coli* O157:H7 strain that naturally produces both toxins.

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