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By

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ELUCIDATING THE IN VIVO TARGETS OF BACTERIAL TOXINS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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DEDICATION

As my mom and John Donne would say, “no man is an island.” Therefore, I wouldn’t be who and where I am today without the help of many. There is no one person to whom I could dedicate this work. All of my family, friends, and colleagues at some point have helped me, encouraged me, and sacrificed for me. These people shared with me the frustrating lows and rewarding highs of graduate school and indeed, science, and to them I will be forever indebted.

ACKNOWLEDGEMENTS

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Fortunately, Jimmy is a rarity in science as he shines with a humility that belies his intellectual brilliance. His proficiency as a scientist, skills as an educator, and talent as a mentor does not surpass his witty, outgoing, and likable personality. Everyday, Jimmy and I would talk not only about research, but politics, religion, books, or what was on the “Daily show” the night before. As my friend, when I was

in the hospital, Jimmy visited me every day and was so understanding of my very slow recovery process. His concern, sympathy, and humor carried me through a very difficult time in my life. His wife (and my doctor) also deserves an acknowledgement for always taking care of me, looking out for me, and being my friend! Furthermore, Jimmy's balance between a successful career and a successful family life will always serve as a reference for my future. But most importantly, he constantly pushes me to take the harder but ultimately more rewarding path and it is because of him that I have the skills, knowledge, passion, and most importantly the confidence that will always help me in all of my endeavors, both personally and professionally. Jimmy also reminds me that, not matter my path, I am not here for myself but to instead perpetuate knowledge. For that, I will be forever in his debt. If I am even a fourth of a scientist, educator, mentor, spouse, parent, and friend that he is, I will consider myself highly successful and completely blessed.

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through my many struggles. He has always been unwavering in his support of whatever new idea or career I have dreamed up. David Nagle shares my passion for science, books, and politics. I never know if an email from him will contain research articles or an enjoyable political commentary. William Ortiz-Leduc will always serve as a teaching role model. He made class something to actually look forward to attending. Marvin Whiteley was the inspiration for the phrase: “jack of all trades.” The man knows something about everything. I would encourage him to go onto jeopardy but I would hate for the world to lose a great scientist after he became a billionaire. To my committee: Thanks for all of your support throughout my career.

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Mere words cannot express what my family has done for me but I shall try.

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My brother James is really the one who should have be in science in the first place. He always bragged about me and my accomplishments to anyone who would listen and he didn’t even have to fake enthusiasm when he listened to my latest projects and experiments. Even though he always claimed that I was adopted, I couldn’t have asked for a better brother. I love you, James. I hope I will be as supportive to you in your medical career (but it is important to know that I got to be a doctor before you…HA)!

Unlike the Beatles, I got by with A LOT of help from my friends.

To Kelli. You are one of those friends that I can truly say “I don’t know where I would be without you.” As a friend, you are a source of strength and as a colleague, a wealth of knowledge. “Thanks” is not enough. To Alison. With a friend like you, I have never needed a sister. You are always there for me in sickness and in health. Your friendship has been a blessing to my life. I love you.

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But, with co-workers like these…I never needed friends.

To Amy. I wouldn't have made it without you. You were my tutor, guide, and friend from graduate school to now. To Dan. You were so damn perfect I should have hated you. But thank god I didn't, as I would have missed out in having an amazing colleague and even better friend. You also made me realize that scientist and Christian aren't mutually exclusive. Thanks for "wild-card poker" coffee and for making me miss my brother a little less! To Kevin. From relationship advice to hospital food, you were always there for me. Thanks for letting me steal all your

buffers and for ordering things overnight! To Matt. Thanks for introducing me to new vocabulary words, Irish whiskey, NASCAR beer, and frozen Twix. Most importantly, thanks for your great advice on science and life. To Maen, who first taught me how to purify TcdB and how to address the Prince of Jordan before we met him. To Katie and Salika. Thanks putting up with me and for feeding me when I became cranky. I am glad that I haven't scared you both away from science! Thanks to Isabelle, Jason, the Whiteley lab, and the Tweten lab for dealing with me. You deserve a degree just for that.

Finally, I would also like to thank God for giving me strength and for guiding me on my many journeys.

Science for me has been a rollercoaster of negative results, eureka moments, crushing setbacks, and elating discovery. And I am all the luckier for having bought a ticket.

Thank you to everyone who helped me achieve my goal.

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CHAPTER I

INTRODUCTION

Identifying the in vivo targets of bacterial toxins using the zebrafish embryo as a model. (Elaine E. Hamm, and Jimmy D. Ballard). ***Future Microbiology.*** **(2007) 2:1 85-92.**

During infection and disease, bacterial pathogens alter host cell physiology. This alteration of the host allows the pathogen to establish niches for growth, avoid clearance by the immune system, and obtain nutrients. As a consequence, pathogens cause tissue damage that can account for the majority of disease symptoms. For many pathogens, this damage to the host can be attributed to the production of virulence factors such as soluble exotoxins, which target and often kill cells. Thus, studying the virulence factors that alter normal physiological processes in the host and identifying their targets can provide important insight into infectious diseases. In this dissertation, we discuss important gaps in our knowledge of the bacterial toxins and describe a new model for addressing one fundamental problem, the elucidation of *in vivo* targets of these toxins.

Bacterial toxins are soluble proteins produced by a variety of pathogens. As major virulence factors, the establishment and progression of bacterial infections often rely on the production of bacterial toxins. Indeed, isogenic strains with one or more toxin genes interrupted are often substantially reduced in virulence (34, 72, 158); in cases such as bacterial neurotoxins and diphtheria toxin, the major signs and symptoms of disease can be recapitulated by the toxin alone (207). Furthermore, vaccines against diphtheria toxin (70) and tetanus toxin (167) can completely prevent disease initiated by infection with the whole organism. For these reasons, toxins have been studied extensively at the atomic, molecular, and cellular levels in order to gain insight into how bacterial pathogens destroy target cells.

Bacterial toxins are roughly divided into two groups: proteins that disrupt the target cell membrane and proteins that translocate to the interior of the cell and modify substrates. Examples of the former include pore-forming toxins, such as cholesterol-dependent cytolysins (listeriolysin O, perfringolysin O) (201), and phospholipases, such as phospholipase-C (193). Examples of the latter include anthrax toxin, tetanus toxin, diphtheria toxin, and large clostridial toxins (LCTs) (177, 178). The membrane active toxins cause cell death by interrupting the integrity of cell membranes, causing cell lysis or influx of ions that modulate signaling pathways within the cell (201). In contrast, intracellular bacterial toxins

are primarily enzymes that target important substrates within the cytosol and disrupt the cell's capacity to modulate steps in signaling (178).

Both the membrane active toxins and intracellular bacterial toxins have been the focus of substantial studies over the past decades; however, major gaps in our knowledge remain. Mechanisms of toxin gene expression, secretion, membrane interaction, membrane translocation, substrate modification, and their impact on downstream cellular effectors have been defined in exquisite detail for several bacterial toxins (178). Most of these studies utilize *in vitro* systems to define toxin activity. Indeed, almost by definition, studies on intracellular bacterial toxins involve using immortalized cell lines and, more rarely, primary cells in culture. These numerous findings now present the field with a formidable problem: to which cell types should this body of information be applied? That is, what cell types are primarily impacted by bacterial toxins within the host during various stages of disease?

A comprehensive understanding of soluble bacterial toxins may depend on resolving this issue, since it is becoming increasingly evident that the same signaling pathways can modulate many different events in various cell types. Take for example signaling through the small GTPases, which are targeted by LCTs. In some cell types, small GTPases activate apoptosis (32, 49, 93), while in other cell

types, small GTPases prevent apoptosis (228). How then should we interpret findings that LCTs inactivate small GTPases in cultured cells? Is this a pro-apoptotic or anti-apoptotic event? To further emphasize this point, consider pefringolysin-O (PFO), a pore-forming toxin produced *Clostridium perfingens*, a common cause of gas-gangrene. PFO rapidly lysis red blood cells, and is often classified as a hemolysin (202). Yet, hemolysis is not known to occur systemically in patients with gas-gangrene, raising questions about the relevance of studies focused on red blood cells. Certainly, a great deal has been learned about the biophysical aspects of PFO pore-formation, membrane insertion, and cell lysis, through use of erythrocytes (69, 90, 181, 213). But, again, to which cell types should we apply this knowledge? The formation of ion-conducting channels could have a substantially different impact on neurons, as opposed to fibroblasts. For these reasons, the field of bacterial toxins is reaching an important crossroad, if not an impasse in their study. With hundreds of immortalized and primary cells ranging from stem cells to cardiomyocytes now available, selection of the most relevant cell type should be a critical element in the study of particular bacterial toxins.

Neurotoxins such as tetanus toxin and botulinium toxin are excellent examples of bacterial toxins whose *in vivo* cell target is known (183). Both of these toxins have a high affinity to presynaptic membranes of neuromuscular junctions and block the

action of synaptic transmission (176). Because the targeted cell types of these toxins are known, the impact on the host is well-understood, and we have a more comprehensive understanding of the deadly diseases that these toxins cause (3). However, for most bacterial toxins, the *in vivo* targets remain undefined (62). This is especially true for pathogens that cause fulminant disease, where systemic effects and multi-organ damage is evident and the toxin is released at the site of colonization but can function distal from this site.

Clostridium difficile is an example of a pathogen that primarily colonizes and causes disease in the gastrointestinal tract, yet patients suffering with serious and fulminant disease exhibit pathologies outside of the original point of colonization (35, 44, 91, 138, 174). Still, it is unknown as to what extent *C. difficile*'s toxins contribute to the morbidity and mortality of these patients. *C. difficile* produces two toxins, toxin A (TcdA) and toxin B (TcdB) (211). Both of these toxins inactivate the small GTPases Rho, Rac, and Cdc42 via glucosylation, leading to changes in the actin cytoskeleton and eventually cell death via apoptosis *in vitro* (10, 17, 100, 164). However, while TcdA is known to be an enterotoxin, TcdB is a cytotoxin with little enterotoxic activity in the rabbit illiel loop model (46, 221). Thus, TcdB is an example of a bacterial toxin studied extensively *in vitro* but whose *in vivo* activities remain poorly understood.

How can the systemic targets of bacterial toxins, such as TcdB, be identified? Unfortunately, traditional animal models such as rodents and primates are not particularly useful in this regard. These models can be used to follow physiological changes like cardiac and respiratory function, but whether these events are due to direct or indirect effects of the toxin is difficult to determine. Furthermore, direct visualization of the toxin localizing to major organs is limited. Therefore, in order to directly visualize complex anatomical structures and assess toxin localization in real-time, a new model was sought that blended the benefits of traditional models and overcame their inherent obstacles. **To this end, in this dissertation we have utilized the developing zebrafish embryo as an animal model to assess the tissue and organ specific effects of bacterial toxins, including TcdB** (75, 211).

The zebrafish embryo as a model

The zebrafish, *Danio rerio,* is a small teleost fish found primarily in fresh water streams of India, but is also conveniently available through a variety of vendors. Zebrafish diverged from human evolution approximately 450 million years ago, yet the zebrafish genome sequence reveals a remarkable degree of similarity to *Homo sapiens* (112). The overall identity between zebrafish proteins and their human orthologues is approximately 70%, but the degree of homology approaches 100% when comparisons are made between functional domains of important regulatory proteins (113). Even prior to the sequencing of their genome, George Streisinger

recognized zebrafish as a valuable model for the study of development and genetic diseases. From that first insightful description by Dr. Streisinger to now, the zebrafish is a widely accepted model for the study of embryonic development and genetics as well as infectious diseases (39, 73, 122, 150, 162, 163, 203, 204). With now over 8000 publications using zebrafish as a model, clearly Dr. Streisinger's visionary use of this small, tropical fish has led to a better understanding of genetics, cellular differentiation, and development.

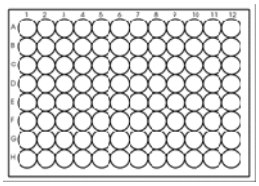
Zebrafish have several characteristics that make them appealing to researchers, including simplicity, fecundicy, transparency, organ similarity, and the availability of many transgenic zebrafish with defined phenotypes. Simplicity: zebrafish and their embryos are small (adults are 4-5 cm and embryos are approximately 3 mm), easy to maintain, and cost effective, making maintenance and experimental set-up straightforward and efficient (Fig. 1). For example, hundreds of zebrafish can be housed in a few aquariums. Additionally, embryo experiments can be performed in 96-well plates with as many as 5 fish per well. This allows for numerous experiments and replicates to be carried out at one time under tightly controlled conditions. Furthermore, because of their small size, pathology and immunohistochemistry can be performed on the whole animal with the entire fish cross-sectioned on the sagittal plane (Fig. 2). Fecundicy: A single mating can provide hundreds of embryos, making it possible to perform high throughput

analysis under a variety of conditions and statistical significance. Transparency: Arguably, the most appealing characteristic of the zebrafish model is that zebrafish

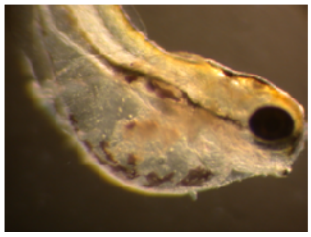
Fig. 1 Zebrafish intoxication assay. Zebrafish are first mated and embryos are collected. Next, prepared embryos are distributed into a 96-well plate containing embryo water, with up to five embryos per well. At selected time points, toxin is added directly to the well and the zebrafish are observed for changes in phenotype and histopathology and/or immunostaining can be performed. Various iterations of this assay include addition of candidate inhibitors against the toxin to test efficacy (76).

Mate and Collect Embryos

Distribute in 96-well plate



Characterize Defects



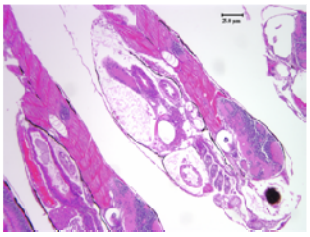
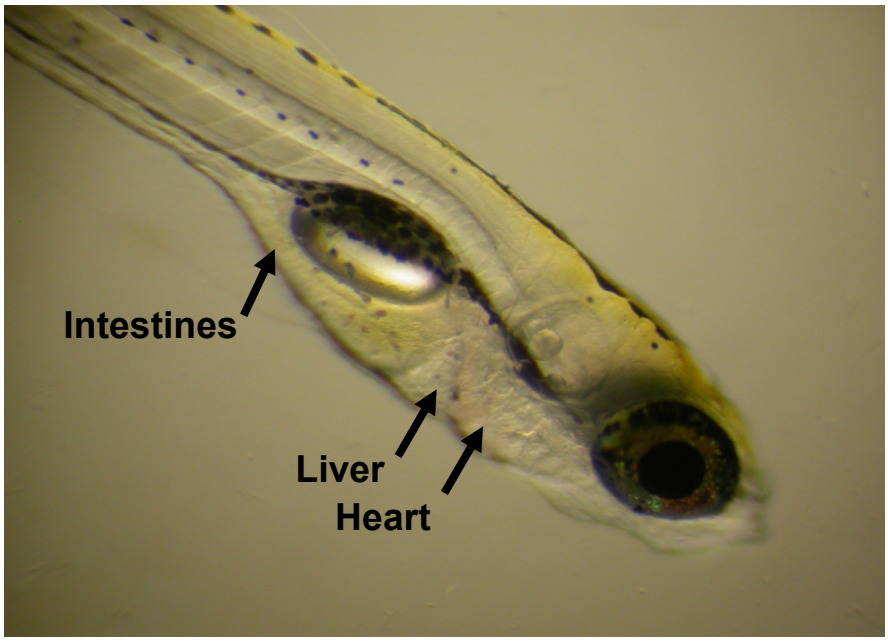


Fig. 2. Histopathology of zebrafish whole-body cross-sections. Shown in the panel are four examples of prepared zebrafish larva cross-sectioned on a sagittal plane and subjected to histopathology. Using this approach it is possible to visualize many or all of the major organs for damage following exposure to bacterial toxins (76).


Dorsal
Anterior
Posterior
Ventral

embryos are optically transparent for the first 10 days of development and growth, circumventing the inherent problems with post-mortem analysis of tissue and organ damage. Such transparency makes it possible to directly visualize the major organs and the vascular system within the zebrafish embryo (Fig. 3). By taking of advantage of this unique quality, following intoxication, researchers can directly determine where labeled toxin localizes, the sequential progression of damage, and which cell types and tissues are damaged in real-time. Organ system similarity: Zebrafish possess many of the same organs as humans, such as heart, gastrointestinal tract, liver, pancreas, gallbladder, and kidneys, to name a few, making the transparent embryos a powerful model to study organogenesis, organ function, and organ-related disorders and diseases, as well as exogenously-induced organ damage (113). Mutant Phenotypes: The zebrafish embryo has been used extensively to identify genes involved in stages of development and function of various organs and systems (73). Because of this, numerous zebrafish phenotypes and their related mutations have been identified, leading to several databases of zebrafish mutant phenotypes with known genetic defects. Overall, with these characteristics combined, zebrafish embryos provide a unique and valuable model to study human disease and have recently been used to characterize the *in vivo* targets of bacterial toxins, such as TcdB. In the following chapter, we highlight an important clinical problem to which the zebrafish model can be applied: *Clostridium difficile*-associated disease.

Fig. 3. Visualization of major organs in the transparent zebrafish embryo. Shown here is a zebrafish embryo 4 days post-fertilization. By simple light microscopy, major internal organs within the fish are easily visualized. Because of this transparency, damage to major organs can be observed in real-time.


Intestines
Liver
Heart

CHAPTER II

LITERATURE REVIEW OF *CLOSTRIDIUM DIFFICILE*

A. *Clostridium difficile*

Over the years, public concern over hospital-acquired infections has primarily focused on methicillin-resistant *Staphylococcus aureus* (MRSA). However, recent reports indicate that *Clostridium difficile*, the causative agent of *Clostridium difficile*-associated disease (CDAD), has caused nearly twice the number of nosocomial infections and related deaths compared to MRSA (139). Outbreaks and epidemics of CDAD have been reported in the United Kingdom, Europe, Canada, and now the United States (138, 156, 210). Coupled with a disturbing and somewhat sudden increase in mortality rate, the once moderate, self-limiting disease has now become an increasing and sometimes fatal problem (139). Even prior to recent media recognition, *C. difficile* has been the leading cause of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC). However, it was not until the late 1970's that *C. difficile* was recognized as a human pathogen, nearly 40 years after its original isolation.

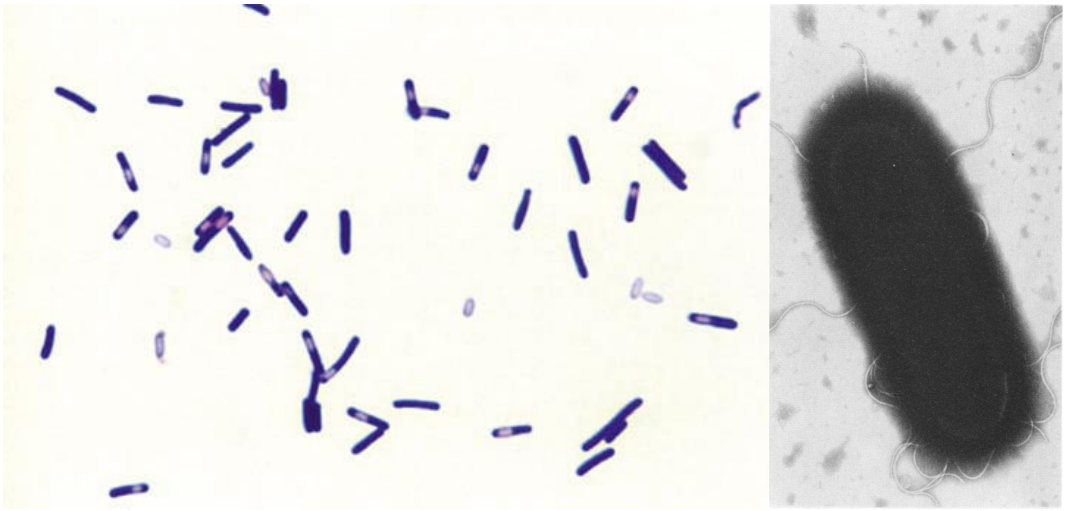
In 1935, Hall and O'Toole isolated *C. difficile* from the meconium and feces of healthy infants and named it *Bacillus difficilis* due to culturing and isolation

difficulties (74). Shortly thereafter, the bacterium was reclassified into the genus *Clostridium* (from greek: small spindle), and thus was renamed *Clostridium difficile* and first listed in the 5th edition of *Bergey's Manual of Systematic Bacteriology* in 1939. *C. difficile* is a Gram-positive, anaerobic rod, approximately 3-5 μm in length, and is motile with peritrichous flagella (Fig. 4). *C. difficile* also forms an oval, subterminal to terminal spore (Fig. 4). Further characterization revealed that *C. difficile* culture filtrates were lethal when injected into animals (74), and in 1937, *C. difficile* antiserum was shown to neutralize toxic activity (184). However, it would be several decades before *C. difficile* was found to cause disease.

B. *Clostridium difficile*-associated disease

Pseudomembranous colitis was first reported in 1893 when pseudomembranous lesions of the colon were observed in a post-operative patient and termed "diphtheritic colitis" (54). However, in 1977, another clostridium, *Clostridium sordelli*, was implicated as the causative agent of PMC. In 1977, Rifkin et al found that antisera to *C. sordelli* neutralized the cytotoxic activity found in the feces of PMC patients (114, 169). Yet curiously, *C. sordelli* could not be isolated from the PMC patient feces while *C. difficile* consistently was. Also in the late 1970's several groups demonstrated that *C. difficile* was the etiological agent of antibiotic-

Fig. 4. Gram-stain of *Clostridium difficile* and example of *C. difficile* with flagella (40).



induced colitis in hamsters (125). These and other studies finally linked the formerly non-pathogenic *C. difficile* to PMC and AAD, decades following its original isolation.

As a nosocomial disease, CDAD is most often found in the hospital or primary care facilities, although there are several cases outside these settings (152). The reservoirs in hospitals are commonly patients, health care workers, and contaminated environments (104). *C. difficile* has been isolated from healthcare workers, patients, hospital therapy dogs as well as fomites including hospital toilets, clothing, carpet, blood pressure cuffs, call buttons, and telephones (51, 94, 104, 108, 116). Of particular concern, *C. difficile* can persist even after typical disinfecting techniques, indicating that standard infection control methods can be ineffective (14). Because it can exist as an inert and hearty spore, this bacterium is the bane of many hospitals, as common techniques of disinfection (i.e. alcohol-based sanitation) are not sporicidial.

As a pathogen, *C. difficile* is unique in that in order for the bacterium to initiate disease the host usually must be exposed to antibiotics (typically clindamycin, cephalosporins, ampicillin, and, more recently, fluoroquinolones) (125, 156). Infection with the bacterium can occur either pre- or post- antibiotic therapy (8). The widely accepted dogma of *C. difficile* infections is transmission of bacterium

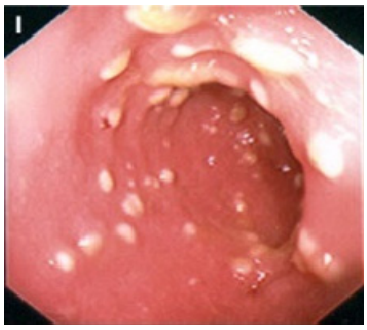
via fecal-oral route, colonization of the gastrointestinal tract, disruption of colonic microflora by antibiotics, and growth and proliferation of *C. difficile* followed by toxin production, and damage to the intestinal mucosa. In addition to antibiotics, other people at risk for CDAD include immunocompromised patients, persons with cystic fibrosis, individuals undergoing chemotherapy, and proton pump inhibitor therapy (7, 42, 88, 133)

Following transmission of *C. difficile* and host exposure to antibiotics, *C. difficile* can proliferate and cause disease (57, 145, 189). The following summary can be found in detail in Kelly, et al 1994 (105). In mild CDAD, diarrhea consists of approximately 3 unformed stools within a 24 h time period and is usually self-limiting. As CDAD worsens, diarrhea becomes more perfuse and contains mucous, abdominal cramping ensues, and a sigmoidoscopy (an internal examination of the colon) may reveal erythematous bowel walls but no pseudomembranes. In addition, patients may complain of symptoms outside of the gastrointestinal system such as nausea, fatigue, low-grade fever, anorexia, and general malaise.

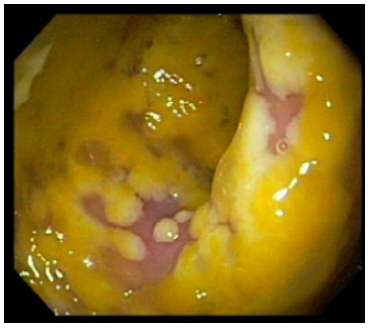
In more serious cases of CDAD, sigmoidoscopy reveals erythematous bowel walls as well as small, raised, yellow-white lesions within the colon (Fig. 5A). In early PMC, these lesions are small (2 to 10 mm) and most of the inflammation is

Fig. 5 Colonic etiology and pathology of CDAD. Panel A and B show the progression of severe CDAD. Panel A illustrates the formation of small white lesions that increase in size and number until they coalesce to form a pseudomembrane within the colon (Panel B). Panel C represents the typical "volcano" lesions in *C. difficile* colitis (104).

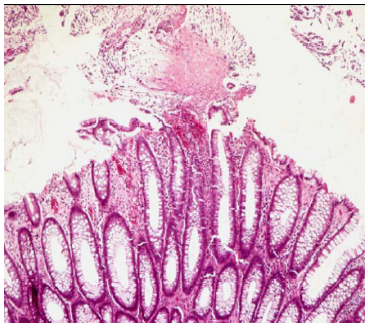
A



B



C



confined to the superficial epithelium. As disease progresses, basal laminia inflammation is extensive and more lesions form and coalesce, creating a "pseudomembrane" of immune cells, mucus, and necrotic tissue within the colon (Fig. 5B). Pathology of PMC reveals eruptive "volcano" lesions caused by the release of mucus, serum proteins, inflammatory cells, and necrotic tissue (Fig. 5C). Patients exhibit persistent diarrhea, high fever, abdominal cramping, diffuse lower quadrant pain, and nausea. Furthermore, leukocytosis can be detected (>20,000/L).

As disease becomes fulminant, abdominal pain can become severe and colonic distension can occur (also referred to as toxic megacolon) (36). Contrary to mild and moderate forms of CDAD, diarrhea at this stage of disease can be minimal as paralytic ileus may develop, causing a loss in the normal contractile movements of the intestinal wall, colonic dysmotility and a pooling of colonic secretions. Because diarrhea is a hallmark of CDAD, lack of diarrhea in these cases can lead to misdiagnosis. Leukocytosis can be >35,000 cells/mm^3 and upwards to 50,000 cells/mm^3 (36). This increase in WBC can be used as an indicator of impending fulminant disease. Patients with WBC of >35,000 cells/mm^3 are associated with a poor prognosis and a high mortality rate (50%) (130). Heart rate in patients with fulminant CDAD is >120 with tachycardia present and patients often require mechanical intubation (36).

In severe cases of CDAD, systemic damage outside of the gastrointestinal tract is detected with documented reports of cardiopulmonary arrest (138), acute respiratory distress syndrome (91), multiple organ failure (44), renal failure (35), and liver damage (174). Although perforations of the colon can occur, there are numerous cases indicating that death occurs prior to free air and perforation of the colon (36). Furthermore, circulating TcdB has been detected in serum and ascitic fluid (166) and patients are known to generate serum antibodies against toxin (206, 215). These data, coupled with the low incidence of *C. difficile* in the bloodstream (52) (personal communication with Stuart Johnson and Dale Gerding, MD VA hospital, Chicago), indicated that systemic events may be due to the release of bacterial toxins into the bloodstream and not sepsis.

Following presentation of the aforementioned clinical symptoms, several methods can be used to diagnose CDAD. The direct fecal cytotoxicity test is the “gold standard” and assays for the presence of toxin by observing specific toxin-induced changes in tissue culture cell morphology (45). This test is not the most sensitive, but it is the most specific as it determines the presence of toxin-expressing strains of *C. difficile*. Conversely, the stool culture test is not the most specific as it does not distinguish between toxigenic and nontoxigenic *C. difficile*; but, it is the most sensitive (136). Sigmoidoscopy is rapid but only detects 50% of

pseudomembranes (15). In general, however, the most frequently used test is the EIA test for TcdA and TcdB that detects the presence of these toxins within the feces of CDAD patients. Because some strains of *C. difficile* do not produce TcdA, it is important that diagnostic tests assay for TcdB (136).

For treatment of CDAD, often discontinuation of the offending antibiotic can result in the resolution of mild cases (105). If this fails, treatment of CDAD routinely consists of oral metronidazole or vancomycin for 7-10 days (105). Metronidazole, which disrupts nucleic acid synthesis, is the first drug of choice primarily because it is the least expensive; however, because of emerging metronidazole-resistant strains of *C. difficile,* a potential decrease in efficacy, and the negative, systemic side effects of metronidazole, vancomycin is also used (5). Vancomycin, which inhibits cell wall synthesis, is very effective in treating CDAD (5). Most importantly, because it is poorly absorbed in the gastrointestinal tract (in contrast to metronidazole), significant luminal levels of vancomycin can be obtained (50-200 fold higher than the MIC) without systemic effects (95). However, vancomycin is expensive and is rarely used at the advice of the Hospital Infection Control Practices Advisory Committee due to an increasing threat of vancomycin-resistant enterococci and staphylococci (although there are contrasting reports as to the relevance of this) (66).

Unfortunately, in addition to emerging antibiotic resistant strains of *C. difficile,* both metronidazole and vancomycin have been associated with *C. difficile* cecitis (12, 13); therefore, new antibiotics are being sought for the treatment of CDAD. Antibiotics such as nitazoxamide (148), rifaximin (175), ramoplanin (58), and teicoplanin (218) have some preliminary success in clinical trials. Additionally, unlike vancomycin and metronidazole, nitazoxamide does not induce *C. difficile* cecitis (140). However, these antibiotics are not yet approved for the treatment of CDAD by the FDA, although nitazoxamide and ramoplanin are currently in phase III trials for FDA approval (11). Teicoplanin is currently used in the UK and Europe for treatment of CDAD but as of yet, is not clinical available in the United States (11).

Because disease symptoms and pathologies are due to the production of toxin, companies have also started developing drugs that are directed against TcdA and TcdB. There are several approaches such as anion-exchange resins and toxin binding polymers as well as antibodies against the toxins. Anion-exchange resins (cholestyramine and cholestipol) are used to bind the toxins (11). Yet, while these resins bind successfully to the toxins *in vitro*, they were found to be ineffective as treatments in the hamster model and clinical trials. Thus these treatments are not recommended for use in severe cases (11, 24). Furthermore, cholestyramine was also found to bind to vancomycin (191).

Tolevamer, a sodium salt of styrene sulfonate polymer, has shown success in hamster infection trials with up to 80% of hamster survival compared to 10% with cholestyramine (123). Although compared to vancomycin in clinical trials, there is no significant difference in initial response; there may be a trend for a faster response with vancomycin but fewer reoccurrence using the tolevamer therapy. Clearly further studies are needed to determine the efficacy of tolevamer. Yet, it appears to be a promising alternative or concomitant therapy to antibiotics and is currently in phase III trails for FDA approval (11).

Animal studies have shown that vaccination with culture filtrates containing inactive TcdA and TcdB provided immune protection against disease (53, 109, 118, 195, 196). Additionally, researchers have shown that antibodies directed against TcdA and TcdB have been somewhat effective at preventing disease in the hamster infection model. In these studies, anti-TcdA alone could provide some protection while anti-TcdB provided little protection; however, both anti-TcdA and anti-TcdB were required for the greatest protection (110). Currently, a toxoid vaccine and monoclonal antibodies to TcdA and TcdB are in phase I trials and phase II trials, respectively (11). It is important to note that all of these toxin-based therapeutics not only provide potential alternatives to antibiotic therapy but also reiterate the important role of *C. difficile*'s toxins in CDAD.

There are also several other alternative approaches to prevention, treatment, and management of CDAD. Probiotics, prophylactics, and immune therapy are just a few alternatives to antibiotics and anti-toxin drugs. Probiotics are defined as supplements (food, drugs, etc) containing live, "beneficial" bacteria (188). This form of therapy is designed to favorably alter the microflora of the gastrointestinal tract in order to promote better digestion and, most importantly, prevent pathogens from causing disease. The most common include *Lactobacillus* sp. and *Saccharomyces boulardii* (188). Although probiotics can be found in drugstores and supermarkets, their place in standard treatment practice is not yet established. Although there appears to be a great deal of promise in using "friendly bacteria" to prevent the colonization and proliferation of *C. difficile,* research in probiotics as therapeutics for enteric disease is mired in single case reports, *in vitro* only studies, and loosely designed clinical studies. Therefore, more research and larger, tightly controlled clinical studies will need to be performed before it becomes standard practice.

Fecal bacteriotherapy, often considered the ultimate human probiotic therapy, has been around for more than 40 years in humans and perhaps as early as the 17th century in animals and has been used to treat not just CDAD but also ulcerative colits, irritable bowel syndrome, inflammatory bowel disease, and even constipation (19). Essentially, 5-300 g of donor stool is collected, screened for

pathogens, suspended in saline, filtered, and administered during a colonoscopy or as an enema or through tubes (enteric, duodenal, jejunium, or nasogastric). Although controversial, fecal implants may prove to be efficacious (in one report, 33 out of 36 (92%) patients with refractory CDAD resolved symptoms following a single fecal implant) and this therapy may be useful in preventing reoccurring CDAD (18, 19, 56, 124, 157, 200). Despite the aesthetics, administration of the therapy, and potential for infection, this area of probiotic therapy is promising; however, more studies are needed with more patients and better controls to fully address the usefulness of this treatment.

Finally, another area in therapy is immune therapy. In order to boost serum antitoxin antibody levels, immune therapy with intravenous immunoglobin (IVIG) has been investigated. In a study by Leung et al., 5 children with recurrent CDAD and low serum IgG antitoxin A were given IVIG every three weeks for 4-6 months. All 5 showed marked improvement and tested negative for TcdB in the stool cytotoxicity assay (117). Other studies also reports IVIG success in treating recurrent CDAD and after no response to antibiotic therapy (11). Patients that suffer from severe and/or recurrent CDAD are often unable to mount a proper immune response and have low levels serum antitoxin antibodies; therefore the use of IVIG may be useful in treating severe and recurrent disease and is currently in Phase IV trials (11).

Recurrent CDAD is a continuing problem and occurs usually 1-10 days post-CDAD-specific antibiotic therapy (11). Recurrent disease occurs frequently (approximately 30% of CDAD patients) and may be attributed to either *C. difficile*'s ability to form a spore or re-infection with a new strain of *C. difficile.* Following cessation of antibiotic therapy and "eradication" of vegetative, active *C. difficile*, inactive and antibiotic resistant spores can still remain in the gastrointestinal tract. If the patients are still immunosuppressed, continuing other antibiotics or immunosuppressive drugs, or the suppressive colonic microflora has yet to re-colonize the gastrointestinal tract, the remaining *C. difficile* spores can become active or a new strain may colonize the patient and cause disease. Probiotics, immune therapy, fecal implants, tapering doses of antibiotic, and pulse antibiotics are all used for treatment of recurrent disease (11).

C. The Toxins of *C. difficile* and their role in disease

Introduction and overview

Following its isolation and identification in 1935, researchers discovered that *C. difficile* culture filtrate caused lesions, respiratory arrest, and death in animals upon injection (74); however, *C. difficile* was not recognized as a human pathogen at this time, thus for decades, little was known about its virulence factors. Interestingly, it was the bacterium's toxins that led to the correlation between PMC and AAD and infection with *C. difficile*, nearly 4 decades after the bacterium's original isolation.

In the 1970s, the feces of PMC patients demonstrated cytotoxic activity (125). Accordingly, researchers began to investigate the possibility of a toxin-producing bacterium as the cause. As a result of this research, it was discovered that the cytotoxic activity was neutralized by a gas gangrene antiserum against a mixture of clostridial species (125). From this mixture, investigators demonstrated that only *C. sordelli* antiserum neutralized the toxin present in PMC fecal samples, originally implicating *C. sordelli* as etiological agent of PMC infections. However, *C. difficile* was soon identified as the causative agent of PMC and that it produced a toxin similar to that of *C. sordelli* (thereby explaining the cross-reactivity of the antiserum) (125) It was not until 1980 that researchers demonstrated that *C.*

difficile actually produced two separate toxins, toxin A (TcdA), an enterotoxin, and toxin B (TcdB), a potent cytotoxin (6, 192).

TcdA and TcdB belong to a larger class of intracellular bacterial toxins termed the Large Clostridial Toxins, or LCTs. These toxins are produced by several pathogenic *Clostridia* species including *Clostridium difficile*, *Clostridium sordelli*, and *Clostridium novyi* and are characterized by their large size and their mechanism of action (16, 177). LCTs are some of the largest bacterial toxins known, with molecular masses ranging from ~250-308 kDa (16, 177). Members of the LCT family are typical act intracellularly as glucosyltransferases, targeting and inactivating proteins in the Rho and Ras family of GTPases via the addition of a sugar moiety within the effector binding region. The disruption of small GTPase signaling in cells results in disaggregation of the actin cytoskeleton and cell death (16, 177, 210).

As members of the LCT family, TcdA and TcdB share sequence homology and are large in size (TcdA = 308 kDa and TcdB = 270 kDa). Additionally, both TcdA and TcdB inactivate Rho, Rac, and Cdc42 by transferring glucose from UDP-glucose to a reactive threonine in the effector-binding region of the GTPases, resulting in their inactivation(16, 55, 98, 99). Despite their similarity, TcdA and TcdB differ in their biological activity *in vitro* and *in vivo*. TcdA is classified as an enterotoxin while

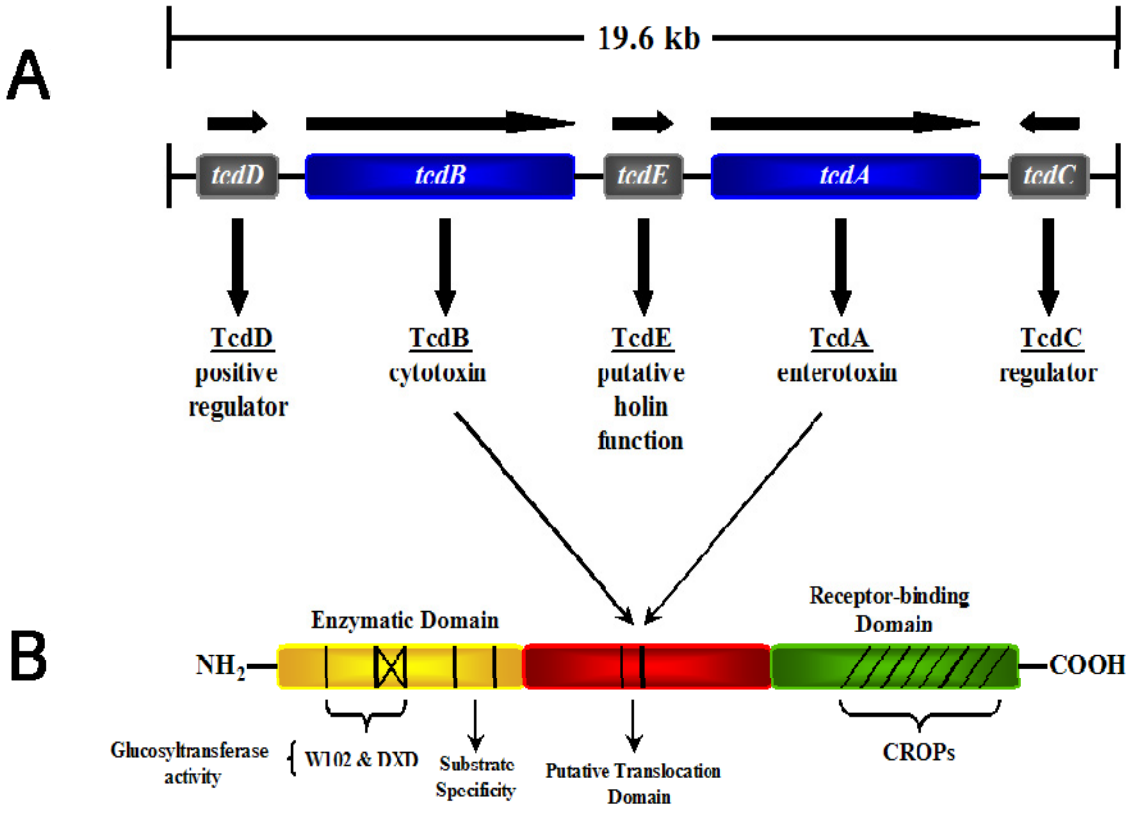
TcdB is a more potent cytotoxin with very little enterotoxic activity (118, 119, 126, 127). Both TcdA and TcdB are important contributors to disease and much is known about these two major virulence factors; therefore, in addition to this dissertation, there are several elegant reviews that detail several aspects of these toxins and their role in CDAD (125, 210).

Genetics of TcdA and TcdB

The genes encoding for TcdA and TcdB are 8133 and 7098 nucleotides in length, respectively, share 66% nucleotide sequence homology, and like the genome of *C. difficile*, *tcdA* and *tcdB* have a low G+C content (<28%) (47). Given their size, sequence homology, functional similarity, and proximal location (only 1350 nucleotides separate the genes), it is thought that these genes may be the result of a duplication event (209). Both of these genes are located on the *C. difficile* chromosome within a 19.6 kilobase pathogenicity locus, along with three other accessory genes *tcdC, tcdD,* and *tcdE*, which are organized as *tcdDBEA* and *tcdC* (Fig. 6A) (77).

The accessory genes *tcdC,D,E*, are believed to be involved in the regulation of TcdA and TcdB production and release. Located downstream from *tcdA*, *tcdC* is divergently transcribed from the toxin genes and hypothesized to negatively

Fig. 6. Pathogenicity island of *C. difficile* and proposed protein domain structures of its toxins, TcdA and TcdB. Panel A represents the genetic arrangement of the toxin genes and their accessory genes, *tcdCDE* within the pathogenicity locus of the *C. difficile* genome. Panel B portrays the 3 domain structure of TcdA and TcdB, including the enzymatic region, the translocation domain, and the receptor-binding domain. Also specified in this drawing are regions of specific activities including the glucosyltransferases activity located at the amino terminus, the region required for receptor binding, and the motifs required for toxin activity, including W102, DXD, and the CROP regions (210).


A
19.6 kb
tcdD
tcdB
tcdE
tcdA
tcdC
TcdD
positive
regulator
TcdB
cytotoxin
TcdE
putative
holin
function
TcdA
enterotoxin
TcdC
regulator
B
Enzymatic Domain
Receptor-binding
Domain
NH2
COOH
Glucosyltransferase
activity
W102 & DXD
Substrate
Specificity
Putative Translocation
Domain
CROPs

regulate the expression of TcdA and TcdB (87). TcdC expression begins in early exponential phase but declines when growth enters into stationary phase. Additionally, during high transcription levels of *tcdC*, there is a corresponding low level of transcription of *tcdA,B,D,E*. Isolations of *C. difficile* strains that have a 18-basepair deletion in their *tcdC* genes or carry a mutation that results in the production of a truncated TcdC protein are found to produce 16 -20 fold more TcdA and TcdB, respectively (214). Collectively, these data suggest that TcdC is a negative regulator of TcdA and TcdB production.

The gene *tcdD* (also known as *txeR*), is located upstream of *tcdB* and is proposed to positively regulate the expression of *tcdA* and *tcdB* (87). Using promoter reporter fusions of the *tcdA* and *tcdB* promoter-binding region, TcdD was found to enhance their expression (146). Furthermore, the protein sequence of TcdD reveals homology to DNA binding proteins, UviA (which regulates the *C. perfringens* UV-inducible bacteriocin gene) (63), as well as TetR and BotR, which positively regulate the expression of tetanus toxin and botulinum toxin, respectively (131, 132). The transcription of *tcdD* also coincides with the expression of *tcdA,B,E* in the late growth phase (87).

The function of TcdE in *C. difficile* is still poorly understood. Also expressed in late growth phase, *tcdE* has homology to several holins (a bacteriophage protein that

disrupts cell membranes) (87, 190). *C. difficile* does not possess a known secretion system; therefore, it has been proposed that TcdE causes the lysis of *C. difficile* resulting in the release its large toxins. Tan, et al demonstrated that TcdE has structure and function similarity to holin proteins; however, the precise purpose of TcdE in *C. difficile* is still currently under investigation (190).

Although it is known that expression of TcdA and TcdB occurs in the late growth phase (87), little is known regarding the environmental signals that regulate toxin expression. Given the association between antibiotics use and CDAD, many researchers have sought to prove that antibiotics directly induce toxin expression. Early studies reported that addition of antibiotics *in vitro* increased the cytotoxic activity of *C. difficile* (65, 149). However, it is important to note that toxin production in response to antibiotics varied from strain to strain and even antibiotic type and does not occur in all toxigenic isolates of *C. difficile* (65, 149). Therefore, while some reports indicate a correlation between antibiotics and toxin expression and production, there has not yet been definitive evidence to support this.

In *C. perfringens* and *C. botulinum*, enterotoxin production has been linked with sporulation (59, 60, 226). Yet, this does not appear to occur in *C. difficile* as mutants deficient in sporulation are still capable of producing toxin and toxin cannot be extracted from spores (106, 107). Numerous studies reveal other

signals that may regulate toxin expression. Yamakawa, et al found that limiting biotin resulted in a 35-64-fold increase in TcdA and TcdB expression, respectively (225). Rapidly metabolizable sugars, such as glucose, are found to repress toxin synthesis (48). Another group reported that toxin synthesis is responsive to certain amino acids, metabolic by-products such as butyric acid and butanol and temperature (101, 102).

Quorum sensing is a means of bacterial cell-cell communication that involves the production, secretion, and detection of extracellular signal molecules called autoinducers (216). Bacteria often use quorum sensing to regulate gene expression, and recently, it has been reported that *C. difficile* uses the quorum-sensing molecule, LuxS/autoinducer-2, to regulate the transcription of *tcdA*, *tcdB*, and *tcdE* (28, 115). Given that: 1) quorum sensing is cell density-dependent, 2) disease occurs following the overgrowth of *C. difficile* in the gastrointestinal system, and 3) the production of toxin occurs in the late-log and stationary phase of *C. difficile* growth, it will be important to further understand the role of quorum sensing in the regulation of *C. difficile* toxin production.

Although numerous environmental stimuli have been shown to influence toxin production, how these signals regulate toxin expression, which genes are specifically involved, and what actually occurs *in vivo* is unclear. Unfortunately,

because *C. difficile* currently lacks a genetic system, it is difficult to fully elucidate the function and role of *tcdABCDE* in pathogenesis and what specifically coordinates the expression of TcdA and TcdB and is clearly an under-studied area of *C. difficile* pathogenesis.

Toxin structure and mechanism of action

Despite their size, TcdA and TcdB, like all LCTs, are single polypeptide proteins and can be roughly divided into three functionally different regions: the enzymatic region, the membrane translocation domain, and the receptor-binding domain (Fig. 6B) (210).

The N-terminal enzymatic domain of TcdA and TcdB

The amino terminal end of TcdA and TcdB contains the enzymatic region, and not surprisingly, this region shares homology between other LCTs and other glucosyltransferases (168). As shown in Fig 6B, the glucosyltransferase activity appears to be within the first 546 residues (84, 186), although Wagenknecht-Wiesner found that only residues 1-467 is required for *in vitro* glucosylation of Rho, Rac, and Cdc42 (212). Within this enzymatic region is a conserved DXD motif, which is important for carbohydrate binding in divalent cation-dependent, sugar-nucleoside diphosphate glucosyltransferases, like the LCTs and numerous other mammalian glucosyltransferase families (220). Indeed, a mutation of the DXD

motif in *C. sordelli* lethal toxin (TcsL) prevented toxin-mediated glucosylation of GTPases *in vitro* (26). Also conserved among the LCTs, tryptophan-102 was found to be involved in UDP-glucose binding, an event required for toxin activity (25). To determine the portion of the amino-terminus that comprises the region of substrate specificity, investigators created chimeric hybrids between TcdB and TcsL, as TcdB glucosylates Rho, Rac, and Cdc42 while TcsL targets Rac, Ras, Rap, and Ral. The construction of these toxin hybrids revealed that the region conferring substrate specificity of TcdB is located at residues 264 to 516 (83).

The C-terminal binding domain of TcdA and TcdB

The highly repetitive C-terminal region and the middle region of the LCTs are required for the internalization of the toxin via receptor-mediated endocytosis. Very little is known about middle region; however, it is proposed to be the membrane-inserting translocation region (208). The C-terminal region is believed to be the receptor-binding region. Frisch, et al found that this region of TcdA is required for endocytosis (61). Furthermore, recombinant fragments of this region and antibodies against this region provide protection against the toxin.

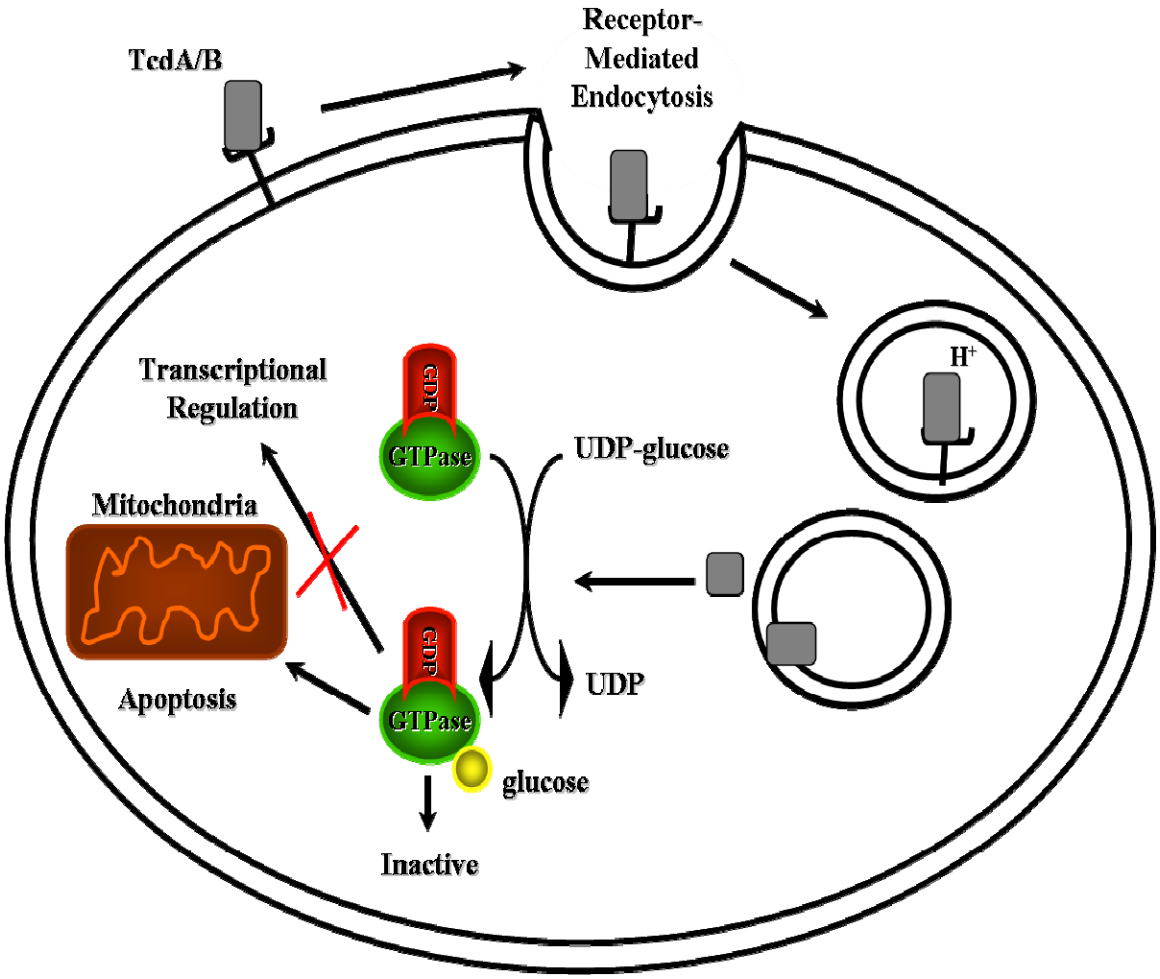
Within the C-terminus are several short (21-50 residues) homologous regions termed the combined repetitive oligopeptides or CROPs (209) (Fig. 6B). TcdA and TcdB both encode for 5 groups of CROPs but TcdB only shares homology with four

of TcdA's CROPs, perhaps explaining the difference in host cell tropism between the two toxins. Although this region varies between LCTs, these toxins are all rich in aromatic amino acids and there is a consensus YYF triad. These repeats are similar to those found in the carbohydrate-binding region of streptococcal glucosyltranserases (224). Importantly, the receptor for TcdA and TcdB, as well as the other LCTs, is believed to be nonproteinaceous, most likely carbohydrate in nature (210). While there are several examples of aromatic amino acids-carbohydrate interaction and they are believed to be important in carbohydrate receptor binding, the specific function of these repeats is still poorly understood (209).

Receptor-binding

Both TcdA and TcdB are intracellular bacterial toxins and thus must gain access to the cell cytosol in order to modify their targeted substrates (See Fig. 7 for general intoxication schematic). To do this, TcdA and TcdB first bind to cell surface receptors, which are believed to be carbohydrate in nature (210). Although TcdB's receptor is currently unknown, TcdA has been shown to bind to Galα1-3Galβ1-4GlcNac carbohydrates; unfortunately, these do not appear to be present on human cells (111). Sucrase-isomaltase may also be another possible receptor for TcdA, yet it too is not found on human colon cells (161). The minimum

Fig. 7. Internalization schematic of TcdA/B. Upon binding to toxin receptor, TcdA/B is internalized into the host cell via receptor-mediated endocytosis. Upon acidification of the endosome, the enzymatic region is released into the cytosol where it can transfer a sugar moiety from UDP-glucose into the effector-binding region of Rho, Rac, and Cdc42, thereby rendering these small GTPases inactive. These events lead to actin condensation, transcription activation, and eventually, cell death, via apoptosis (210).


TcdA/B
Receptor-
Mediated
Endocytosis
H+
Transcriptional
Regulation
GDP
GTPase
UDP-glucose
Mitochondria
Apoptosis
GDP
GTPase
UDP
glucose
Inactive

carbohydrate receptor Galβ1-4GlcNac has been found on the human I, X, and Y blood antigens, but the functional role of this receptor is unknown (198). Therefore, while several carbohydrates have been found to bind to TcdA, a functional human ligand has not yet been definitively elucidated.

Although TcdB's receptor is unknown, experimental data suggest that it is different from the TcdA receptor and, unlike TcdA, which binds to the apical site of colonic T-84 cells, the receptor for TcdB appears to be basolaterally located on T-84 cells (31). Furthermore, because it is able to intoxicate a wide variety of cell types, the TcdB receptor is believed to be ubiquitous (210).

Receptor-mediated internalization of TcdA and TcdB.

Following binding to the receptor, TcdA and TcdB are then internalized via receptor-mediated endocytosis (9, 55, 81). Upon acidification of the endosome, the enzymatic region, of the toxin is released into the cytosol. Endosome acidification is a required step for intoxication as lysosomotropic inhibitors (ammonium chloride, hydroxychloroquine, chloroquine, and bafilomycin A) prevent toxin activity (55). The low pH of the endosome is believed to induce the required structural changes in the toxin, causing the hydrophobic region of the toxin to be exposed for insertion into the target membrane by channel formation (9, 165). Next, like many other AB-toxins, the toxin is cleaved and the first 543 amino acids

of the enzymatic region is released into the cytosol, an event proposed to take place following translocation into the cytosol where it is able to access the toxin targets, Rho, Rac, and Cdc42; however, how and where in the cell this cleavage occurs is not known (173).

Toxin targets and mechanism of action

Following release into the cytosol, TcdA and TcdB target the Rho proteins, Rho, Rac, and Cdc42. These proteins are important for regulation of wide variety of cellular activities. Rho has been found to be involved in the regulation of stress fiber formation, focal adhesions, and cell contractility (151). Rac and Cdc42 is important for the regulation of lamellipodium and filopodium formation (151, 153). Members of the Rho family are regulated by a GTPase cycle. The following summary can be found in detail in a thorough review by Jaffe et al (92). The GDP-bound Rho, Rac, and Cdc42 proteins are inactive and regulated by guanine nucleotide dissociation inhibitors (GDIs). These proteins block the activity of the guanine nucleotide exchange factors (GEFs) and maintain the proteins in the cytosol. Following dissociation of the GDIs, Rho is then cycled to the membrane, where GEFs then assist in the dissociation of GDP, allowing Rho to bind to GTP and become activated. In the active, GTP-bound form, the Rho proteins are able to interact with numerous downstream targets and effectors. Following the hydrolysis of the γ-phosphate of GTP to GDP, which is facilitated not only by the

intrinsic activity of Rho proteins but also by GTPase activating proteins (GAPs), the active state is terminated and the proteins return to the inactive, GDP-bound state.

TcdA and TcdB glucosylate Rho, Rac, and Cdc42 at a reactive threonine residue: Thr37 of Rho and Thr35 in Rac and Cdc42 (99, 100). The addition of glucose to these residues could have several outcomes. Reports indicate that the glucosylation of Rho/Ras proteins prevents them from directly interacting with their downstream effectors (82, 180). In studies with Ras proteins, it was shown that glucosylation prevents the structural change from the inactive to the active conformation (68, 205). Furthermore, glucosylation may also prevent Rho proteins from becoming activated by GEFs (82, 180).

Another consequence of glucosylation by TcdA and TcdB is the up-regulation of RhoB, which remains in the activated state while only a small pool of RhoB is partially glucosylated by the toxin (67). RhoB is up-regulated during cellular stress and is also known to be involved in apoptotic pathways and may be due to the glucosylation and inactivation of proteins such as Rac1 (67). Therefore, in addition to inactivating Rho, Rac, and Cdc42, these toxins also are capable of upregulating RhoB, which may contribute to the detrimental effects of the toxins.

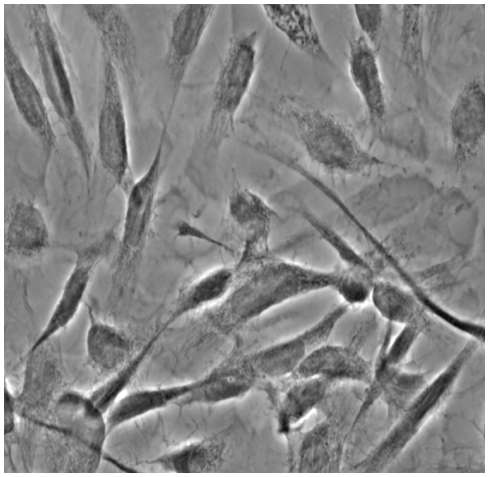
The biological activity of TcdA and TcdB

The most prominent effect of TcdA and TcdB on the cell is the drastic changes in cell morphology, an unsurprising result given the role of Rho, Rac, and Cdc42 in the regulation of the actin cytoskeleton. Following intoxication, TcdA and TcdB induce a type of cytopathic effect (CPE) where the body of the cell retracts, but radiating, neurite-like cellular protrusions remain, an effect referred to as actinomorphism or arborization (Fig. 8) (143). This appears to be substrate-specific as a variant TcdB from *C. difficile* 1470 which inactivates Rac, Ras, Rap, and Ral, produces a cell rounding effect similar to that of TcsL, which targets the same substrates (30).

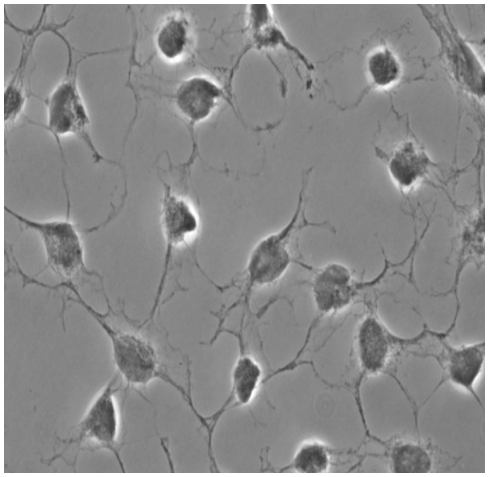
In addition to cell rounding, TcdA and TcdB have also been found to increase paracellular permeability and disrupt epithelial membrane integrity (80, 153). Tight junctions are important for the cell-to-cell association and barrier function (71) and, in an intestinal T84 monolayer model, treatment with toxin resulted in the disruption of epithelial tight junctions and changes in F-actin organization in the apical and basal membranes (153). This event was also associated with the dissociation of tight junction proteins, ZO-1, ZO-2, and occludin (153). These proteins are important for not only forming the scaffolding between cells but also regulating the paracellular barrier (135). Therefore, disruption of these important

Fig. 8. Representative photographs of Pac2 zebrafish fibroblasts. The left panel shows zebrafish fibroblasts treated with heat-inactivated TcdB. The right panel reveals zebrafish fibroblasts 2 hours post-treatment with TcdB. Intoxicated cells undergo morphological changes termed “arborization” or “actinomorphic,” where the cell body retracts but retains “neurite-like” protrusions.

Heat-inactivated TcdB



TcdB



cellular properties and increases in paracellular permeability would clearly contribute to the pathophysiology of CDAD patients.

Although the targeted substrates of TcdA and TcdB are similar, TcdB is 4-fold to 200-fold more cytotoxic than TcdA, depending on cell type (210). Several investigations have revealed that TcdA has a lower enzymatic activity (31) and V_{max} (33) compared to TcdB and that following microinjection into the cell, TcdB demonstrated a significant difference in cytotoxicity compared to TcdA (31). The difference in cytotoxicity can also be attributed to the differences in the TcdA and TcdB receptor-binding domain and cell-surface receptor availability (210).

Both TcdA and TcdB induce cell death via apoptosis (22, 164). Members of the Rho GTPase family are known to regulate cell growth and survival pathways and thus their inactivation can promote cell death pathways via apoptosis (154, 179). In addition to inducing cell death via the inactivation of small GTPases, both toxins can cause cell death by directly interacting with the mitochondria. TcdA induces apoptosis by localizing at the mitochondria and disrupting this important organelle, an event that appears to occur prior to the glucosylation of Rho proteins (79). Recent studies by Matarrese, et al, found that TcdB also directly impacted the mitochondria by causing mitochondrial hyperpolarization. Using isolated mitochondria, TcdB was also found to cause mitochondrial swelling and release of

cytochrome c, a pro-apoptotic factor (134). Other investigations indicate that TcdB is capable of inducing caspase 3-dependent apoptosis as well as caspase-independent apoptosis (164). Interestingly, studies indicate that cell death does not occur until 24 h post-intoxication (164), well after changes in the actin cytoskeleton occur; however, most likely both the cytopathic and cytotoxic effects of the toxins are important contributors to the pathology of CDAD.

TcdA and TcdB in disease

TcdA and TcdB are the major virulence factors of *C. difficile* and are examples of toxins that alone can recapitulate many of the disease symptoms (210). The toxin-related effects on the actin cytoskeleton, tight junctions, and cell viability together with immunopathological events (most of which are toxin-mediated as well) illicit the typical pathology of *C. difficile* colitis: massive inflammatory infiltration, damage to the intestinal mucosa, and fluid secretion (210).

A hallmark of CDAD is an intense, acute inflammatory response in the intestines, which can be attributed both directly and indirectly to the toxins. TcdA and TcdB have been shown to directly activate monocytes and stimulate the release of interleukin-8 and tumor necrosis factor-α (TNF-α) (120, 185). Toxin-activated macrophages also release macrophage inflammatory protein 2 (MIP-2) and substance P, both of which have been shown to stimulate fluid secretion and

contribute to inflammation (29, 210). In studies by Kelly et al, TcdA was shown to directly bind to neutrophils (103). Interestingly, mice that are deficient in mast cells are less sensitive to TcdA and demonstrate a reduction in neutrophil recruitment and fluid accumulation (Wershil, 1998) and TcdA was also shown to cause the degranulation mast cells, which in turn produces TNF-α (27). All of these events contribute to the massive neutrophil recruitment, inflammation, and extravasations of the colon, a typical pathology of a CDAD patient.

TcdA acts as an enterotoxin, causing a fluid accumulation within the colon. However, unlike the rice-water fluid induced by other enterotoxins, such as cholera toxin, TcdA induces hemorrhagic fluid production and substantial mucosal edema (126, 144, 159). Also in contrast to cholera toxin, TcdA causes gross tissue damage by damaging the microvillus brush border, resulting in the shedding of cells into the lumen and the formation of shallow ulcers on the mucosa membrane (126, 144). In addition to tissue damage, following luminal application of TcdA in rats, a release of substance P and calcitonin gene-related peptide occurred (160). Both of these peptides are implicated in the production of fluid and diarrhea (170).

There are limited reports of TcdB effects on cells of the gastrointestinal tract, and, in general, TcdB has very little enterotoxic activity and is considered to be a potent cytotoxin (210). Interestingly, TcdB was found to be only effective when given

intragastrically with low doses TcdA or when the intestines were mechanically damaged (127). This suggests a synergy between TcdB and TcdA or perhaps other host-damaging *C. difficile* factors. It also suggests that TcdA may facilitate the systemic release of TcdB.

CHAPTER III

RATIONALE

Because bacterial toxins are important contributors of disease, these virulence factors have been studied extensively over the last century. While much is known about toxin mechanism of action and even toxin structure, the cells and organs targeted by many bacterial toxins during disease are unknown, leaving their overall impact on the host poorly understood. *Clostridium difficile* toxin B (TcdB) is an example of a bacterial toxin studied extensively *in vitro*, but whose *in vivo* activities are poorly understood (210). TcdB is a potent cytotoxin with a broad cell tropism and therefore is able to intoxicate a wide variety of cell types *in vitro* (16, 38, 121, 137, 194, 217). Yet, whether any of these cell types are impacted during disease is unknown.

In general, *Clostridium difficile*-associated disease (CDAD) is a considered to be a disease of the gastrointestinal tract; however, in severe cases of CDAD, patients experience symptoms outside the colon (35, 44, 91, 138, 174). Furthermore, despite the theoretical eradication of the organism in the gastrointestinal tract via effective antibiotic therapy and complete colectomies, patients suffering with fulminate CDAD exhibit systemic damage outside of the gastrointestinal tract and

can still succumb to the disease, evidence suggestive of released soluble factors such as TcdB (78, 155). Because circulating TcdB can be detected in serum and ascitic fluid, we hypothesize that TcdB could contribute to the systemic damage observed in patients with fulminate CDAD (166). Therefore, in order to fully understand TcdB's role in disease and design effective, toxin-specific CDAD therapeutics, it is necessary to determine the cell and organ tropism of TcdB *in vivo*. Identifying systemic targets of TcdB (and other toxins) has been limited since it is difficult to directly visualize the impact of these proteins on major organs in real-time.

To address this problem, zebrafish embryos are used herein to determine the *in vivo* targets of TcdB in real-time. Unlike other vertebrates, zebrafish embryos are transparent and major organs can be visualized by standard light-microscopy (219). Thus, zebrafish embryos provide a unique system for directly visualizing the effects of a TcdB intoxication event. Additionally, experiments are performed using the established models of TcdB intoxication (mouse) and *C. difficile* infection (hamster), further confirming any results discovered in the zebrafish model. The findings provide important insight into the *in vivo* activities of TcdB, and will present the zebrafish embryo as a model for determining the systemic targets of bacterial toxins.

CHAPTER IV

IDENTIFICATION OF *CLOSTRIDIUM DIFFICILE* TCDB CARDIOTOXICITY USING A ZEBRAFISH EMBRYO MODEL OF INTOXICATION

Identification of *Clostridium difficile* TcdB Cardiotoxicity Using a Zebrafish Embryo Model of Intoxication. (Elaine E. Hamm, Daniel E. Voth, and Jimmy D. Ballard). ***Proceedings of the National Academy of Sciences.*** **(2005). 104:14176-81.**

Abstract

Clostridium difficile TcdB has been studied extensively using cell-free systems and tissue culture; but, like many bacterial toxins, the *in vivo* targets of TcdB are unknown and have been difficult to elucidate with traditional animal models. In the current study, the transparent *Danio rerio* (zebrafish) embryo was used as a model for imaging of in vivo TcdB localization and organ-specific damage in real-time. At 24 hours post treatment, TcdB was found to localize at the pericardial region, and zebrafish exhibited the first signs of cardiovascular damage, including a 90% reduction in systemic blood-flow and a 20% reduction in heart-rate. Within 72 h of exposure to TcdB, the ventricle chamber of the heart became deformed and was unable to contract or pump blood, and the fish exhibited extensive pericardial

edema. In line with the observed defects in ventricle contraction, TcdB was found to directly disrupt coordinated contractility and rhythmicity in primary cardiomyocytes. Furthermore, using a caspase-3 inhibitor we were able to block TcdB-related cardiovascular damage and prevent zebrafish death. These findings present the first insight into the *in vivo* targets of *C. difficile* TcdB, as well as demonstrate the strength of the zebrafish embryo as a tractable model for identification of *in vivo* targets of bacterial toxins and evaluation of novel, candidate therapeutics.

Introduction

Protein toxins are produced by bacterial pathogens during disease and have evolved different functions ranging from pore-formation in plasma membranes to enzymatic activities that alter intracellular signaling, cell cycle, apoptosis, and protein synthesis in targeted cells (178). Mechanisms of receptor-binding, cell entry, membrane insertion, and enzymology are routinely determined using a broad range of cell types *in vitro*, yet for many toxins the cell types targeted during disease are unknown (62).

Clostridium difficile toxin B (TcdB) is an example of a bacterial toxin studied extensively *in vitro*, but the *in vivo* activities remain poorly understood (210). TcdB is a potent (LD_{50}= 200 ng/kg) intracellular bacterial toxin; the protein enters cells via receptor-mediated endocytosis, translocates to the cytosol, hydrolyzes UDP-glucose, and transfers the liberated sugar to a reactive threonine in the effector binding loops of the small GTPases Rho, Rac, and Cdc42 (16, 55, 98, 99). As a result, cultured cells treated with TcdB exhibit changes in cell morphology and undergo apoptosis, eventually leading to death of the cell (10, 17, 164). TcdB intoxicates numerous cell types *in vitro* including fibroblasts, neuronal cells, epithelial cells, endothelial cells, lymphocytes, and hepatocytes (16, 38, 121, 137,

194, 217), yet whether any of these cell types are targeted during *C. difficile* associated disease (CDAD) is unknown.

CDAD involves extensive gastrointestinal damage, however these pathologies appear to be caused by TcdA, a similar toxin encoded on the same pathogenicity locus as TcdB (46, 127). This raises further questions about the role of TcdB in disease. Previous studies have shown that TcdB is only effective when the intestinal mucosa is damaged (221), suggesting that the intestinal effects of TcdA facilitate the entry of TcdB into the bloodstream. In life-threatening cases of CDAD, systemic complications include cardiopulmonary arrest (96), acute respiratory distress syndrome (91), multiple organ failure (44), renal failure (35), liver damage (174), and each could be due to the systemic effects of TcdB. Hence, identifying the cells targeted *in vivo* by TcdB is needed in order to gain relevant insight into the disease-related activities of this toxin, and advance our understanding of CDAD.

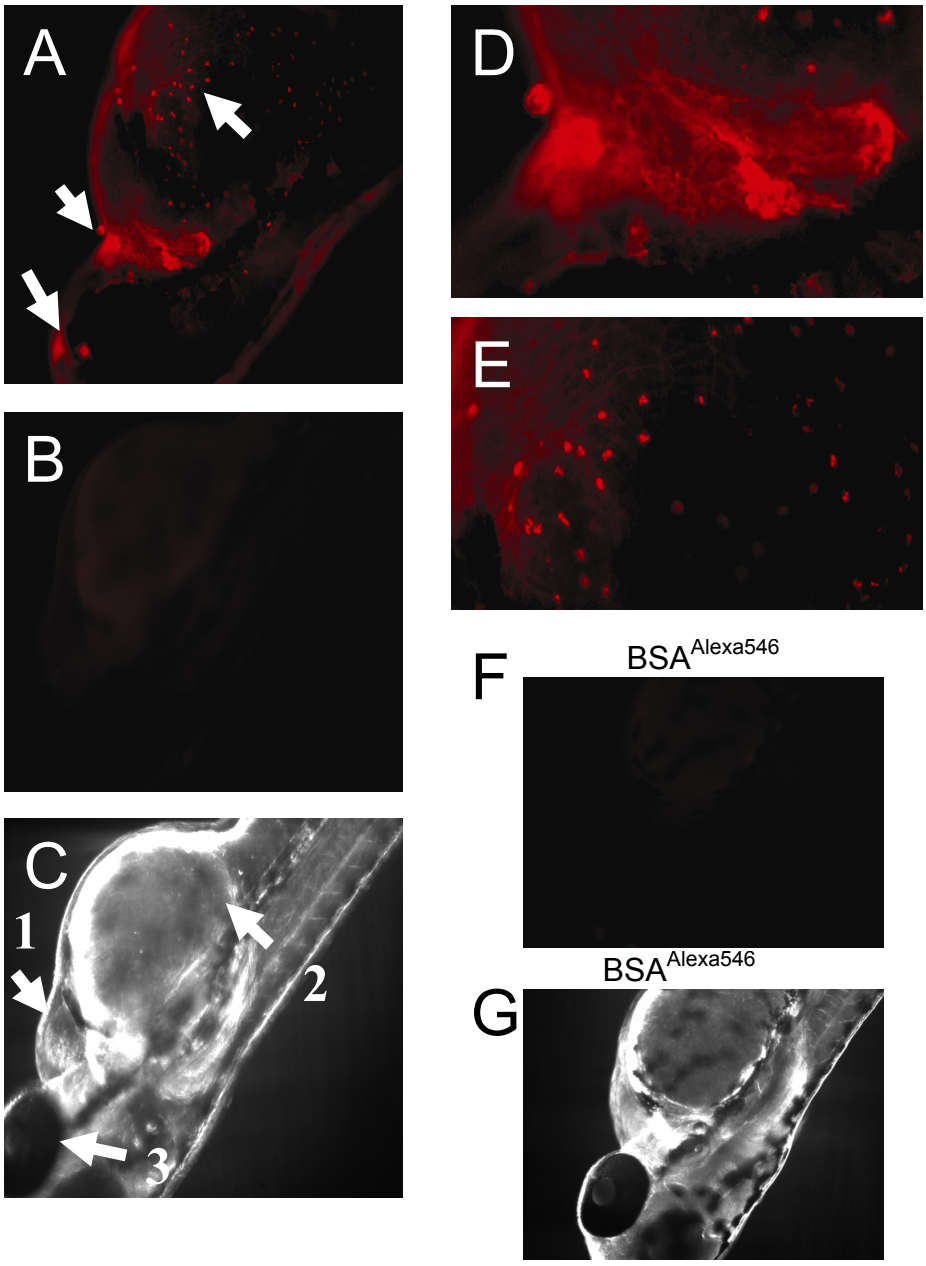
Identifying systemic targets of bacterial toxins such as TcdB has been limited since it is difficult to directly visualize the impact of these proteins on major organs in real-time. To overcome this problem, zebrafish embryos were used herein to characterize the systemic impact of TcdB in real-time. Unlike other vertebrates, zebrafish embryos are transparent and major organs can be visualized by

standard light-microscopy (219). Thus, zebrafish embryos provide a unique system for directly visualizing the temporal and spatial effects of TcdB intoxication. By using the zebrafish embyro as a model, in the current work we have found that TcdB functions as potent cardiotoxin, reducing blood-flow and ventricle contraction. Furthermore, corresponding to TcdB's known pro-apoptotic activity, a caspase-3 inhibitor was found to alleviate the cardiotoxic effects of TcdB. These findings provide important insight into the *in vivo* activities of TcdB, and present the zebrafish embryo as a model for determining the systemic targets of bacterial toxins.

Results

Localizaton of TcdB in zebrafish embryos. To determine the localization of TcdB, zebrafish were treated with fluorescently-labeled (Alexa-Fluor 546) TcdB ($TcdB^{Alexa\text{-}546}$) approximately 48 hours post-fertilization (hpf) and examined by fluorescence microscopy for sites of toxin tropism. As shown in Fig. 9, following a 24 h treatment with the toxin, $TcdB^{Alexa\text{-}546}$ localized at the frontal ventral portion of the fish, with specific foci formed within the pericardial region. Localization of $TcdB^{Alexa\text{-}546}$ was also observed in an anatomical region corresponding to the outflow chamber of the heart (see arrow in Fig. 9A). Magnified views, as shown in Fig. 9D and E, reveal intense localization near the cardiac region. In contrast, the negative control, fluorescently-labeled bovine serum albumin (BSA) $BSA^{Alexa\text{-}546}$, did not show detectable anatomical localization within the zebrafish (Fig. 9F-G).

Fig. 9. TcdB localization observed in zebrafish. (A) Zebrafish (48 hpf) treated with 37 nM $TcdB^{Alexa\ 546}$ for 24 h. Arrows indicate toxin accumulation around the pericardial sac as well as distinct foci on the yolk sac and upper cranial region. **(B)** Zebrafish treated with 37 nM $TcdB^{Alexa\ 546}$ and 370 nM TcdB receptor binding domain (RBD). **(B)** Exhibited a substantial decrease in fluorescence, indicating that that the RBD can competitively reduce TcdB binding and TcdB localization is specific. Images were acquired and processed using identical intensity and contrast settings for each sample. **(C)** Brightfield image of zebrafish treated with Zebrafish treated with 37 nM $TcdB^{Alexa\ 546}$ and 370 nM TcdB receptor binding domain (RBD). Arrow 1 points to the heart, arrow 2 indicates the gastrointestinal tract, and arrow three points to the eye **(D)** Magnification of the pericardial region and **(E)** the yolk sac of a zebrafish treated with $TcdB^{Alexa\ 546}$. **(F)** Zebrafish treated


A
B
C
1
2
3
D
E
F
BSA Alexa546
G
BSA Alexa546

To further demonstrate specificity of TcdB localization, competition experiments were performed using the putative receptor binding domain (RBD) of TcdB. As shown in Fig. 9B, co-treatment with 30 fold molar excess of the TcdB receptor binding domain reduced the detectable levels of labeled toxin to that observed in the BSA control. Collectively, these observations suggested TcdB exhibits specific tissue tropism in the zebrafish, with the toxin primarily localizing to the yolk-sac, pericardial, and cardiac region of the zebrafish.

Treatment of zebrafish embryos with TcdB results in damage to the cardiovascular system. Experiments were performed to determine the effects of TcdB on zebrafish physiology. For initial analysis, zebrafish embryos were collected at 24 hpf and exposed to TcdB, heat-inactivated TcdB, or buffer alone. Treatment with doses ranging from 0.037 nM to 0.37 nM did not cause detectable damage to the zebrafish embryos (data not shown). However, exposure of the embryos to doses of toxin ranging from 3.7 nM to 37 nM resulted in distinct, dose-dependent changes in zebrafish physiology and anatomy.

The time-course and specific changes in physiology are summarized in Table 1. A decrease in heart rate was the first physiological change observed; 72 ± 6 beats/30s in the control compared to 57 ± 3 beats/30s in TcdB-treated fish ($p < 0.001$). Corresponding to the reduced heart rate, a visible reduction in blood flow

HPT[a]	System/Organ Impacted[b]	Observed Phenotype
24	Cardiovascular	Reduced blood flow
	Heart	Decreased beats per minute and arrhythmia
24-48	Heart	Decrease in chamber contraction Elongated ventricle and atrium Changes in heart morphology Mild Pericardial edema
48-72	Heart	Arrhythmia Necrotic Ventricle and Atrium Ventricle unable to contract Severe pericardial edema
	Gastrointestinal	Necrosis
	Liver	Discoloration
72-96	Heart	Complete loss of morphology Atrium unable to contract
	Gastrointestinal	Severe necrosis
	Epithelium	Degeneration of the epithelium

a. 48 hpf treated with 37 nM TcdB
b. Observed by standard light microscopy at 100X magnification

Table 1. Summary of Temporal Systemic Effects of TcdB

(calculated as the red blood cell (RBC) perfusion rate) was also observed. In control fish, RBC perfusion rate within the intersegmental veins was 402 ± 10.6 RBC/30s at 24 h post-treatment. In comparison, in TcdB-treated zebrafish, the RBC perfusion rate within intersegmental veins was 44 ± 1.49 RBC/30s ($p < 0.0001$) (See Fig. 10 and supplemental Movie 1-2 on the PNAS web site). Reduced blood-flow was observed in the caudal and intersegmental veins, and appeared to occur in the absence of detectable damage to the vascular endothelium. To confirm this, fli1::EGFP zebrafish, which express GFP in endothelial cells, were utilized to assess vein integrity following treatment with TcdB. As shown in Fig. 10B and C, despite a loss in blood flow, the veins of toxin-treated zebrafish appeared to be intact.

TcdB-treated zebrafish were examined for cardiac damage, as a possible explanation for the reduction in blood-flow. Between 24-48 h post-treatment, there was a decrease in ventricle chamber contractility and loss in heart looping (see Fig. 11 and the supplemental Movies 3-4). As shown in Fig. 11, in control fish, the ventricle exhibited a dynamic change in size of 20% during contraction and expansion (see supplemental Movie 3). However, treatment with TcdB substantially reduced the change in ventricle size during beating (see Movie 13), indicating the heart was unable to contract and expand in a normal fashion. At approximately 48 h post-treatment, both the atrium and ventricle were deformed

Fig. 10. RBC perfusion rate and vein integrity of TcdB-treated zebrafish. (A) Red blood cell perfusion (RBC) rate was used as a measurement of blood flow and calculated as the average # of RBC/30 s in the intersegmental veins (n=27) with error bars representing standard error. Zebrafish treated with TcdB had a reduced RBC rate compared to the heat-inactivated TcdB control. Transgenic fli1::EGFP embryos were treated with TcdB and, after a loss of blood flow became apparent, were examined for changes in vein ultrastructure. Images of veins of control heat-killed zebrafish (B) and veins of TcdB-treated zebrafish (C). Images are viewed at 40X magnification using a Leica® confocal microscope and were processed using Leica® Confocal Software.

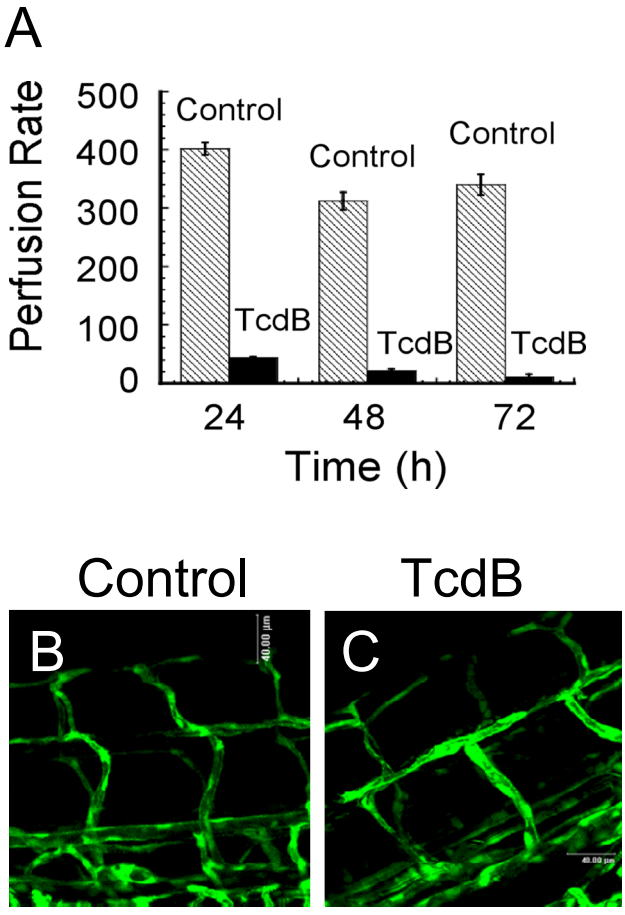
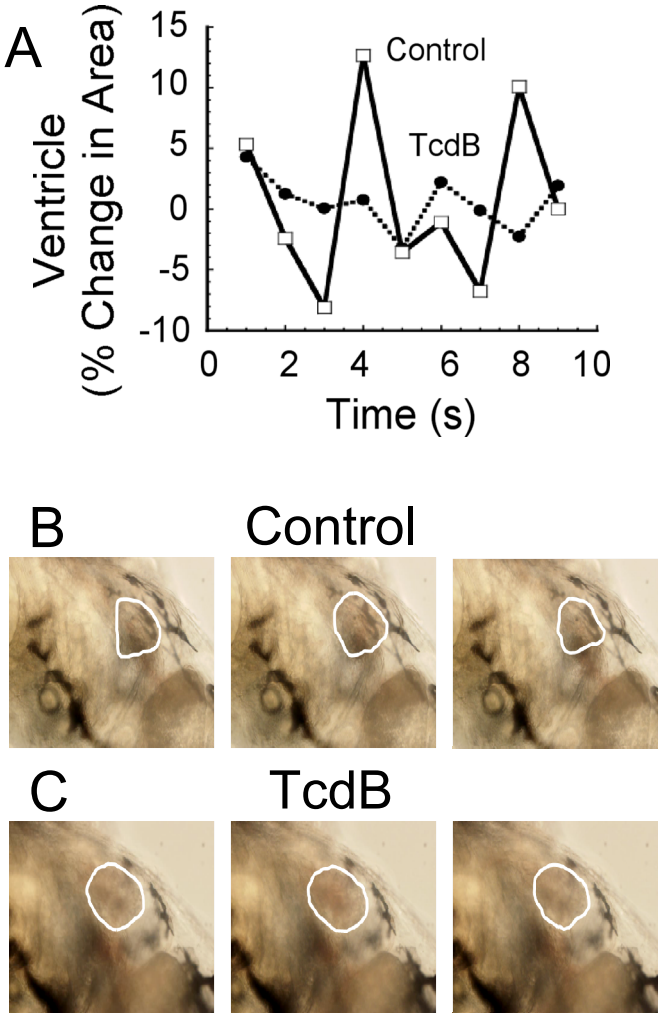

A
Perfusion Rate
500
400
300
200
100
0
Control
TcdB
Control
TcdB
Control
TcdB
24
48
72
Time (h)
Control
TcdB
B
C

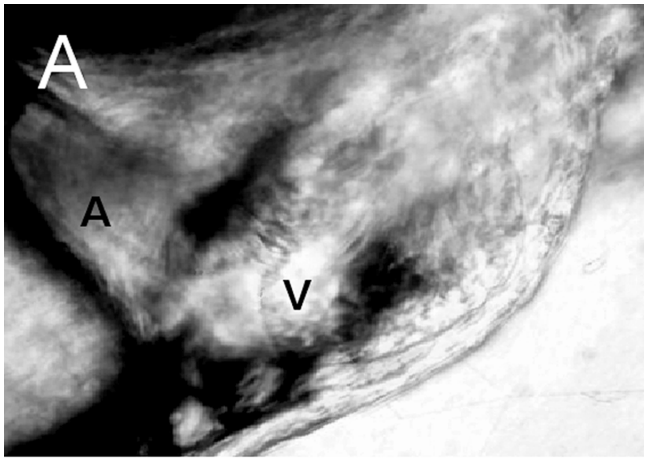
Fig. 11. Comparisons of contraction and expansion dynamics of the ventricle in control-and TcdB-treated zebrafish embryos. (A) Plot of relative changes in ventricle size in heat-inactivated TcdB (control) (black line) and TcdB-treated (dashed line) embryos over at 10 s time-period. TcdB-treated zebrafish had a marked decrease in overall ventricular size. **(B)** Panels showing incremental contraction and expansion in control zebrafish embryos. **(C)** Panels showing absence of contraction and expansion in TcdB-treated embryos. Ventricle is denoted in white.


A
Ventricle
(% Change in Area)
15
10
5
0
-5
-10
0
2
4
6
8
10
Time (s)
Control
TcdB
B
Control
C
TcdB

(Fig. 12). By 7 days post-treatment, 100% of TcdB-treated fish exhibited pericardial edema, with 70% developing whole-body edema (Fig. 13). TcdB-treated fish survived between 7-10 days after initial exposure to TcdB, but by 11 days post-treatment, 100% of the fish died. Similar cardiovascular defects were observed in fish treated with TcdB 24, 72, and 96 hpf, indicating that toxin effects were not dependent upon treatment at a particular stage of development (data not shown). Collectively, these observed defects indicated TcdB disrupts cardiac function.

Delivery of the TcdB enzymatic domain with a surrogate system results in systemic damage. Experiments were next performed to determine if the cardiotoxicity of TcdB was due to heightened sensitivity of this organ to the toxin's enzymatic activity, or if this effect results from a preferential localization of the toxin to the heart. Arguably, if TcdB cardiotoxicity were due to specific toxin tropism, then delivery of the enzymatic domain with a system that targets multiple tissues, should reduce these effects, as the enzymatic domain would be distributed throughout the body. Alternatively, if cardiotoxicity were due to heightened sensitivity of cardiac tissue to the enzymatic activity of TcdB, then the heart should be preferentially impacted despite localization of the enzymatic domain to multiple tissues. To address this issue we took advantage of a

Fig. 12. Morphological changes in zebrafish heart following exposure to TcdB. (A) Zebrafish embryos treated with 37 nM heat-inactivated TcdB at 37 hpf. (**B)** Zebrafish embryos (37 hpf) treated with 37 nM TcdB at 37 hpf (n=50). Images are of live embryo hearts at 48 h post treatment. A=Atrium, V=Ventricle


A
A
V

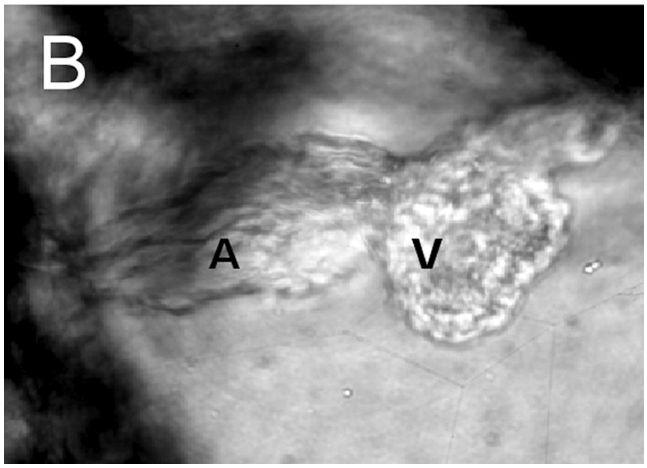
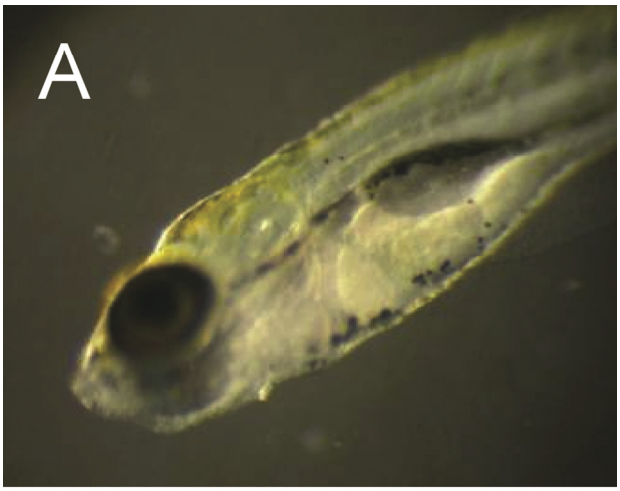
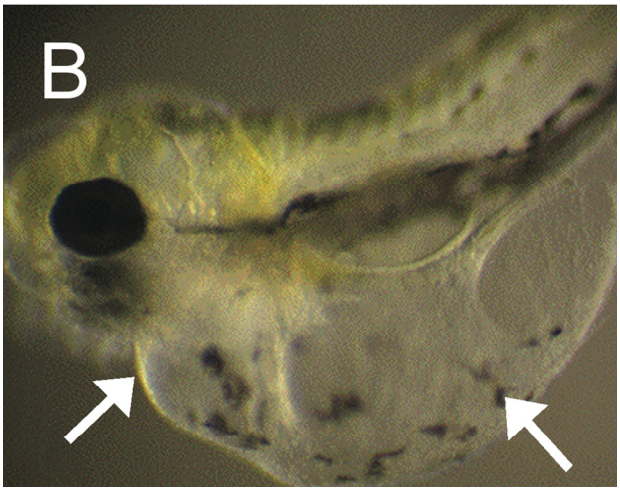

B
A
V

Fig. 13. Representative photographs of zebrafish following exposure to TcdB. (A) Control zebrafish treated heat-inactivated TcdB (37 nM). **(B)** Defects observed in cleavage stage-treated fish 144 h post treatment treated with 37 nM TcdB (n=50). Arrows indicate regions of massive edema observed following cardiac damage.


A


B

previously described heterologous delivery system derived from the cell entry components of anthrax lethal toxin, which consists of *Bacillus anthracis* protective antigen (PA) and LFnTcdB$^{1-556}$, a fusion protein consisting of the translocation active, non-toxic 255 amino-terminal residues of anthrax toxin lethal factor (LFn) and the enzymatic glucosylation region of TcdB (amino acids 1-556) (4, 142, 186). Previous studies have shown that PA can target multiple tissues within the zebrafish (197, 211). As shown in Fig. 14, zebrafish embryos treated with PA and LFnTcdB$^{1-556}$ exhibited widespread tissue damage, which differed substantially from that observed in TcdB-treated zebrafish. These findings suggest that TcdB-related cardiac damage may involve a specific tropism for cardiac tissue.

TcdB modulates cardiomyocyte physiology. Results from the zebrafish treatments indicated that TcdB could influence cardiac function, perhaps by directly targeting functional cells of the heart. To further elucidate TcdB-cardiotoxic effects, experiments next sought to determine if this toxin were capable of disrupting the physiology of cardiomyocytes. In these experiments, cultured cardiomyocytes were treated with TcdB and examined for changes in overall morphology, contraction, and viability. Within two hours of toxin treatment, the cardiomyocytes exhibited morphological changes. Phalloidin staining of actin in TcdB-treated cardiomyocytes revealed distinct changes in cellular structure when

Fig. 14. Representative photographs of zebrafish following exposure to LFn-TcdB$^{1-556}$. **(A)** Zebrafish treated with LFn-TcdB$^{1-556}$ alone resembled untreated zebrafish. **(B)** Zebrafish treated with PA and LFn-TcdB$^{1-556}$ (n=50). Arrow indicates tissue damage (visualized as tissue discoloration).

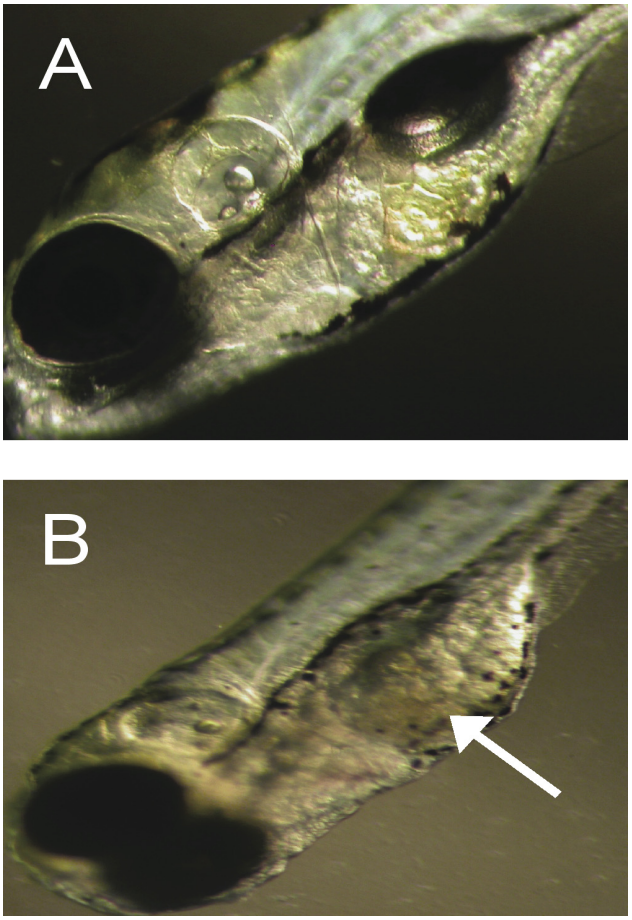
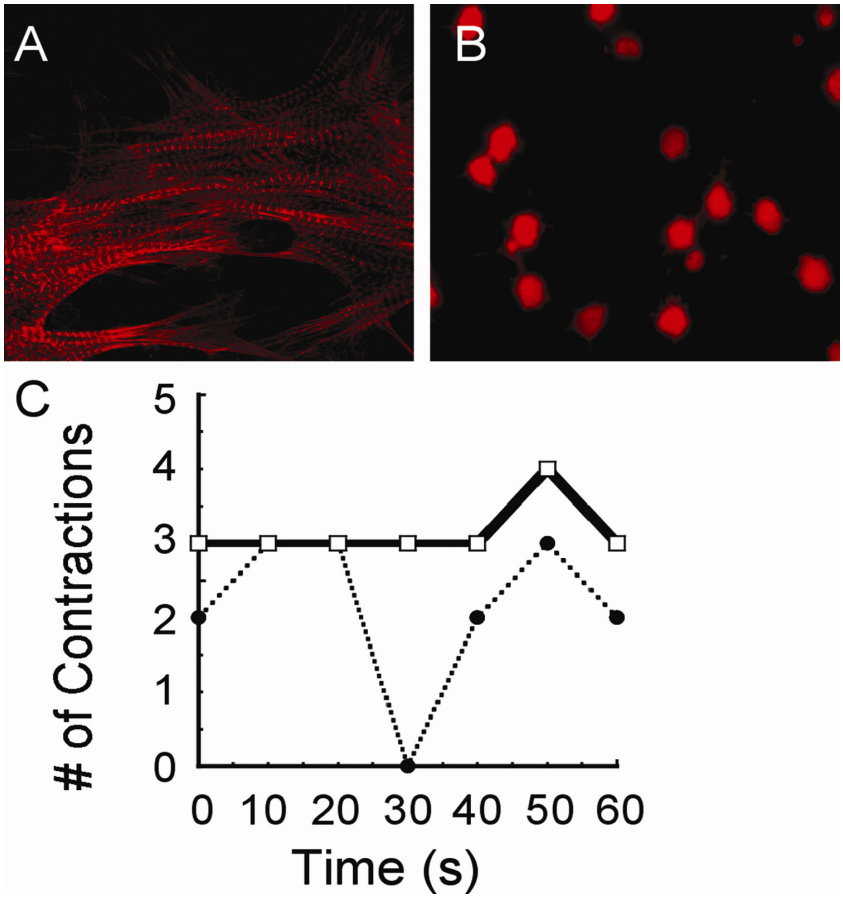

A
B

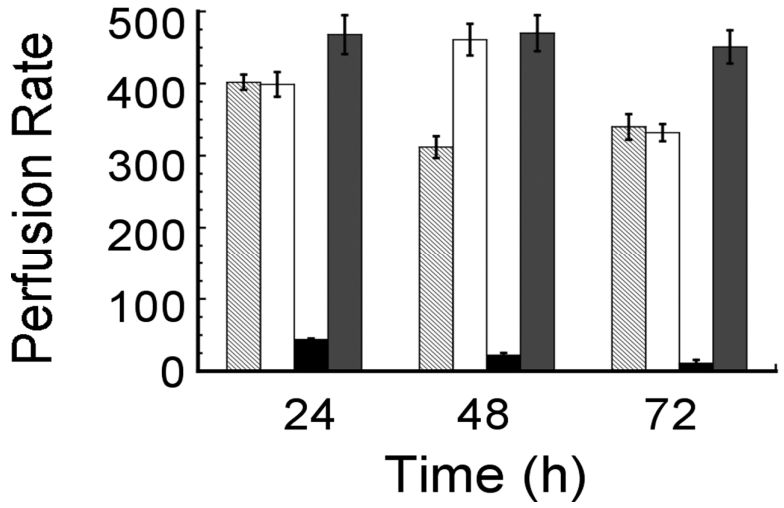
Fig. 15. TcdB intoxicates cultured primary rat cardiomyocytes (RCm). (A) Rhodamine phalloidin actin stain of RCm treated with heat-inactivated TcdB (7.4 nM). **(B)** RCm treated with TcdB (7.4 nM) and stained for actin 4 hpt. **(C)** Changes in the number of contractions over time were also explored. Heat-inactivated TcdB-treated is represented by the solid, black line while TcdB-treated RCm is denoted by the dashed line. Contractions were measured 4 h post treatment as number of contractions per 60 s.


A
B
C
# of Contractions
0
1
2
3
4
5
0 10 20 30 40 50 60
Time (s)

compared to cardiomyocytes treated with heat-inactivated TcdB (Fig. 15A and B). Control cells had numerous dense bodies of fibrils and bundled Z-bands (Fig. 15A), while these structures were undetectable in cardiomyocytes treated with TcdB (Fig. 15B). By 5 h post-treatment, the cardiomyocyte contractions were less coordinated and arrhythmic (Fig. 15C, see supplemental Movie 5-6). Finally, 48 h post-treatment, there was a 60% decline in cardiomyocyte viability (data not shown).

Caspase-3 inhibitor reduces cardiovascular damage in TcdB-treated zebrafish. TcdB is known to cause cytotoxicity via apoptosis, and blocking caspase activity in cell culture slows the rate of death following treatment with TcdB (164). Hence, experiments were designed to determine if cardiotoxicity correlates with the ability of TcdB to induce apoptosis, and whether inhibition of apoptosis could provide protection against the systemic effects of this toxin. In these experiments, zebrafish embryos were treated with TcdB and co-treated with Ac-DMQD-CHO, a water-soluble tetrapeptide inhibitor of caspase-3. TcdB-treated and control zebrafish were observed for changes in heartbeat and overall blood flow. As shown in table 2 zebrafish treated with TcdB exhibited a significant decrease in heartbeats/30s as compared to the control; yet, zebrafish co-treated with caspase-3 inhibitor presented heart rates similar to control zebrafish along with a normal RBC perfusion rate (Fig. 16). The events quantified

Fig. 16. Caspase-3 Inhibitor reduces the cardiotoxic effects of TcdB. Following concomitant exposure to TcdB and caspase-3 inhibitor, zebrafish were examined for reduction of TcdB-related phenotype, specifically decrease in heart function. The RBC perfusion rate of treated zebrafish over time (stripped bar = heat-inactivated TcdB, white bar = caspase-3 inhibitor IV, black bar = TcdB, and dark grey = TcdB + caspase-3 inhibitor IV. RBC perfusion rate is calculated as the average # of RBC/30 s in the intersegmental veins (n=27) with error bars representing standard error.


Perfusion Rate
500
400
300
200
100
0
24
48
72
Time (h)

Treatment	Heartbeat per 30 s, 72 h after treatment (± standard deviation)
Heat-inactivated TcdB	72 ± 6
Caspase-3 inhibitor	76 ± 7
TcdB	57 ± 3
TcdB + caspase-3 inhibitor	78 ± 2

Table 2. Heart rate of treated zebrafish

in Fig. 15 can be viewed as video (see Movie 7) in the supplementary material on the PNAS web site. Furthermore, the addition of caspase-3 inhibitor decreased the frequency and severity of damage observed in the TcdB-treated zebrafish (Fig. 16 and 17). These results indicate the cardiotoxic effects of TcdB can be alleviated by a caspase-3 inhibitor.

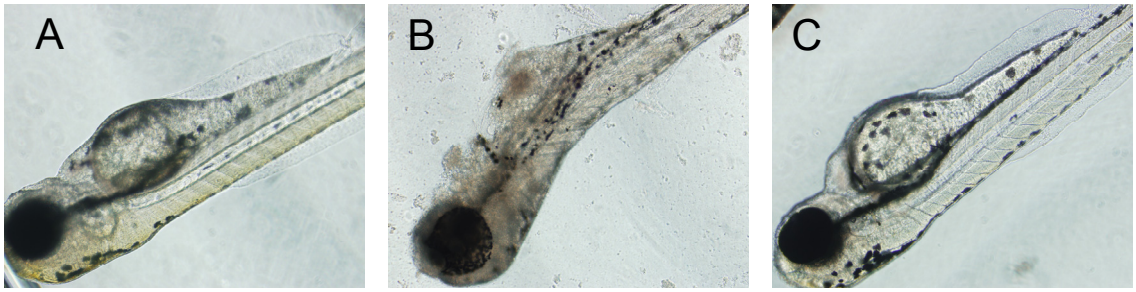
Discussion

Tissue damage and inactivation of specific cell-types by bacterial toxins is an integral part of many infections and is important for colonization, immune evasion, and progression of disease. Thus, toxin immunogenicity and mechanisms of action have been studied for over a century, leading to new vaccines and an understanding of these virulence factors at the molecular level. Yet, despite numerous advances in the study of bacterial toxins, little is known about cell types targeted *in vivo* (62). In particular, and important to the current study, although *C. difficile* TcdB is a potent cytotoxin, has a low LD_{50}, and causes rapid death in animal models, the overall physiological systems impacted by this toxin have not been identified.

While *in vitro* studies have provided important insight into TcdB's mechanisms of cell entry, membrane translocation, and enzymatic activity, it is difficult to apply

Fig. 17. Caspase-3 inhibitor prevents TcdB-related damage in zebrafish. Zebrafish embryos were exposed to heat-inactivated TcdB, TcdB, or TcdB plus a caspase-3 inhibitor 72 hpf. Approximately, 12 h post-treatment, zebrafish were observed for changes in morphology. **(A)** Zebrafish treated with a high dose of heat-inactivated TcdB resembled untreated zebrafish. **(B)** Zebrafish exposed to a high dose of TcdB demonstrated massive tissue degeneration while **(C)** zebrafish treated with TcdB and caspase-3 inhibitor resembled control zebrafish.

Control TcdB



this knowledge to the *in vivo* setting. *In vitro* analysis cannot mimic toxin receptor availability within the host nor reflect overall organ sensitivity to TcdB. Therefore, in order to characterize TcdB's systemic effects, we sought an animal model that would allow direct, in vivo visualization of events leading to death following exposure to the toxin. Contemporary models, such as higher order primates, rodents (23), *Drosophila melanogaster* (37, 43, 129), and *Caenorhabditis elegans* (1, 50) were considered for assessing the systemic effects of TcdB, but these lacked many of the qualities needed for the current study. It is difficult to directly visualize all the major organs in higher order models, and the fruit-fly and nematode systems lack the organ complexity needed for a thorough study of systemic damage. In contrast, the zebrafish embryo provides several distinct advantages over these traditional models. In addition to having many of the major organs found in humans, zebrafish embryos are transparent, which allows direct visualization of labeled toxin and toxin-induced changes in anatomy and physiology (219). Indeed, these same characteristics have made the zebrafish a widely accepted model for the study of embryonic development and genetics (73) and infectious diseases (39, 141, 150, 162, 163, 203, 204).

The phenotypes of TcdB-treated zebrafish support the notion that intoxication with TcdB leads to cardiovascular damage. Indeed, many of the changes observed in the TcdB-treated embryos have been reported in mutant lines of zebrafish

defective in genes necessary for a functional heart. For example, *dead beat* (*ded*)(171, 187) and *heartstrings* (*hst*)(64) are documented to possess cardiac defects that result in poor contractility and pericardial edema (*ded*) or stretching and loss of function of cardiac chambers as well as a loss in circulation (*hst*). These phenotypes were observed in the TcdB-treated embryos, further suggesting that the toxin impacts cardiac function. Additionally, results from the toxin localization experiments using TcdB labeled with a trackable marker, and the delivery of the TcdB enzymatic domain with PA and LFn, support the idea that the toxin preferentially localizes to the heart. A specific *in vivo* affinity for cardiac tissue may explain why TcdB can damage many cell types *in vitro*, but primarily impacts the heart in the zebrafish.

In the zebrafish embryo studies, loss in chamber contractility was observed, allowing for the identification of a relevant candidate cell type, cardiomyocytes, for further studies in cell culture. Whether cardiomyocytes are the only cardiac cells impacted by TcdB is not known; however, loss of cardiomyocytes will have the most dramatic impact on the heart and may provide an explanation for TcdB-related defects in contractility. These cells are central to heart contraction and the movement of blood, thus even subtle intoxication events could have dramatic effects. Furthermore, unlike cardiac fibroblasts and endothelial cells, cardiomyocytes are not renewable, and loss of these cells can result in chronic

heart problems (89, 128). It seems reasonable to predict that if other cells are impacted by TcdB within the heart, death of cardiomyocytes would have a more severe and sustained effect on the host.

In cardiac diseases that involve activation of apoptosis in cardiomyocytes, caspase inhibitors have been promoted as promising treatments (85, 86, 227). Moreover, prior work has shown that inhibitors of apoptosis, caspase inhibitors in particular, reduce the cytotoxic effects of TcdB (164). Thus, it was hypothesized that inhibition of caspase-3 would alleviate the cardiac damage caused by this toxin. The results of our study show that TcdB's damaging and fatal cardiotoxic effects could be prevented through the use of a caspase-3 inhibitor. To our knowledge, this is the first example in which a caspase inhibitor blocked *in vivo* effects of a bacterial exotoxin. Moreover, these results suggest these compounds and other modulators of apoptosis could be promising therapeutics for treating advanced CDAD.

It is important to consider the current findings in the context of CDAD in humans. Cardiotoxicity could explain many of the observed clinical signs of serious CDAD. Patients with advanced CDAD experience multi-organ failure, and decreases in cardiac function could be one of the factors contributing to this event (44). Death of CDAD patient has also been directly associated with cardiac arrest (21, 96, 182,

199). Collectively, these data suggest that inactivation of Rho, Rac, and Cdc42, by TcdB leads to altered cardiac activity and cell death in the heart of CDAD patients. Ongoing studies assessing cardiac damage in infectious models of CDAD will help in directly addressing this hypothesis.

In summary, results from the current study now provide insight into a possible mechanism by which *C. difficile* causes severe systemic damage during CDAD. By functioning as a cardiotoxin, TcdB may directly or indirectly cause much of the systemic damage observed in CDAD patients. These findings also indicate that the zebrafish embryo is a valuable model for identifying systemic targets of bacterial virulence factors and that this model is useful in the *in vivo* assessment of toxin therapeutics.

Materials and Methods

Protein isolation. TcdB was isolated as previously described (165). *Bacillus anthracis* protective antigen (PA) and LFnTcdB$^{1\text{-}556}$ were isolated as previously described (4, 142, 186). The 2165 nucleotides from the 3' end of the *tcdb* gene, which encode for the putative receptor binding domain (RBD) of TcdB, were cloned in-frame into the pET15b plasmid (Novagen). All recombinant proteins were

expressed as a His_6 fusion in *Escherichia coli/*BL-21 DE3 and isolated using Ni^{2+} affinity chromatography according to the manufacturer's protocol (Novagen).

Fluorescent labeling of protein. TcdB and BSA were labeled with a reactive fluorescent dye Alexa-Fluor^{546}, according to manufacturer's instructions (Molecular Probes, Invitrogen). The relative activity of labeled TcdB to unlabeled TcdB was determined using a standard cytoxicity assay. Labeling of TcdB did not reduce the effective cytotoxic dose of the toxin by more than 20%.

Zebrafish maintenance and care. Wild-type zebrafish were obtained from Aquatic Eco-system (Apopka, FL) and mutant fli1::EGFP fish were obtained from ZFIN (University of Oregon, Eugene, Oregon). Zebrafish were maintained at 28.5ºC on a 14 h light/10 h dark cycle in a Z-plex unit (Aquatic Habitats) and matings, embryro collection, and preparation were performed as previously described (219).

In vivo toxin localization studies using fluorescently labeled TcdB. Zebrafish embryos were placed into a 96-well plate (5 embryos/well) 24 h post fertilization (hpf) and allowed to incubate with TcdB$^{Alexa\ 546}$ (3.7 nM-100 nM) or TcdB $^{Alexa\ 546}$ (3.7 nM-100 nM) and 30 fold molar excess TcdB RBD for 24 h. Control zebrafish were incubated with 100 nM BSA$^{Alexa\ 546}$. Subsequently, zebrafish were rinsed 10 times

in embryo water for 20 min and visualized using an Olympus BX81 epifluorescent microscope. Images were captured and processed using the Nikon Spot Software.

Treatment of zebrafish embryos with TcdB and LFnTcdB$^{1-556}$. For TcdB studies, zebrafish embryos were placed (5 embryos/well) into a 96-well plate and treated with 37 nM of TcdB, heat-inactivated TcdB, or 20 mM Tris-HCl buffer in replicates of 10 (50 embryo/experimental condition). Similarly, LFnTcdB$^{1-556}$–treated zebrafish were placed (5 embryos/well) into a 96-well plate and treated with 0.42-0.85 nM PA and LFnTcdB$^{1-556}$ in replicates of 10 (50 embryos/treatment). Controls included 0.85 nM PA or LFnTcdB$^{1-556}$ and 20 mM Tris-HCl. The embryos were observed for seven days post-treatment for morphological changes using a SZX-7 microscope with a DP70 camera (Nikon). All still and video images were captured and processed using the DP controller and DP manager software (Nikon). To calculate blood circulation, the red blood cell (RBC) perfusion rate was measured using the SZX-7 Nikon microscope with a DP70 video camera and is recorded as the number of blood cells detected within the intersegmental veins over a 30 s time period. RBC perfusion rate was measured in replicate countings in three separate veins in three separate fish and is reported as # of RBC/30 s.

Treatment of rat cardiomyocytes with TcdB. Primary rat cardiomyocytes (RCm) cells (Cell Application) were seeded into 96-well plates and treated in triplicate with 7.4 nM TcdB or heat-inactivated TcdB for five hours, and subsequently stained with rhodamine phalloidin according to the manufacturer's instructions (Molecular Probes, Invitrogen). Images were acquired using a TCS NT confocal microscope (Leica) and processed using Confocal Software (Leica). RCm cell viability post-TcdB treatment was quantified across a 72 h time-period using the Cell Counting Kit-8 (CCK-8, Dojindo).

Caspase-3 inhibitor assays. Zebrafish embryos were placed into a 96-well plate (at 5 embryos/well) in sterile embryo water and were treated concomitantly with TcdB (37 nM) and caspase-3 inhibitor IV (Calbiochem) (500 μM) and were observed up to one week post-treatment. Controls for this experiment included 500 μM caspase-3 inhibitor IV, TcdB (37 nM), and heat-inactivated TcdB (37 nM). The embryos were examined for changes in heart rate and RBC perfusion rate, as well as phenotype using SZX-7 Nikon microscope with a DP70 camera. All still and video images were captured and processed using the DP controller and manager software.

Statistical analysis of data. All statistical data was calculated using a Student's 2 tailed t-Test.

CHAPTER V

ELUCIDATING THE CARDIC DAMAGE OF FULMINATE *CLOSTRIDIUM DIFFICILE*-ASSOCIATED DISEASE: CONSIDERATIONS BEYOND THE GASTROINTESTINAL TRACT

Abstract

Clostridium difficile is the etiological agent of *Clostridium difficile*-associated disease (CDAD). This disease is primarily confined to the colon; however, patients with fulminant disease exhibit signs of severe systemic damage in addition to damage to the gastrointestinal tract. Because previous studies indicate that *C. difficile*'s exotoxin, TcdB, can act as a potent cardiotoxin, the current study investigates the potential cardiac damage from a TcdB intoxication event and *C. difficile* infection in rodents and in human patients. Results from rodent studies reveal that systemic TcdB intoxication event and infection with *C. difficile* cause massive myocardial infarct as well as edema, hemorrhaging, and marked vein dilation in the examined heart tissue. In addition, in a preliminary, retroactive review of patient autopsy data revealed that patients who died with CDAD also demonstrated massive damage to heart tissue with edema, hemorrhaging, lymphocytic infiltrates, and damage to myocardial fibers, similar to what was observed in the rodent disease models. In total, data from the current

investigation indicate that CDAD in animals and humans may result in severe damage to the heart.

Introduction

Clostridium difficile-associated disease (CDAD) impacts 250,000-1,000,000 people annually in the United States, making *C. difficile* a significant cause of infectious disease (222). Unfortunately, the number of cases of CDAD has increase in the past few years, with the US Nosocomial Infection Surveillance System reporting a twice the number of cases of CDAD since 1986 (2). More concerning is the increase in morbidity and mortality associated with CDAD. In one hospital setting, the mortality rate of CDAD patients increased from 3.5% to 15.3% from 1994-2000 (147), while in hospices the mortality rate has now approached 25% (41). The increase in cases, morbidity, and mortality of CDAD can be attributed, in part, to the emergence of hypervirulent strains of *C. difficile* that produce high levels of toxin and have more resistance to clindamycin and fluoroquinolines (97).

CDAD symptoms of this disease range from the mild, with self-limiting diarrhea and abdominal cramping, to the severe; with development of pseudomembranous colitis, paralytic colon, and toxic megacolon. While the etiology of the colonic disease has been well-studied, little is known about the cause of systemic complications in severe CDAD, making it difficult to treat patients with fulminant disease. Although in general CDAD is a disease confined to the gastrointestinal system, patients suffering from life-threatening cases of CDAD exhibit signs of damage outside of the colon (35, 44, 91, 96, 174). Additionally, in some cases, a

fatal outcome occurs despite total colectomy and eradication of the organism (78, 155); further suggesting systemic effects of *C. difficile* or soluble virulence factors released from this bacterium.

The major virulence factors of *C. difficile* and the main contributors to CDAD symptoms and pathology are its toxins, toxin A (TcdA) and toxin B (TcdB) (125). Because toxin has been detected in serum and ascitic fluid of CDAD patients and patients develop serum antibodies to the toxins, it is possible that circulating toxins may contribute to the systemic complications in severe CDAD (166, 206, 215). Previous studies, also featured in Chapter IV of this dissertation, determined that TcdB localizes to the pericardial region and causes a disruption in cardiac function, resulting in decrease in chamber contraction, loss in blood flow, cardiac tissue damage, and death of zebrafish embryos (75). Results from this investigation indicate that TcdB is a potent cardiotoxin capable of reducing heart contraction and hindering blood-flow. Further studies using cardiomyocytes revealed that TcdB altered cell physiology and disrupted coordinated cellular contractions. Collectively, these and previous findings, suggest more insidious, systemic events outside of the gastrointestinal tract (notably heart damage) as a cause for patient morbidity and mortality.

In order to further investigate the potential cardiac damage in CDAD and confirm findings in the zebrafish model, we sought to analyze the effects of a systemic TcdB intoxication event and *C. difficile* infection using rodent models and collected CDAD patient data. Corresponding to the cardiac damage in the zebrafish model, both mice intoxicated with TcdB and hamsters infected with *C. difficile* exhibited signs of massive cardiac damage including damaged myocardial cells and myocardial fibers, edema, hemorrhaging, vein dilation, blocked blood vessels, and lymphocytic infiltration (Fig. 22 and 23, respectively). In order to relate these findings to human disease cardiac, histopathology was obtained from patients who died with CDAD and examined for signs of tissue damage in a preliminary, retroactive study. Importantly, in this initial investigation, patients who died with CDAD demonstrated signs of severe cardiac damage similar to that of our animal models with damage to myocardial tissue, edema, hemorrhaging, and lymphocytic infiltration (Fig. 24). These findings present the first comprehensive investigation into the systemic events in fulminant CDAD and confirm one of the main organ targets of the major virulence factor, TcdB.

Results

The cardiac tissue of mice intoxicated with TcdB revealed signs of cardiac damage. Mice were injected with a dose range of TcdB intravenously and following their death, their organs were examined via histopathology. Treatment with lethal (>50 ng) and sublethal doses of toxin resulted in damage to cardiac tissue. The pathology revealed signs of myocardial infarct (Fig 18), damaged myocardial cells, edema, hemorrhaging, and lymphocytic infiltrates. Mice treated with lethal doses of TcdB exhibited minimal signs of morbidity prior to death.

Hamsters infected with C. difficile demonstrated detectable damage to heart tissue. Hamsters were infected with 10^4 *C. difficile* and examined for signs of cardiac damage. Approximately 36-48 hours post infection, hamsters demonstrated few signs of morbidity, produced minimal diarrhea (appearing as "wet tail"), and then died quietly (no signs of distress or convulsions). Immediately, following disease-related death, heart tissue was collected and examined via histopathology. Compared to control hamsters, the cardiac tissue of hamsters with CDAD revealed cardiac damage including damage to myocardial fibers (as visualized as loss of striations in myocardial fibers), hemorrhaging, edema, and lymphocytic infiltrates (Fig 19).

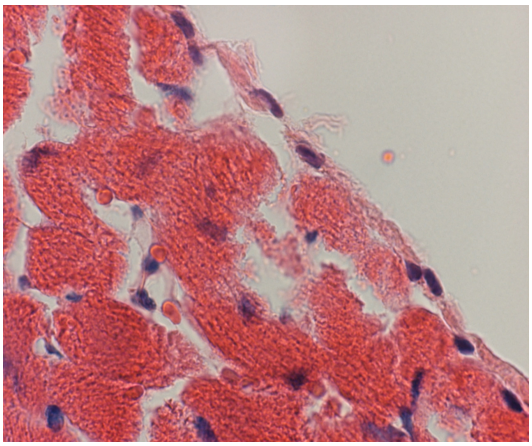
Patients that presented with CDAD upon death exhibited signs of cardiac damage. Following animal studies with TcdB and *C. difficile,* hearts from patients

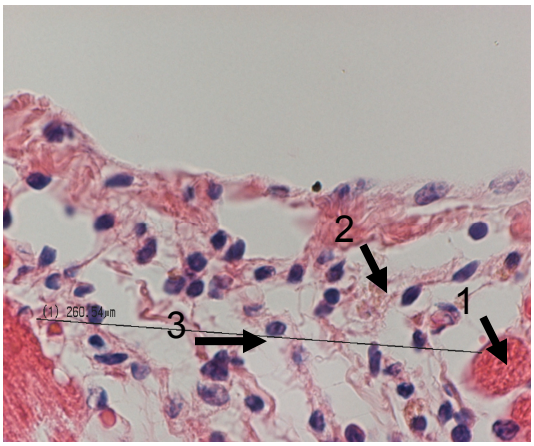
with CDAD were examined. In this initial, retrospective study, cardiac tissue was obtained via autopsy from 5 patients that died with CDAD, and were analyzed for signs of cardiac tissue damage via histopathology. Although in addition to CDAD these patients had varying pre-existing conditions, they all demonstrated similar signs of cardiac damage including damage to heart tissue, hemorrhaging, edema, and lymphocytic infiltrates (Fig. 20).

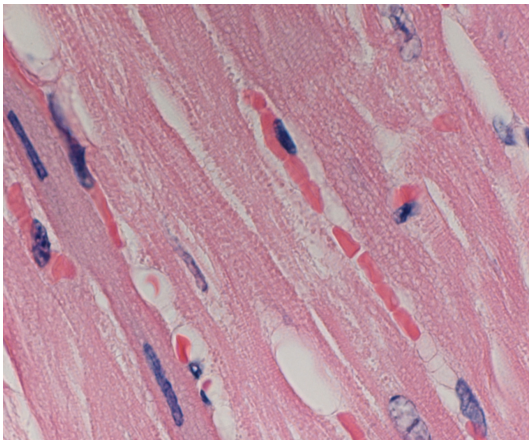
Fig. 18. Representative heart tissue following systemic intoxication with TcdB. Mice were injected intravenously with buffer control or TcdB and their heart tissue was examined for signs of damage via histopathology. Compared to control mice (left panels), mice injected with TcdB (right panel) demonstrated signs of massive myocardial infarct: damaged myocardial cells (arrow 1), edema (arrow 2), lymphocytic infiltrates (arrow 3), and hemorrhaging and capillary congestion (arrow 4).

Control

TcdB







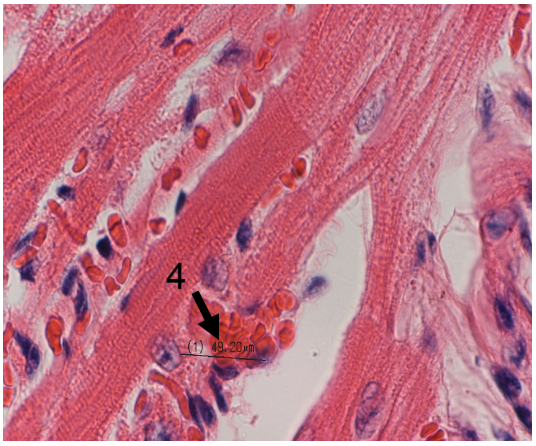
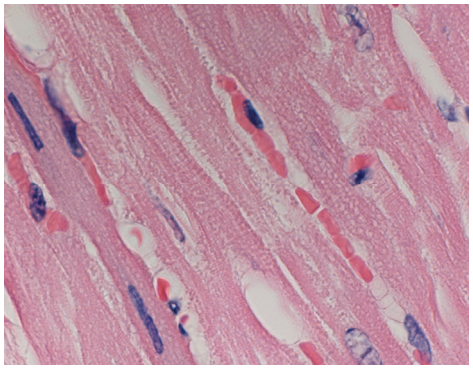
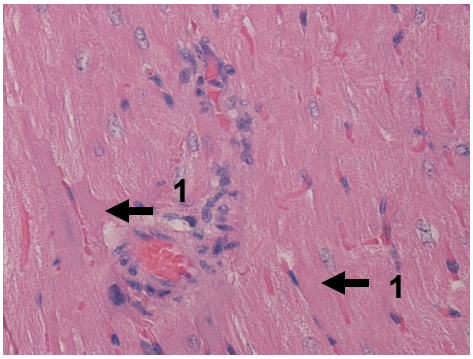


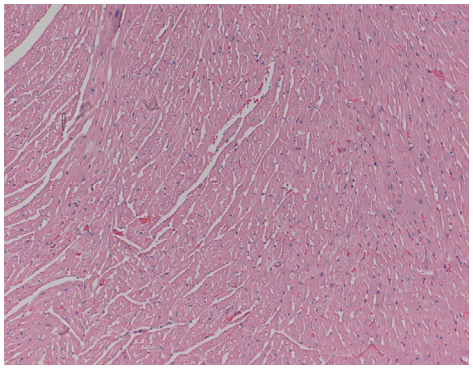
Fig. 19. Representative heart tissue from hamsters with CDAD. Hamsters were infected with buffer control or TcdB and, following disease-related death, heart tissue was examined for signs of damage via histopathology. Compared to control hamsters (left panels), hamsters with CDAD (right panel) demonstrated signs of massive heart damage: loss in striated myocardial fibers (arrow 1) and vein dilation (arrow 2).

Control

C. difficile infected







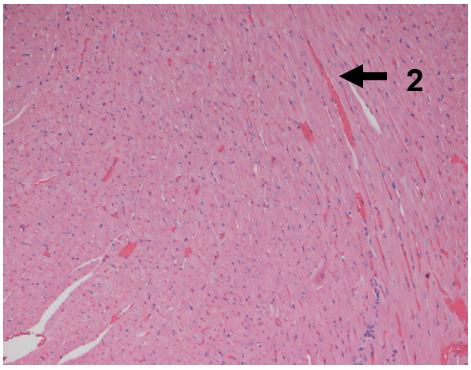
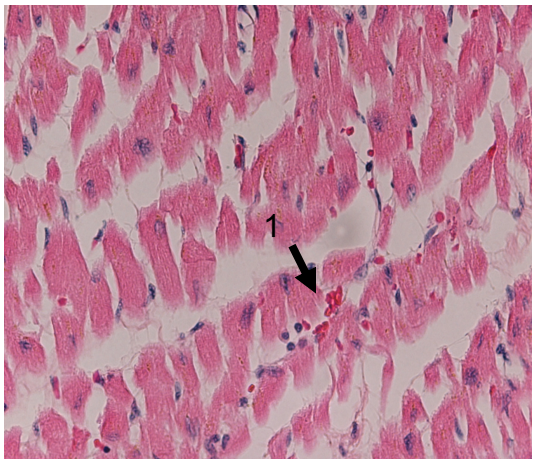
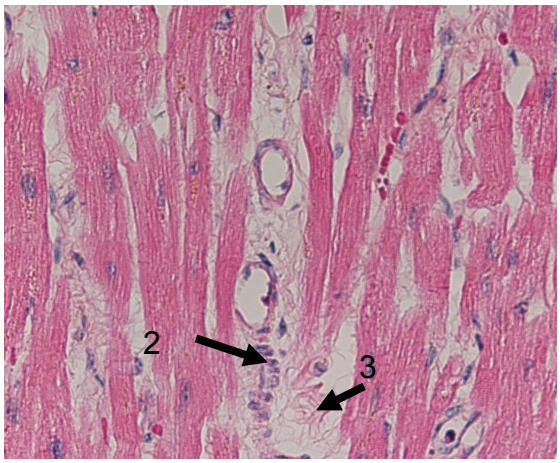


Fig. 20. Representative heart tissue of patient with CDAD. In a retroactive study, patients that died with CDAD were examined for signs of damage via histopathology. Cadaver heart tissue demonstrated signs of heart damage: hemorrhaging (arrow 1), lymphocytic infiltrates (arrow 2), and edema (arrow 3).

Heart with lymphocytic infiltrate and hemorrhage



Heart with lymphocytic infiltrate and edema



Discussion

C. difficile is one of the leading causes of hospital-associated disease and is the primary cause of antibiotic-associated diarrhea (210). The pathogenesis of *C. difficile* within the intestines has been extensively studied, and several events are known to contribute to the inflammation and pseudomembrane formation (125, 210). Of particular importance is the release of two exotoxins, which contribute to the pathologies observed in CDAD. The two major *C. difficile* toxins are TcdA and TcdB, which share similar mechanisms of action but differ in their ability to cause disease. In general, TcdA, with its limited cell tropism and *in vivo* enterotoxic activity, is considered to be the enterotoxin, while TcdB, possessing a very broad cell tropism and limited enterotoxic activity is considered to be a potent cytotoxin (6, 192). Results from several studies indicate TcdA contributes to intestinal damage and modulates the extensive inflammatory response observed in CDAD patients, while, the vast majority of the reports implicate TcdB as a cytotoxin and a potential contributor of the systemic events during fulminant disease (6, 125, 192, 210) .

Although the cellular and physiological events leading to intestinal pathology in CDAD patients have been defined, little is known about the systemic events in these patients. However, in life-threatening cases of CDAD, major organs are impacted. Acute respiratory distress syndrome (91), multiple organ failure (44), cardiopulmonary arrest (96), chronic renal failure (35), as well as liver abscesses

(174) have all been described in CDAD. Sepsis does not appear to be the likely cause of death in fulminant CDAD. In a thorough literature review and personal communication (Stuart Johnson MD and Dale Gerding MD Hines VA, Chicago, IL), *C. difficile* is rarely cultured from the blood of patients (52) and, in laboratory studies, blood from hamsters infected with *C. difficile* (unpublished data) are culture negative. These data, coupled with the low incidence of *C. difficile* in the bloodstream (personal communication with Stuart Johnson and Dale Gerding, MD VA hospital, Chicago), indicates that systemic events may be due to the release of bacterial toxins into the bloodstream and not sepsis. Furthermore, in some cases, a fatal outcome occurs despite total colectomy and eradication of the organism via antibiotic therapy (78, 155) and in one report, death generally occurred prior to colon perforation and was a rare event (2/64 patients) (36). These data indicate that systemic events may be due to the release of bacterial toxins into the bloodstream and not sepsis.

It has been reported previously and in this dissertation that in zebrafish, TcdB localizes to the pericardial region and causes cardiac damage including reduction in chamber contractility and degeneration of heart tissue as well as loss in blood flow resulting from the potential disruption of cardiomyocyte physiology and cell death (75). Consistent with this report, results from the current study indicate that in higher order animals and patients with CDAD all demonstrate signs of cardiac damage. Mice systemically intoxicated with TcdB demonstrated no signs of severe morbidity prior to death, yet necropsy revealed signs of acute

myocardial infarct and hemorrhaging and edema within the heart, suggesting heart failure as a potential cause of death. Similarly, hamsters with a *C. difficile* infection within the colon also demonstrated minimal signs of distress and minimal diarrhea TcdB also results in severe cardiac damage, also suggestive of insidious, systemic events involving the heart. In three separate animal models systemic intoxication and infection with *C. difficile* resulted in damage to cardiac tissue, thus leading us to investigate the potential heart damage in humans with CDAD. In a retrospective histopathological examination of patient autopsy data, heart tissue from patients who died with CDAD also revealed demonstrated substantial damage similar to that observed in the animal models. Although due to the nature of this initial analysis, it is difficult to discern whether this damage can be directly attributed to TcdB, the preliminary human pathology further indicates that systemic, cardiotoxic events outside of the gastrointestinal tract can occur in patients with severe CDAD.

CDAD is a complex disease with both colonic and systemic etiologies. However, the systemic events of this disease are not well-understood. Yet, clearly, extracolonic activities can be the major contributors to patient morbidity and mortality. Thus, understanding the systemic events is necessary so that more comprehensive treatments can be established, especially in light of the association of this disease with antibiotic therapy and antibiotic resistant strains. Treatment approaches must therefore extend beyond antibiotic regiments and

certainly beyond the colon in order to treat the short term and potentially long term effects of this increasingly fatal disease

Materials and Methods

Purification of TcdB. TcdB was isolated as previously described (165). Briefly, *C. difficile* VPI 10463 was grown in dialysis tubing suspended in brain heart infusion broth (BHI) and grown at 37°C for 72 h. The culture was then centrifuged and TcdB was purified from the collected supernatant using sequential chromatography steps including anion-exchange (Q-Sepharose) chromatography and high resolution anion-exchange (Mono-Q) chromatography. TcdB was de-salted into 20 mM Tris-HCl, pH 8.0 and the protein concentration was determined via the Bradford method (20).

Spore preparation. For spore preparation, *C. difficile* VPI 10463 was grown in sporulation broth (For 1 L, 90 g Trypticase Peptone, 5.0 g Proteose Peptone, 1.0 g $(NH_4)_2$ SO_4, 1.5 g Tris to 1000 ml H_2O, pH= 7.4) (223) in hanging dialysis tubing for 2 weeks. Spores were centrifuged and washed in deionized H_2O, and heat-inactivated via incubation at 72°C for 20 min. Following heat-inactivation, spores were washed twice in cold, deionized H_2O, and incubated at 16°C, shaking at 300 rpm for 72 h. Spores were washed in cold, deionized H_2O until vegetative cells and debris was removed and the final spore sample was filtered through a 3.1 mm filter. Spore purity was determined via the Wirtz-Conklin spore

stain and spores were collected at no less than 90% purity. Following spore preparation, spores were innumerated on BHI agar plates (37°C, anaerobic conditions) and reported as colony-forming units (CFU).

Mouse intoxication studies. For TcdB intoxication experiments, 6 week BALB-c mice (Charles River) were injected intravenously with TcdB (1.0 ng-1.0 μg). Controls were injected with 20 mM Tris-HCl buffer. Immediately following death, organs were collected in 10% buffered formalin and processed for pathology. Mice injected with sublethal doses of TcdB (<50 ng) (10 ng and 20 ng), were euthanized 72 h post-intoxication via CO_2 asphyxiation and organs were immediately harvested and processed for pathology.

Hamster infection studies. Male Golden Syrian hamsters (Charles River) were injected intraperitoneally with clindamycin (30 mg/kg) (Sigma). Two days post-antibiotic therapy, hamsters were orally administered 10^4 *C. difficile* spores via gavage needle. Immediately following death, organs were collected in 10% neutral buffered formalin and processed for pathology.

Mouse and hamster histopathology. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm for routine staining. Sections were deparaffinized and rehydrated through three changes of xylene and graded alcohol and stained with hematoxylin and eosin. Slides were coverslipped and examined for histopathology. This portion of the experiment

was performed in collaboration with Ms. Megan Lerner, research associate in the pathology department of the VA hospital in Oklahoma City, Oklahoma.

Human autopsy pathology. Pathology was performed on cadaver tissue from patients who all presented with CDAD upon autopsy. This retroactive investigation was carried out as part of a collaborative effort with Dr. Stan Lightfoot. Credit for this preliminary investigation should be given solely to Dr. Stan Lighfoot, Dr. Brandon Guthrey, and Ms. Megan Lerner.

CHAPTER VI

SUMMARY

The goal of the studies presented herein is to further understand the role of a bacterial toxin during the disease process. In 1888, researchers Emile Roux and Alexandre Yersin demonstrated that the systemic symptoms of diphtheria could be recapitulated using cell-free filtrates; an observation that lead to the discovery of diphtheria toxin and the beginning of bacterial toxin research (172). Since that seminal study, the mechanism of action and even the structures of most bacterial toxins have been elucidated. However, despite advancements in the field of toxin research, the organs targeted by many bacterial toxins during disease are unknown, leaving the overall impact of these major virulence factors on the host poorly understood. **The purpose of this research was to develop a new animal model that enabled the determination of *in vivo* targets of bacterial toxins and to specifically investigate the targeted organs of one of the major toxins from *C. difficil*e, TcdB**.

Although traditional animal modes have provided a wealth of information in toxin research, they have also presented numerous obstacles. However, the zebrafish embryo model provides a solution to many of these major issues. Besides the obvious appeal of the zebrafish embryo (size, population number, expense, care, and maintenance), the zebrafish embryo transparency circumvents the inherent problems with post-mortem analysis of tissue and organ damage. By taking

advantage of this unique characteristic, researchers can intoxicate the animal and then, using simple light microscopy, determine which cell types are damaged in real-time and not following animal death. Other experiments using labeled toxin can easily be performed to determine the anatomical localization of toxin *in vivo*, an appealing advantage over traditional models. Therefore, if damage to the liver was observed histopathologically but only showed localization to the heart, this information could be used to ascertain the direct and indirect effects of the toxin. Additionally, because the immune system of zebrafish develops at distinct time points, intoxication of the animal can be performed in the absence of an active and fully developed immune system, thereby negating the issue of nontoxin-related tissue damage due to an inflammatory response. Overall, the transparent zebrafish embryo model allows for the direct visualization of toxin localization and activity and helps distinguish between toxin-related damage and secondary damage (i.e., post-mortem necrosis and inflammation). By exploiting a simple fish commonly found in pet stores, a new animal model has emerged to investigate the *in vivo* effects of bacterial toxins.

Chapter IV describes the development and the use of the zebrafish embryo as a model to determine the targeted organs of bacterial toxins. Employing this model, this chapter investigates the *in vivo* targets of TcdB, the major cytotoxin of *C. difficile.* TcdB is an example of a bacterial toxin whose *in vivo* targets are unknown, thereby limiting our knowledge of *C. difficile* pathogenesis. CDAD is a complex disease that has, in recent years, become an increasing cause of

severe disease and death. Although its other major toxin, TcdA, is known to be an enterotoxin, TcdB has very little enterotoxic activity and is a broad cytotoxin known to hit a variety of cell types. Therefore, the organs targeted by TcdB during severe disease are unknown and, consequentially, TcdB's role in CDAD is unidentified. This gap makes it difficult to discern the cascade of events leading to the serious, systemic damage observed in fulminant disease and, more importantly, has hindered physicians' ability to provide targeted and meaningful appropriate short and long term treatment regimen to CDAD patients.

Prior to this work, TcdB was known only as a broad cytotoxin. Using the zebrafish as a model, we determined the localization of TcdB and the phenotypic changes induced by TcdB. From this information, we were then able to investigate the mechanism of organ damage via the disruption of cardiomyocyte physiology. Also using the model we were able to test a potential toxin therapeutic.

First, embryos were treated with fluorescently-labeled TcdB and observed for localization of toxin. Approximately 24 hpt, punctuated sites of toxin were observed throughout the zebrafish body; however, the greatest accumulation of toxin was found within the pericardial region of treated zebrafish. In order to determine whether this was a toxin specific event, zebrafish were treated with a 30-fold molar excess of the receptor-binding domain of TcdB. The receptor

binding domain was found to competitively prevent the localization of labeled TcdB, indicating that pericardial localization of toxin was a TcdB-specific event.

To investigate the real-time impact of TcdB on the animal, zebrafish embryos were treated with toxin and changes in phenotype were observed. The phenotype of intoxicated zebrafish was obvious; following intoxication, the animals developed pericardial edema that eventually resulted in whole-body edema. Using a database of zebrafish mutants with established phenotypes, it was discovered that zebrafish with mutations in genes required for a functioning cardiovascular system exhibited a phenotype similar to that of the zebrafish treated with TcdB, thus prompting investigations into the effect of TcdB on the cardiovascular system.

Because zebrafish are optically transparent, blood-flow of the animal can be observed via simple light microscopy. Approximately 24 hpt with TcdB, there was a noticeable decline in the red blood cell (RBC) perfusion rate, which, over time, continued to decrease until flow rate ceased. A possible explanation for this phenomenon is the destruction of vein integrity via TcdB; however, red blood cells were still capable of moving through blood vessels, albeit at a reduced rate. To further confirm vein integrity, transgenic zebrafish that expressed GFP within endothelial cells were used to assess the impact of TcdB on vein structure by observing any loss in ultrastructure or decrease in GFP expression. Using this

approach, it was determined that TcdB caused no discernable loss in vein integrity.

The heart was examined next as a possible source of a decrease in RBC perfusion rate. Between 24 and 48 h following treatment with TcdB, there was a decrease in heart rate and loss in cardiac rhythmicity. Furthermore, there was a noticeable decrease in ventricle chamber contractility, impeding the overall flow of blood through the heart and into the rest of the animal. As damage was allowed to progressed, the heart underwent drastic changes in morphology and the tissue appeared necrotic. Finally, the heart was unable to contract and subsequently blood flow ceased.

In order to determine the mechanism by which TcdB caused cardiac damage, it was necessary to determine whether TcdB had a specific tropism for the heart or if cardiac tissue was simply more sensitive to inactivation of Rho, Rac, and Cdc42. This was accomplished using a surrogate system to delivery TcdB into mammalian cells. Briefly, the enzymatic domain of TcdB was fused to a truncated form of lethal factor that is translocation active but enzymatically inactive. Therefore, the enzymatic region of TcdB was delivered into the cell using the anthrax toxin receptor, which is widely distributed throughout zebrafish tissue (197). If cardiac tissue was more sensitive to the inactivation of Rho, Rac, and Cdc42, then the heart would be preferentially impacted despite localization of the enzymatic domain to multiple tissues. However, if TcdB has a specific

tropism for the heart, then delivery of the TcdB enzymatic domain via the widely-distributed anthrax toxin receptor would result in non-specific tissue damage. Using this surrogate delivery system, TcdB was found to cause widespread tissue degeneration, indicating that TcdB has a cardio-specific tropism, confirming the localization studies.

To further determine the cause of cardiac damage by TcdB, the effect of TcdB on cardiac cell physiology was explored. Cardiomyocytes are the functional cell of the heart and control the contraction and expansion of cardiac tissue. Following intoxication, cardiomyocytes not only underwent changes in cell morphology but also in cell physiology, with a decrease in rhythmicity and coordinated contraction. Loss in cardiomyocyte function could be a possible explanation for the decrease in heart rate, rhythmicity, and chamber contraction. Although cardiac fibroblasts could be targeted by TcdB as well, the loss in cardiomyocyte function would have the most drastic impact on heart physiology. Overall, because of the zebrafish model, we were able to closely examine the effects of TcdB on a relevant cell-type, and found that the disruption of cardiomyocyte physiology may be a mechanism by which TcdB disrupts cardiac function.

Overall, Chapter IV provides evidence that TcdB acts as a potent cardiotoxin. Not only does TcdB have specific tropism for the heart in zebrafish, but it also disrupts basic cardiac function. Furthermore, TcdB drastically alters the physiology of cardiomyocytes, the functional cells of the heart. These data

provides the first insight into the *in vivo* targets of TcdB and a possible role for this toxin in fulminant disease. Finally, this chapter also introduces the zebrafish as a useful model for determining the *in vivo* targets of bacterial toxins.

Using the zebrafish model of intoxication, the heart was identified as a target of TcdB, allowing for more focused examination into the causes of severe CDAD. Chapter V further describes the cardiac damage found in severe disease using both rodent and patient studies. First, the systemic impact of TcdB on the heart was investigated using the murine model. Mice were injected with lethal and sublethal doses of TcdB and then their organs were examined for damage. Administration of both lethal doses and sublethal injection of TcdB resulted in cardiac damage that included damage to myocardial cells and fibers, edema, hemorrhaging, and infiltration of lymphocytes, indicating that systemic intoxication with TcdB can result in massive myocardial infarct and cardiac tissue damage.

In order to correlate the zebrafish and mouse toxin data to the systemic effects of a severe *C. difficile* infection, a *C. difficile* infection model was used. Following treatment with clindamycin, hamsters were orally administered *C. difficile* and, upon disease-related death, pathology was performed on the collected organs. Similar to the TcdB intoxication models, hamsters with CDAD also exhibited damage to their hearts, including myocardial infarct, edema, hemorrhaging, damage to myocardial cells and fibers, as well as infiltration of lymphocytes.

Finally, patient autopsy reports were analyzed to determine whether patients who died with CDAD exhibited signs of cardiac damage. In a preliminary study of 5 autopsies, substantial damage to cardiac tissue was detected in all 5 cases. Although it remains unknown whether this damage was due to prior conditions or to severe CDAD, this initial analysis indicates that CDAD may contribute to heart damage. Clearly, a more extensive panel of patients will be required for a full, comprehensive investigation into the heart damage in human CDAD; however, results from this initial study appear promising.

Overall, the research herein presents not only a new animal model to study bacterial toxins but also a four-arm approach to pathogenesis disease research. Using a combination of tissue culture, zebrafish, rodent, and patient studies, we were able to gain meaningful, more inclusive insight into a complex and serious disease, *C. difficile*-associated disease. Initial studies in zebrafish and in tissue culture indicate that TcdB has a specific tropism for cardiac tissue and disrupts cardiomyocyte physiology, ultimately resulting in damage to the heart and disruption of heart function. Further studies revealed that a systemic TcdB intoxication event and severe *C. difficile* infection also caused heart damage. Using information for these studies, we were able to more closely examine the hearts of patients who died with CDAD. In an initial, retrospective study, all five patients exhibited massive cardiac damage detected similar to that revealed in the rodent and zebrafish model. Collectively, these data validate the robustness

of the developing zebrafish embryo as a model to study the *in vivo* activity and specific targets of bacterial toxins and further emphasizes the need to fully understand the systemic events and cardiotoxicity during CDAD.

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