CLOSTRIDIUM DIFFICILE TOXINS A AND B: EXPLORING
THE POSSIBLE MECHANISM OF ACTION

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(ABSTRACT)

Clostridium difficile is a common cause of antibiotic-associated diarrhea and occasionally causes the life-threatening disease pseudomembranous colitis. The pathogenicity of the organism has been attributed to the production of two large exotoxins, toxin A (308,000 daltons) and toxin B (269,000 daltons). Toxin A is a powerful enterotoxin and is generally thought to play the more important role in the pathology of the disease. Toxin B may exert its effect after the initial tissue damage by toxin A. Both toxins cause rounding of mammalian culture cells by disrupting the cytoskeletal system. The similar biological activities and high percentage of sequence homology between the two toxins suggest that they have a similar mechanism of action. I found that purified preparations of both toxins cleave skeletal muscle actin at a single site, producing a 38,000 dalton actin fragment, and that the toxins are capable of autodigestion. The proteolytic activity may be involved in the mechanism of action of the toxins. I also analyzed an aberrant strain of C. difficile which reportedly lacked the gene for toxin B. Such a strain would be very useful for the study of the mechanism of toxin A. I concluded however, that the strain contained the genes for both toxin A and
toxin B. The toxin genes and resulting proteins appear, however, to be slightly different from those of other strains.
Dedication

This work is dedicated in loving memory to my mother Patricia and my brother Eric, both of whom had an insatiable hunger for knowledge, and for whom learning was a means to deepen and strengthen the soul. My mother drove me to break free from any gender stereotypes, to pursue my ambitions, and to strive to fulfill my potential. My brother, with seemingly unending energy to learn and to love taught me to be more understanding and giving.
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Section I. Literature Review.

Pathogenesis of Clostridium difficile

History of Pseudomembranous Colitis

In 1893, Dr. Finney was the first to document a case of pseudomembranous colitis (1). He described a young female patient who complained of vomiting and had noticed a lump on her abdomen. Dr. Finney performed gastrointestinal surgery and found that the pylorus was obstructed by scar tissue. He removed part of the obstruction from the pylorus and observed the condition of the patient. Ten days after the surgery, the patient began to suffer from diarrhea. The symptoms of diarrhea gradually worsened and the stools became bloody. Fifteen days after the surgery, the patient died. The autopsy revealed a swelling and reddening of the mucus membrane of the large intestine, as well as patches of blood and fibrin. These symptoms are characteristic of pseudomembranous colitis.

Cases of pseudomembranous colitis (PMC) which occurred after gastrointestinal surgery were occasionally documented following Finney's report, but reports of the disease were infrequent until the late 1940s when the use of antibiotics became common (2). Clindamycin was introduced in 1969 (3) and was the first antibiotic that was associated with the onset of PMC (4, 5, 6). Later, lincomycin, ampicillin and erythromycin were also implicated (7, 8, 9, 10). It was not known whether the onset of colitis after antibiotic therapy was due to a bacterial infection, an allergic response to the antibiotic, or direct toxicity of the antibiotic (11).
In 1977, research on the cause of antibiotic-associated colitis began to flourish. Allen et al. reported that a loss of the normal flora in the colon was concomitant with the onset of antibiotic-associated pseudomembranous colitis (12). Larson et al. detected a toxin in fecal extracts from patients with antibiotic-associated colitis which was cytopathic for HeLa cells and Rhesus monkey kidney cells (13). Shortly thereafter Larson and Price (14) and Rifkin et al. (15) discovered that the cytotoxin which was responsible for the rounding of tissue culture cells was neutralized by \textit{Clostridium sordellii} antitoxin. \textit{Clostridium sordellii}, however, could not be cultured from the fecal specimens containing the cytotoxin. Bartlett et al. (16) presumed that if the cytotoxin was not being produced by \textit{C. sordellii}, than the antitoxin must have been cross-reacting with a toxin of similar origin. In searching for the source of the cytotoxin, Bartlett et al. were able to isolate several clostridial species from stool specimens from patients with PMC. They found that \textit{C. difficile} was the only clostridial species which produced a cytotoxin and that this cytotoxin was neutralized by \textit{C. sordellii} antitoxin. This finding was confirmed by George et al. in 1979 (17), and \textit{Clostridium difficile} was subsequently implicated as the causative agent of antibiotic-associated PMC.

\textbf{Pathogenesis of \textit{C. difficile}}

It has been reported (18) that \textit{Clostridium difficile} is only present in a small percentage (0-3\%) of normal, healthy adults (excluding those who are taking antibiotics). These statistics suggest that \textit{Clostridium difficile} is not a part of the normal flora of the colon, but an opportunistic pathogen which is only able to colonize the colon when the normal flora is disrupted by antibiotic therapy (19). The onset of the disease caused by \textit{C. difficile} may occur during antibiotic therapy or after the therapy has been discontinued, but symptoms usually begin within two weeks of receiving antibiotics (20, 21). The most common form of the disease is self-limiting diarrhea, characterized by watery or bloody stools, elevated temperature, and often times, leukocytosis (22, 23, 24, 25). Generally the
only action necessary for antibiotic-associated diarrhea is the discontinuation of the antibiotic and replacement of lost fluids and electrolytes. Once the patient stops the antibiotic therapy, the normal flora should become re-established and *C. difficile* will again be excluded from the colon. In more serious cases of diarrhea, and cases where the discontinuation of antibiotics does not result in a rapid recovery, vancomycin or metronidazole can be administered.

In about 5-10% of the cases of enteritis caused by *C. difficile*, the symptoms are much more severe and the disease manifests itself as PMC. Pseudomembranous colitis is characterized by the formation of yellow plaques on the colonic mucosa. These plaques are composed of fibrin, dead epithelial cells, mucus, and polymorphonuclear leukocytes (26, 19, 27, 28). Pseudomembranous colitis can be effectively treated with vancomycin or metronidazole. Vancomycin is poorly absorbed along the gastrointestinal tract and is therefore able to reach a high concentration in the large intestine. Relapses occur in 20-50% (29) of the cases in which vancomycin or metronidazole are administered because the antibiotics do not allow for the normal flora to become re-established. Fecal enemas containing bacteria from healthy individuals have also been used successfully in an attempt to re-establish the normal flora of the intestine (30, 31).

Pseudomembranous colitis is primarily a nosocomial infection. Heard et al (32) reported that in normal circumstances, approximately 2.5% of hospital patients acquire *C. difficile* during their stay, and that during an outbreak, up to 21.5% of the patients will acquire the organism. The disease is often acquired in hospitals for several reasons: 1) the bacteria produce spores which are released by infected patients during bouts of diarrhea, 2) the hospital environment is often contaminated with spores, 3) hospital workers often carry the organism on their hands, and 4) neonates provide a reservoir for the organism. Infection by *C. difficile* is particularly a problem in the geriatrics ward. Elderly patients have more trouble resisting infection and often display more serious symptoms than do younger adults.

As stated above, neonates act as a reservoir for *C. difficile* in hospitals. It has
been reported (33, 34) that up to 50% of newborns are asymptomatic carriers of the organism. The presence of *C. difficile* in infants has been well documented but is poorly understood (35, 36, 37, 38). Newborns may either be exposed to *C. difficile* as they pass through the mother’s vaginal canal, or they may be exposed during handling by hospital workers who carry the organism on their hands. Presumably *C. difficile* is able to colonize the colons of newborns because they have not yet developed a normal flora. It is not known, however, why infants do not exhibit the symptoms of *C. difficile*-associated diarrhea or PMC.

**Toxins of *C. difficile***

**History of the toxins**

It was thought originally that *C. difficile* produced a single toxin which was responsible for both the cytotoxicity exhibited by patient fecal samples and for the symptoms of PMC. In the early 1980s, Taylor et al. (39, 40) and Banno et al. (41) demonstrated that *C. difficile* produces two toxins which are separable by ion-exchange chromatography. The toxins were designated toxins A and B by Taylor et al. (39, 40) and toxins D-1 and D-2 by Banno et al. (41). The toxins were so named because toxin A (toxin D-1) was found to have a much lower binding affinity for the ion-exchange resin than toxin B (toxin D-2), and was therefore the first to elute during chromatography. Differences in the biological activities of the two toxins were observed immediately. Taylor et al. and Banno et al. both reported that toxin A was more active in the mouse lethality assay and that, unlike toxin B, it was enterotoxic. The toxins were also found to be antigenically distinct (42).
Physicochemical properties of the toxins

There was a great deal of controversy in the literature regarding the molecular weight of toxins A and B. Molecular weight estimates of toxin A have ranged from 52,000 to 600,000 daltons (41, 43, 44). Estimates of the molecular weight of toxin B have ranged from 50,000 to 550,000 daltons. In 1990 Sauerbrey et al. (45) and Dove et al. (46) sequenced the entire toxin A gene and Barroso et al. (47) sequenced the toxin B gene. According to their nucleic acid sequences, the molecular weights of the toxins should be approximately 308,000 daltons for toxin A and 269,000 daltons for toxin B. The discrepancies in the molecular weight estimates may have been due to post-transcriptional modification of the toxins but there is no evidence to support this. More likely, the discrepancy was caused by contaminating proteins in toxin preparations.

The two toxins share a high percentage of sequence homology (Fig. #1). The amino acid sequences of both of the toxins contain a region near the N-terminus, which resembles a typical ATP-binding motif. Close to the center of the primary sequence of both toxins is a region consisting of 50 hydrophobic amino acid residues (46). Near the carboxyl terminus, both toxins have a series of repeating units; toxin A contains 38 repeating units and toxin B contains 24 repeating units. The toxins contain four cysteine residues which are located in almost identical positions in both toxins, but it is not known if any of these cysteines are involved in disulfide bonds.

Biological Activity

Toxin A is a powerful enterotoxin and as little as one microgram will induce a fluid response in ligated ileal loops (48). The mucosa of the ileum becomes swollen and damaged, and epithelial cells die and slough off. The fluid that accumulates in the ligated ileal loops contains blood and mucus. The toxin elicits a similar but milder response in the colon. The mechanism of toxin A seems to be very different from other "classical"
Figure 1: Structure of *C. difficile* toxins A and B. This diagram illustrates the basic structures of the toxins and emphasizes their homology.
enterotoxins such as the cholera toxin. The cholera toxin stimulates adenylyl cyclase leading to an increase in the concentration of cyclic adenosine 5'-monophosphate within cells. The increase in intracellular CAMP levels inhibits the uptake of water by cells in the intestinal lumen and leads to fluid accumulation. Since the cholera toxin does not damage the cells themselves, the fluid that accumulates does not contain mucus or cellular debris. Toxin A does not increase intracellular CAMP levels, but rather the fluid accumulation appears to be a consequence of the tissue damage. Toxin B does not have any enterotoxigenic activity.

Both toxins A and B are cytotoxic, but toxin B is 100 to 1,000 times more cytotoxic than toxin A. The toxins disrupt the cytoskeletal structure of cultured fibroblast cells causing them to round up. The fibroblasts also exhibit nuclear polarization and eventually die.

Toxins A and B are lethal to mice with a minimum lethal dose of 50 ng (48, 49). Neither the exact cause of death nor the target of the toxin is known, but if the toxins are injected intravenously or intraperitoneally into an animal, its respiration quickly ceases.

Toxin A agglutinates rabbit erythrocytes by binding to the carbohydrate receptor Galα1-3Galβ1-4GlcNAc present on the cell surface (50). The hemagglutinating activity of toxin A is temperature-dependent, the toxin binds to erythrocytes readily at 4°C but very poorly at 37°C. Toxin B does not have hemagglutinating activity.

Toxin Purification

Many different methods have been devised for the purification of Clostridium difficile toxins A and B. A large portion of the toxins that are produced by C. difficile is released into the growth medium, so the toxins are generally purified from the culture filtrate. Toxin production can be maximized by growing the cells in a dialysis tube in an anaerobic flask containing brain heart infusion broth (26). The dialysis flask was devised by Sterne and Wentzel (51) for the production of botulism neurotoxin and works equally
well for the production of *C. difficile* toxins. The dialysis flask acts as an initial concentration step because it contains a small amount of media relative to the culture flask. The dialysis tube permits the slow but steady entry of nutrients from the broth while excluding large molecules that might interfere with the purification procedure. It is well documented that the slow growth induced by the dialysis culture increases toxin production but it is not known why.

Shortly after *C. difficile* was determined to be the cause of antibiotic-associated pseudomembranous colitis in 1977, researchers began to characterize the cytotoxin it produced. In 1981, during attempts to purify toxin from *C. difficile* using ion-exchange chromatography, Banno et al. (41) and Taylor et al. (40) discovered that the bacteria produced not just one toxin as was originally suspected, but two. Based on the order in which they eluted during ion-exchange chromatography, Banno et al. designated the toxins D-1 and D-2 and Taylor designated them A and B. Banno et al. partially purified the toxins from culture filtrate sequentially by 70% ammonium sulfate precipitation, anion-exchange chromatography using a Sephacryl S-300 column, and anion-exchange chromatography using a DEAE Sephadex A-25 column. Taylor et al. purified the toxins by a similar method, but the initial concentration step was accomplished using ultrafiltration rather than ammonium sulfate precipitation. This was followed by the separation and partial purification of the toxins by two anion-exchange chromatography steps using a Sephadex G-200 column and a DEAE-Sepharose CL-6B column. In 1982, Sullivan et al. (52) were the first to use the dialysis flask method to grow *C. difficile*. In the purification method they described, the culture filtrate was initially concentrated by ultrafiltration and the toxins were separated and partially purified by anion-exchange chromatography using a DEAE-Sepharose CL-6B column. Toxin A was further purified by isoelectric precipitation at pH 5.5.

In 1984, Rihn et al. (53) attempted to develop a simple two-step method for the purification of toxin A. In this method, toxin A was purified by fast protein liquid chromatography using a Mono Q anion exchange column followed by chromatofocusing.
using a Mono P column. The protein that Rihn et al. purified was only 50,000 daltons and was later shown to be enolase. The enolase purified by Rihn et al. probably contained a small amount of toxin A and therefore displayed biological activity similar to that of the toxin.

Lyerly et al. (54) built upon the methods of Banno and Sullivan and developed a purification procedure which is still commonly used. The purification procedure that he described began with an ammonium sulfate precipitation. The ammonium sulfate concentration used in this procedure was lower (40% saturation for toxin A and 55% saturation for toxin B) than that used by Banno so more proteins were excluded in this initial step. The second step in the purification was ion-exchange chromatography using a DEAE-Sepharose CL-6B column. Toxin A was further purified by isoelectric precipitation at pH 5.6.

In 1987, Krivan and Wilkins (55) developed a one-step method for the purification of toxin A which was based on the specific, temperature-dependent binding of toxin A to the carbohydrate sequence Galα1-3Galβ1-4GlcNAc. This sequence is present on bovine thyroglobulin. Thyroglobulin was covalently bound to an affinity column which was used to bind toxin A at 4°C. The column was washed at 4°C and purified toxin was eluted from the column at 37°C. This method works well for purifying small quantities of toxin A, but the method of Lyerly et al. is more effective for purifying toxin A from large amounts of culture filtrate.

Immunoochemical properties of the toxins

It was the discovery that the cytotoxin found in stools from patients with antibiotic-associated pseudomembranous colitis, was neutralized by C. sordellii antitoxin, that led to the implication C. difficile as the causative agent of the disease (14, 15, 16). Consequently, studies were conducted in order to determine the reason for the cross-reactivity between C. sordellii antitoxin and C. difficile toxins A and B. It has since been
reported that the physicochemical properties as well as immunochemical properties of *C. difficile* toxin A closely resemble those of the hemolytic toxin (HT) of *C. sordelli* and that toxin B closely resembles the lethal toxin (LT) of *C. sordelli* (56).

In 1985, Lyerly et al. (57) produced both monoclonal and polyclonal antibodies specific for toxin A. The monoclonal antibody (MAb) was designated PCG-4. The MAb PCG-4 immunoprecipitated toxin A and inhibited the enterotoxic activity but failed to inhibit cytotoxic activity. The polyclonal antibody was purified from antiserum against a highly toxigenic strain of *C. difficile* using a toxin A affinity column. The polyclonal antibody inhibited both the enterotoxic and cytotoxic activities of toxin A.

A polyclonal antibody specific for toxin B has also been produced (58). The antibody was purified from *C. difficile* antiserum by affinity chromatography. This polyclonal antibody inhibits the cytotoxicity of toxin B. The toxin B-specific polyclonal antibody does not neutralize the cytotoxic or enterotoxic activities of toxin A. Similarly, neither the polyclonal toxin A antibody nor MAb PCG-4 neutralizes the cytotoxic activity of toxin B. Western analysis also reveals very little cross-reactivity of the toxin antibodies (58), suggesting that the two toxins are immunogenically distinct. In 1986 Lyerly et al. (59) produced the monoclonal antibody G-2, which reacted with both toxins A and B and so they concluded that the toxins have a similar immunoreactive site. However, in 1989, Lyerly et al. (60) reported that toxins A and B would react nonspecifically with many monoclonal antibodies and that the binding of the monoclonal antibody G-2 to toxins A and B was not a true immune reaction.

**Possible mechanism of action**

A great deal is known about the physicochemical properties and biological activities of toxins A and B. To date, however, neither the molecular mechanism of the cytotoxic activity of the toxins, nor the mechanism of the enterotoxicity of toxin A, has been proven. One factor that makes the characterization of the mechanism difficult is that
the nucleic acid sequences of the toxins have no homology to other known enzymes or toxins. The large size of the toxins also complicates the characterization of the mechanism. It is possible that the toxins contain different regions with different functions, or that the toxins must be processed into smaller proteins before they are active.

Florin and Thelestam observed in 1981 (61), that *C. difficile* toxin added to tissue culture cells will elicit a cytotoxic effect even if the cells are thoroughly washed after a brief exposure to the toxin. They postulated that the toxins bind to and are internalized by their target cells. Several years later Henriques, Florin, and Thelestam (62) observed that chemicals which inhibit the acidification of endosomes in cells reduced the cytotoxic affect of toxin A. The effects of these chemicals could be overcome by lowering the extracellular pH. They also observed that mutuant Chinese hamster ovary cells which were defective in acidification of their endosomes were more resistant to the cytotoxic affects of toxin A. They hypothesized that once internalized, toxin A is processed in the endosome and that the processing is dependent upon low pH. This hypothesis has some experimental support (62, 63, 64, 65) but the possibility remains that the toxins are internalized by general pinocytosis or that the toxins are not internalized at all.

The cytotoxic effect of toxins A and B is characterized by rounding up of the cells and eventually detachment from the substrate. On the macromolecular level, the effect is seen as a disruption of the cytoskeletal structure, involving the rearrangement of actin filaments. Researchers have tried to pinpoint a particular cytoskeletal protein as the target of the toxins (66, 67, 68, 69). In 1987, Mitchell (66) reported that toxin B affected the ability of actin to polymerize in cells but did not show a direct affect of the toxin on actin polymerization *in vitro*. He therefore concluded that toxin B did not directly affect actin but that it affected some cellular protein which was responsible for actin polymerization. Ottlinger (67) disputed the speculation that the depolymerization of actin is the cause of cell-rounding in toxin B-treated cells. He reported that the relocalization, not the depolymerization, of filamentous actin was the effect induced by toxin B in tissue culture cells.
The research cited above has shown that the toxins affect the cytoskeleton of tissue culture cells but by an indirect, rather than a direct effect on the cytoskeletal proteins. Florin and Thelestram reported in 1986 (70) that toxin B was responsible for the ADP-ribosylation of some protein in tissue culture cells but later found that the ADP-ribosylation was not dependent on the toxin (71). Ciesielski-Treska et al. reported in 1991 (72), that toxin B altered the phosphorylation of several cellular proteins including tropomyosin and vimentin. They suggested the possibilities that toxin B may affect a protein kinase, a phosphatase, or the release of a second messenger, but the exact target of the toxin was not determined. This finding was somewhat supported by a later study conducted by Shoshan et al. (73) who suggested that toxins A and B, although not themselves kinases, play some role in the protein kinase C pathway. The authors are not steadfast in their conclusions, but the overall sense from the studies conducted by Ciesielski-Treska et al. (72), and Shoshan et al. (73, 74) is that the primary molecular mechanism of action of the toxins is not the phosphorylation of some cellular protein.

Recently, Just et al. (75) reported that toxin B affects the GTP-binding Rho proteins that control the polymerization of actin. The unmodified Rho protein causes monomeric actin to polymerize. The *Clostridium botulinum* C3 enzyme normally catalyzes the ADP-ribosylation of the Rho proteins. When Rho is ADP-ribosylated, it causes filamentous actin to depolymerize. The C3 enzyme subsequently elicits its cytotoxic effect by causing the depolymerization of actin. Florin and Thelestram (71) reported that toxin B does not possess ADP transferase activity so it presumably does not directly ADP-ribosylate Rho. Just et al. investigated the possibility that the toxin affects the ADP-ribosylation of Rho in a different manner. The ADP-ribosylation of Rho in lysates from toxin B-treated CHO cells was quantified using the C3 enzyme. Just et al. reported that toxin B prevented the ADP-ribosylation of Rho by the C3 enzyme. They also measured the effect of toxin B on the ADP-ribosylation of purified recombinant Rho and found that the presence of CHO cell lysate was critical to the inhibition of ADP-ribosylation. They concluded that toxin B directly or indirectly affects the Rho protein.
in some way which results in the inability for Rho to become ADP-ribosylated, and that the affect on Rho, is associated with the cytotoxic effect.

Determining the mechanism of action of the toxins in the intestine is an even more complex task than determining the mechanism of cytotoxicity. Toxins A and B elicit a single major response in tissue culture cells, disruption of the cytoskeletal structure; toxin A, however, elicits at least two major responses in the intestine. Toxin A is responsible for direct damage of tissue in the intestine which seems to be the cause of the influx of fluid into the intestinal cavity, but it also seems to have a powerful effect on the host immune response. Leukocytosis and inflammation are commonly associated with pseudomembranous colitis (76, 77) and the toxins have been shown to be chemotactic for leukocytes in vitro (78). Several researchers have attempted to characterize the complex effect that the toxins have on the immune response (79, 80, 81). To date, however, the effect is poorly understood.

**Literature Cited**


Section II. Analysis of Proteolytic Activity in Purified Toxin Preparations.

Introduction

*Clostridium difficile* is a frequent cause of antibiotic-associated diarrhea and is an important cause of the rare but serious disease, pseudomembranous colitis. The symptoms associated with the colonization of the colon by *C. difficile*, are caused by two large exotoxins, toxin A (308,000 daltons) and toxin B (269,000 daltons). The complete nucleic acid and amino acid sequences of toxins A and B are known (1, 2). The toxins share a high percentage of homology. Both toxins contain a potential nucleotide-binding site in the N-terminal region, a hydrophobic region, and a repeating region at the C-terminal end of the protein. Since the toxins share a high percentage of sequence homology and are located concurrently in the genome, it is believed that they are products of gene duplication. Both toxins are cytotoxic, causing rounding but not immediate death of tissue culture cells. Toxin B is the more potent of the two cytotoxins in tissue culture assay. Toxin A is also capable of inducing a fluid response in ligated ileal loops, whereas toxin B lacks this activity. The difference in toxicity of the two toxins may be due to a difference in the recognition of the toxins by cell membrane receptors or it may be due to a difference in the mechanism of action of the toxins once inside the cytosol of cells.

The toxins are thought to exert their toxicity in the cytosol of target cells and seem to enter cells either by receptor-mediated endocytosis or by generalized pinocytosis. During the process of receptor-mediated endocytosis, macromolecules such as proteins and lipoproteins bind to receptors present on the surface of certain cells. Once the
macromolecule has bound to the receptor, the cell membrane begins to form a vesicle around the it. The vesicle enters the cytosol and undergoes a drop in intravesicular pH which disrupts the ligand-receptor complex. The vesicle, now called an endosome, fuses with a lysosome. At this point the protein may be nicked or completely digested by cathepsins or other lysosomal proteases (3).

Several well-characterized toxins enter cells by receptor-mediated endocytosis and undergo proteolytic nicking within the endosome which converts the toxin to its active form. One such toxin is the diphtheria toxin (4). In order for the diphtheria toxin to become biologically active, it must be proteolytically nicked. Nicking of the diphtheria toxin by a trypsin-like protease within the endosome produces two fragments, the A-fragment and the B-fragment, that remain connected by a disulfide bond. The nicked toxin, once acidified in the endosome, undergoes a conformational change. The conformational change exposes a hydrophobic region and allows the B-fragment to insert into the endosomal membrane. Once the B-fragment is inserted into the endosomal membrane, the A-fragment is able to escape the endosome, and enter the cytosol of the cell. The A-fragment of the diphtheria toxin exerts it effect in the cytoplasm of the cell by inhibiting protein synthesis which leads to cell death.

There is some evidence to suggest that C. difficile toxins A and B enter target cells by endocytosis and are processed within the endosome in a manner similar to the diphtheria toxin. Certain chemicals such as monensin, chloroquine, and ammonium chloride, are believed to interfere with endosomal processing by disabling the acidification process that occurs inside the endosome. It has been shown that Chinese Hamster Ovary (CHO) cells that have been treated with these chemicals are resistant to the cytotoxic affects of toxin A (5, 6), suggesting that the acidification of the endosome is an important step in the internal processing of toxin A. Similar to the diphtheria toxin, the acidification of toxin A may lead to a conformational change which exposes the hydrophobic region and allows the toxin to enter the cytosol. Alternatively, the acidification may expose a site in the toxin which is susceptible to cleavage by a
lysosomal protease which converts the toxin to its active form. Presumably, once the toxin is inside the cell and in its active form, it disrupts the cytoskeletal structure of the cell causing the observed change in cell morphology. That toxins A and B are taken up by cells by endocytosis is strongly supported (5, 6, 7, 8). However, the mechanism of action of the toxins once inside the cells has not been proven.

Many different mechanisms have been proposed for the disruption of the cytoskeletal structure by the toxins. Several researchers have reported that filamentous actin is disrupted by the action of the toxins and that the redistribution or depolymerization of filamentous actin results in cell rounding (9, 10). The affect on the actin network may be an indirect one, and the toxins may have a different target. It has recently been proposed by Just et al. that toxin B affects the GTP-binding Rho proteins (11). The Rho proteins play a role in the polymerization of actin. The unmodified Rho protein causes monomeric actin to polymerize, forming filamentous actin. When Rho is ADP-ribosylated, it induces the depolymerization of actin. The C. botulinum C3 enzyme catalyzes the ADP-ribosylation of the Rho proteins and can therefore be used in vitro to stimulate the depolymerization of actin. Just et al. reported that, in the presence of cell lysate, toxin B prevents the ADP-ribosylation of Rho by the C3 enzyme. They propose that the effect of the toxin on Rho somehow results in the breakdown of the cytoskeletal structure. These findings do not reveal the precise target of toxin B. The toxin may affect Rho directly, or it may alter some other enzyme which affects Rho.

One possible mechanism by which the toxin may prevent the ADP-ribosylation of Rho, is the proteolytic nicking of Rho itself, or of another protein that affects Rho. It has been shown that several other clostridial toxins are proteases that exert their effect on cells by cleaving a specific target protein. Some clostridial toxins are metalloproteases and require the association of a Zn atom with the protein in order to cleave their substrates. Among these Zn-dependent, proteolytic toxins are the neurotoxins type B and E (BoNT B and BoNT E) produced by Clostridium botulinum, and the tetanus toxin (TeTx) produced by Clostridium tetani (12, 13). The toxins BoNT E, BoNT B, and TeTx
are produced aszymogens and must be activated before they are able to cleave their substrates. When a toxin is produced as a zymogen, its proteolytic activity may escape detection by the investigator. Often, the first indication that a toxin is a protease is its ability to autodigest. An indication that a toxin is a Zn metalloprotease is the presence of a Zn-binding motif, HEXXH, in the amino acid sequence or the direct detection of Zn in a solution of purified toxin. Clostridium difficile toxins A and B display no obvious proteolytic activity but do appear to autodigest. The amino acid sequences of toxins A and B do not contain the Zn-binding motif but metal ion analysis shows that purified toxins contain elevated levels of Zn (Lyerly et al. unpublished). The purpose of this study was to determine if toxins A and B are proteases and if their putative proteolytic activity is somehow involved in the mechanism of action.

**Materials and Methods**

**Purification of toxin A.** Toxin A was isolated from C. difficile VPI strain 10463 culture filtrate by either of two methods. Toxin A was purified sequentially by 40% ammonium sulfate precipitation, anion exchange chromatography on DEAE Sepharose CL-6B (Sigma Chemical Co., St. Louis MO.), and isoelectric precipitation at pH 5.6 as previously described by Lyerly et al. (14) and by Sullivan et al. (15). Toxin A was also purified by bovine thyroglobulin affinity chromatography according to Krivan and Wilkins (16). The latter purification is based on the specific binding of a site located in the repeating region of toxin A to the carbohydrate ligand Galα1-3Galβ1-4GlcNAc. Binding of the toxin to this carbohydrate ligand is temperature-dependent so the toxin may be bound to insolubilized thyroglobulin at 4°C and eluted at 37°C.

**Purification of toxin B.** Toxin B was purified from the supernatant fluid of the culture filtrate following the removal of toxin A. The toxin was purified sequentially by
55% ammonium sulfate precipitation and anion exchange chromatography on DEAE Sepharose CL-6B as described by Lyerly et al. (14).

**Analysis of toxin purity and concentration.** The purity of both toxins was determined by SDS and native PAGE electrophoresis according to Laemmli (17). Toxins were layered onto a discontinuous gel containing 4% acrylamide in the stacking gel and 7.5% acrylamide in the separating gel. Separation was carried out at 30 mA per gel for 90 minutes. Proteins were visualized by staining gels with Coomassie blue R-250 and removing the excess stain with 7% glacial acetic acid and 5% methanol.

Purity was further analyzed by crossed immunoelectrophoresis performed in 1.2% low electro-endosmotic agarose (Sigma Chemical Co.) in 0.0125 M Tris-tricine buffer, pH 8.6, containing goat antisera against *C. difficile* (Ehrich et al., 18) in the second dimension. The basic method of Axelsen et al. (19) was followed.

Protein concentration was determined using a Coomassie protein assay from Pierce (Rockford, IL). A 2 mg/ml bovine serum albumin standard was provided by Pierce. This standard was diluted with water to concentrations ranging from 0.125 mg/ml to 0.500 mg/ml in order to develop a standard curve. Absorption of light, wavelength 595 nm, was monitored using a DU-70 Spectrophotometer from Beckman Instruments Inc. (Fullerton, CA).

**Western analysis of toxins A and B.** The toxins were analyzed by Western blotting. The toxins were layered onto a discontinuous SDS PAGE gel containing 4% acrylamide in the stacking gel and 7.5% acrylamide in the separating gel. Separation was carried out at 30 mA per gel for 90 min. Proteins were transferred from the gels onto nitrocellulose paper using a TE22 Mighty Small Transphor Tank transfer unit from Hoefer Scientific Instruments (San Francisco, CA). Samples were blotted onto the nitrocellulose for 4 hours at 50 V in 0.025 M Tris-0.192 M glycine buffer, pH 8.3, containing 20% methanol. The nitrocellulose membrane was then rinsed in 50 mM Tris-HCl, pH 7.5,
containing 0.15 M NaCl (TBS) and blocked at room temperature in 3% gelatin for 1 hour. To detect toxin A, the membrane was incubated for four hours in a 1:5,000 dilution of PCG-4, a monoclonal antibody against the repeating units of toxin A. Ascites fluid containing high levels of PCG-4 MAb was prepared as described by Lyerly et al. (20). To detect toxin B, the membrane was incubated for four hours at room temperature in a 1:2,500 dilution of affinity-purified, polyclonal antibody against toxin B. The toxin B antibody was prepared according to the method of Lyerly et al. (21). The membrane was washed three times with TBS before the secondary antibody, rabbit anti-mouse for PCG-4 or goat anti-rabbit IgG-horseradish peroxidase conjugate for affinity B was added. After 1 hour, the membrane was washed again and the substrate, Opti-Mist horseradish peroxidase reagent (TSI, Boston, MA), was added. The was incubated with the substrate for 5 minutes during which a blue color developed. The membrane was then rinsed with deionized water, and allowed to air-dry.

**Analysis of autodigestion of the toxins.** Toxins A and B were subjected to SDS PAGE electrophoresis as described above. Three lanes were loaded with each toxin. The gel was sliced vertically to separate the three lanes. The first lane was subjected to Western blot analysis using PCG-4 MAb. The second lane was stained with Coomassie blue R-250 to visualize the high-molecular-weight toxin band which presumably contains the undigested toxin. This band was excised from the third gel lane and eluted into Tris buffer (50mM pH 7.5) overnight at 4°C. The protein that eluted from the acrylamide band was then subjected to electrophoresis a second time. The second SDS PAGE gel was analyzed by Western blot with PCG-4 MAb or affinity-purified, polyclonal toxin B antibody and observed for breakdown of the toxin. In a similar experiment the toxins were subjected to SDS PAGE electrophoresis, and the entire lane containing the toxin was excised and placed perpendicular to a second separating gel which had un polymerized stacking gel on top. The stacking gel was allowed to polymerize around the excised lane to create a seal between the lane and the separating gel. This second gel was then
subjected to electrophoresis and analyzed by Western blot.

Assay for actin-cleaving activity. All toxin preparations, culture filtrates, and other proteases were tested for the ability to cleave actin in the same manner. The substrate, rabbit skeletal muscle actin (Sigma Chemical Co.), was solubilized in deionized water to a concentration of 1 mg/ml and combined with an equal volume of the test sample. Purified toxins were diluted to 0.3 mg/ml in 50 mM Tris pH 7.5. Culture filtrates and cell lysates were tested for protease activity without dilution. The reaction was either reduced with 15mM dithiothreitol or left unreduced. The reaction mixture was incubated at 37°C for 4 hours. The sample was subjected to SDS PAGE in a 4% stacking/12.5% separating gel. The gel was stained with Coomassie blue, and the cleavage of actin was monitored by the generation of lower molecular weight protein bands.

Actin degradation was also monitored by subjecting the test sample to denaturing capillary electrophoresis (CE). Sodium dodecyl sulfate CE was performed on a Beckman PACE 2200 system by using the eCAP SDS-200 kit according to the manufacturer's instructions (Beckman Instruments, Fullerton, Calif.). Samples were injected by using a high pressure injection for 30 seconds and were separated at 14.4 KV for 30 min. Protein peaks were monitored at 214 nm.

Immunological detection of actin cleavage. Cleavage of actin by culture filtrates and cell lysates was detected by Western blotting. Test samples were subjected to SDS PAGE in a 4% stacking/12.5% separating gel. Gels were then electro-blotted onto nitrocellulose for 4 hours at 50 V and analyzed by Western blotting as described above. The primary antibody used in immunodetection was an affinity-purified, polyclonal rabbit antibody specific for the C-terminal end of actin (Sigma Chemical Co.). The membrane was blocked and incubated in primary antibody for 4 hours. The membrane was washed in TBS, incubated in goat anti-rabbit IgG peroxidase conjugate for 1 hour, and washed
in deionized water as described above, and the horseradish peroxidase substrate was added.

**Zymogram analysis of protease activity.** Protease activity was analyzed using the zymogram method described by Garcia-Carreño and Haard (22). Test samples were diluted to 0.1 mg/ml and 10 μl were subjected to non-reducing SDS PAGE electrophoresis in a 4% stacking/7.5% separating gel. After electrophoresis the gel was incubated for one hour at 4°C in 2% casein in 50mM Tris-HCl (pH 7.5). During incubation, the gel to absorb the substrate while the activity of the protease was low. The substrate-laden gel was incubated at 37°C for 4 hours, stained with Coomassie Blue R-250 for 3 hours and destained with 7% glacial acetic acid/5% methanol. The gel was observed for bands of clearing (digested casein) against a dark blue background.

**Production of recombinant lysates.** The recombinant toxin clones, pCDtoxA.03 and pCDtoxB were constructed as described by Phelps et al. (23) and Johnson et al. (24). The recombinant toxin clones pCDtoxBP-BRU and pCDtoxB-R73 were constructed as described by Barroso et al. (25). These recombinants, as well as the pUC19 host vector, were transformed into *E. coli* DH5α host cells (Figure 1). The clones were grown in 40 ml Terrific broth (26) containing potassium phosphate supplement and ampicillin for 4 hours at 37°C with shaking. The cultures were induced at 37°C for 18 hours with 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) from United States Biochemical Corp., Cleveland, OH. All subsequent steps were carried out at 4°C. Cultures were centrifuged at 7,500 rpm for 10 minutes, the cell pellets were washed with Tris-buffered saline (TBS) and centrifuged a second time. Cell pellets were suspended in 1 ml TBS and lysed with glass beads using the mini-beadbeater from Biospec products (Bartlesville, OK). Lysates were centrifuged and the supernatants were filtered through a 0.45 micron filter to remove the glass beads and cellular debris.
Figure 1: Cleavage of actin by recombinant lysates. The toxin genes and fragments of the toxin genes shown above were cloned into pUC19 and expressed in *E. coli* DH5-a. Cell lysates were added to actin and actin-cleaving activity was monitored by Western blot analysis. All of the lysates were found to degrade actin into multiple fragments ranging from 10,000 to 25,000 daltons.
N-terminal Sequencing of the actin cleavage site. Reaction mixtures containing 50 μl of actin (1 mg/ml), 25 μl of purified toxin A (0.3 mg/ml), and 15 mM dithiothreitol were incubated at 37°C for 4 hours. The cleaved actin was then subjected to SDS PAGE electrophoresis as described above. The gel was electro-botted onto a polyvinylidene difluoride membrane (Immobilon from Millipore, Bedford, MA) at 0.5 A for 20 minutes in 10 mM CAPS, pH 11 containing 10% methanol. The fragment of actin produced by proteolytic cleavage with purified toxin A was prepared for N-terminal sequencing as previously described by Matsudaira (27). Sequencing was performed at the Department of Biology at Virginia Polytechnic Institute and State University by automatic Edman degradation using a model 477A Protein Sequenator from Applied Biosystems (Foster City, CA).

Inhibition of protease activity with PCG-4 MAb. Varying amounts of PCG-4 monoclonal antibody were added to purified toxin A (0.3 mg/ml) and the mixture was incubated at 37°C for 3 hours to allow the antibody to complex with the toxin. The reaction mixtures were assayed for residual protease activity as described above and analyzed by SDS capillary electrophoresis.

Affects of various protease inhibitors on actin-cleaving activity. Protease inhibitors were purchased from Sigma Chemical Co. unless otherwise indicated. Purified toxins and culture filtrates were combined individually with each of the following protease inhibitors and incubated for 30 minutes at 37°C: 40 mM O-phenanthroline from 200 mM stock in methanol, 20 mM EDTA in Tris, 10 mM Captopril in Tris buffer, 1 mM Phosphoramidon in Tris buffer, 1 mg/ml AEBSF (ICN Biochemicals Aurora, OH) in water, Pepstatin (0.01 mg/ml) in Tris, Leupeptin (0.01 mg/ml) in Tris, (0.01 mg/ml) iodoacetic acid in water. The proteolytic activity of the toxin/inhibitor mixtures was assayed as described above.
Affects of other reagents on toxin A. Different proteases and chemical reagents were added to freshly purified toxin A. All proteases and reagents were purchased from Sigma Chemical Co. The toxin was treated with the following proteases: trypsin, chymotrypsin, *S. griseus* pronase E, pepsin, collagenase, Factor Xa, cathepsin B, cathepsin C, cathepsin D, cathepsin G, proteinase K, thermolysin, and human furin (kindly provided by Dr. Stephen Leplla at National Institutes of Health, Bethesda, Md.). Proteases were added to the toxin at a 1:100 protease:toxin (wt/wt) ratio (except furin which was added 1:4, vol/vol ratio). The mixtures were incubated at 37°C for 30 minutes. After incubation, the toxin/protease mixtures were analyzed by the zymogram method described above. Trypsin/toxin mixtures were treated with agarose-coupled soybean trypsin inhibitor to remove trypsin, and assayed for the ability to cleave actin. Cathepsin C and cathepsin D are not able to cleave actin, so toxin/cathepsin mixtures were also assayed for the ability to cleave actin.

The affect of various detergents on the protease activity of the toxin was analyzed by incubating freshly purified toxin A in a 1% or a 10% (wt/vol) solution of the following detergents at 37°C for 4 or 60 hours: CHAPS, deoxycholic acid, SDS, SLS, Triton X-100, Triton X-14, nonidet P-40, Tween 80, Tween 20, Lubrol-PX, and Brij-35. Reaction mixtures were tested for their ability to cleave actin according to the method described above. The affect of various metal ions on protease activity was investigated by adding varying concentrations (10⁻³ to 10⁻⁸ M) of the following metal chloride salts to toxin A and incubating the reaction at 37°C for 2 or 18 hours: HgCl₂, CaCl₂, FeCl₂, ZnCl₂, MgCl₂, and MnCl₂. The effect of nucleotides and electron donors on the protease activity of toxin A, was also analyzed. ATP, CTP, GTP, UTP, NAD, or FAD was added to purified toxin (0.3 mg/ml) for a final concentration of 10 mM, and the mixture was incubated 2 hours at 37°C. The toxin was then assayed for actin-cleaving activity as described above. The toxin mixtures were also analyzed by the zymogram method described above.
Results

**Purity of Toxin preparations.** Regardless of how carefully a protein is purified, it can only be considered as pure as the limit of detection of the analytical method used. When an investigator observes a certain activity in a purified protein preparation, the preparation must be analyzed carefully and consideration must be given to the possibility that any activity observed is due to a contaminant. For these reasons, the purity of toxins A and B was carefully evaluated.

Purified toxin A preparations were examined by native polyacrylamide gel electrophoresis (Figure 2). The concentrations of different toxin preparations used in the analyses ranged from 0.25 mg/ml to 1.9 mg/ml. When the PAGE gel was stained with Coomassie Blue, a single band was observed at the top of the gel that reacted with PCG-4 MAb, a MAb specific for the repeating region of toxin A. Purified toxin A preparations were also subjected to denaturing PAGE electrophoresis. One major band at about 300,000 daltons and multiple faint bands, ranging from 250,000 to 50,000 daltons were observed. When the SDS PAGE gel was subjected to Western analysis, the major 300,000 dalton band and all of the bands ranging from 250,000 to 50,000 daltons reacted with the monoclonal antibody, PCG-4. When the purified toxin A was analyzed by crossed immunoelectrophoresis, using goat antiserum against *C. difficile* in the second dimension, a single peak appeared corresponding to the peak that appears when rabbit antiserum against the repeating region of toxin A is used (Figure 3).

Purified preparations of toxin B were also examined by native PAGE electrophoresis, and this revealed a single band at the top of the gel when stained with Coomassie Blue (Figure 2). When the preparations were subjected to Western analysis using affinity B polyclonal antibody, a similar band was observed. When the preparations of purified toxin B were examined by denaturing PAGE and stained with Coomassie blue, a major band of about 250,000 daltons and multiple faint bands ranging from 230,000 to 50,000 daltons were seen. When the SDS PAGE gel was subjected to Western analysis,
Figure 2: Analysis of purified toxin preparations by non-denaturing polyacrylamide gel electrophoresis. Purified preparations of toxins A and B were electrophoresed in a 4% stacking/7.5% separating native PAGE gel. The gel was stained with Coomassie blue. Native PAGE shows the presence of a single band in both toxin preparations. Lane 1, culture filtrate from *C. difficile* VPI strain 10463. Lane 2, purified toxin A (7.5 μg). Lane 3, purified toxin B (5.0 μg).
Figure 3: Analysis of purified toxin preparations by crossed immunoelectrophoresis. Crossed immunoelectrophoresis using goat antiserum against *C. difficile* VPI strain 10463 shows the presence of a single peak in purified toxin A and B preparations. Plate 1, culture filtrate from *C. difficile* VPI strain 10463. Plate 2, purified toxin A (3 μg). Plate 3, purified toxin B (2 μg).
the major band and the bands ranging from 230,000 to 50,000 daltons all reacted with affinity purified B antibody. When the toxin preparations were subjected to crossed immunoelectrophoresis, using goat antiserum against *C. difficile* culture filtrate in the second dimension, a single, faint peak was seen corresponding to the peak seen when affinity B antibody is used in the second dimension (Figure 3). The protein concentrations of toxin B preparations used in the analyses ranged from 0.19 mg/ml to 0.25 mg/ml.

**Analysis of autodigestion.** Purified preparations of toxins A and B subjected to SDS PAGE electrophoresis separated into multiple protein bands (Figure 4). The smaller molecular weight bands were faint when visualized by staining but were easily seen on a Western blot. All of the bands that were observed by Coomassie Blue staining of the toxin A preparations reacted with MAb PCG-4. Likewise, all of the proteins present in the toxin B preparations reacted with affinity-B antibody. The toxin fragments ranged in size from 250,000 daltons to 50,000 daltons. The high molecular weight toxin band from the SDS PAGE gel containing toxin A was excised and eluted. The protein that eluted from the high molecular weight band was electrophoresed in a second SDS PAGE gel and the multiple banding pattern was again observed. This experiment was repeated in a slightly different way. Toxin A was electrophoresed in an SDS PAGE gel and the entire lane was cut from the gel. The lane was then placed perpendicular to a second SDS PAGE separating gel and the proteins were electrophoresed a second time. The high molecular weight band again degraded but the smaller bands did not (Figure 5).

**Digestion of actin by purified toxins and culture filtrates.** Actin was used as a potential substrate in testing toxins A and B for proteolytic activity for two reasons. First, evidence strongly suggests that toxins A and B somehow affect the arrangement of actin in tissue culture cells, and second, it was shown that botulism neurotoxin type E cleaves actin. Assays for actin-cleaving activity were performed on ten different lots of
Figure 4: Denaturing PAGE of purified toxin A showing autodigestion. This is a 4% stacking / 7.5% separating SDS PAGE gel which has been stained with Coomassie blue. Purified toxin A was electrophoresed. The high molecular weight band was excised from the gel, eluted and electrophoresed a second time in the gel shown above. The gel shows that purified toxin A degrades when it is subjected to SDS PAGE and that the excised toxin A band degrades when it is electrophoresed a second time. Lane 1, high molecular weight standards from Bio-Rad: myosin 194,000, β-galactosidase, 116,000, bovine serum albumin, 85,000, ovalbumin, 49,000. Lane 2, culture filtrate from VPI strain 10463. Lane 3, purified toxin A (7.5 μg). Lane 4, eluate from 300,000 dalton toxin A band.
Figure 5: Western blot analysis showing autodigestion of purified toxin A. Purified toxin A (7.5 μg) was electrophoresed in a 4% stacking / 7.5% separating SDS PAGE gel. The lane containing the toxin was cut and placed perpendicular to a second SDS PAGE gel such that the proteins top of the first gel would migrate along the left side of the second gel and the proteins at the bottom of the first gel would migrate along the right side of the second gel. The protein in the second gel was electroblotted onto nitrocellulose. Western analysis using the monoclonal antibody for toxin A, PCG-4, was performed on the nitrocellulose. The high molecular weight toxin A band (the left-most band on the blot shown above) broke down into multiple bands whereas the smaller bands did not.
toxin A (Table 1). Six lots of purified toxin A which had been in storage at 4°C for less than six months cleaved actin when the reaction mixture was reduced with dithiothreitol (DTT). The proteolytic cleavage occurred at a single, N-terminal site between the histidine\(^{40}\) and the glutamine\(^{41}\) (Figure 6) resulting in a 38,000 dalton fragment (Figure 7). A 5,000 dalton fragment which would correspond to the N-terminal region that was removed from actin was not seen on the gel. Actin was not cleaved in the absence of DTT. In test samples containing 25 µl of toxin A at 0.3 mg/ml, 25 µl of actin at 1 mg/ml and 15mM DTT, only about 50% of the actin was cleaved regardless of whether the incubation time was 4 hours or 72 hours. These results indicate that 1 mole of toxin A is able to cleave approximately 10 moles of actin. The toxin A concentration had to be increased to 2 mg/ml or higher in order to cleave 100% of the actin. These results seem to indicate that 1 mole of toxin A is only able to cleave 3.5 moles of actin.

Toxin A purified by thyroglobulin affinity chromatography cleaved actin in the same manner as toxin purified by sequential ammonium sulfate precipitation, anion-exchange chromatography, and isoelectric precipitation. Purified toxin A which had been in storage at 4°C for six months or longer, possessed an elevated proteolytic activity and did not require reduction with DTT. When 0.07 mg/ml of one of these "older" toxin preparations was added to 1 mg/ml of actin, 100% of the actin was cleaved. This suggests that 1 mole of "old" toxin A is able to cleave approximately 100 moles of actin. The actin was also cleaved at multiple sites by the "older" preparations of toxins (Figure 7). The addition of DTT restricted the cleavage of the actin to a single site. A time study of the cleavage of actin by the "older" toxin preparations was performed employing SDS capillary electrophoresis to monitor the breakdown. These studies showed that approximately 50% of the actin was broken down after 1 hour of incubation with the toxin and that 100% of the actin was broken down by 2 hours.

Six lots of purified toxin B were tested for their ability to cleave actin. Freshly purified toxin B did not cleave actin under reduced or non-reduced conditions (Table 1). Purified toxin B which had been in storage (4°C) for one year or longer cleaved actin at
Figure 6: N-terminal sequence of actin fragment produced by toxin A. Purified toxin A (25 ul at 0.3mg/ml) was added to rabbit skeletal muscle actin (50 ul at 1mg/ml). The mixture was incubated at 37°C for 4 hours and subjected to SDS PAGE(a). The cleaved band was sequenced by Edman degradation (b). Figure 6b shows the 9 amino acids that were sequenced (underlined) and the site in actin that is cleaved by freshly purified toxin A (arrow).
**Figure 7 : Western blot analysis of actin cleavage.** Reaction mixtures were electrophoresed in a 4% stacking / 12% separating SDS PAGE gel. Proteins were electrobotted onto a nitrocellulose membrane. Western analysis was performed on the nitrocellulose using a polyclonal antibody directed towards the C-terminal region of actin. Lane 1, low molecular weight standards from Bio-Rad: phosphorylase B (142,000), bovine serum albumin 97,200, ovalbumin 50,000, carbonic anhydrase, 35,100, and soybean trypsin inhibitor 29,700. Lane 2, rabbit skeletal muscle actin (10 μg). Lane 3, actin + "fresh" toxin A (3 μg) + DTT (15 mM). Lane 4, actin + "old" toxin A (3 μg) + Tris buffer. Lane 5, actin + "fresh" toxin B (3 μg) + DTT. Lane 6, actin + "old" toxin B (3 μg) + Tris buffer. Lane 7, actin + culture filtrate from C. difficile VPI strain 10463 (25 μg) + DTT. Lane 8, actin + culture filtrate from VPI strain 11186 (25 μg) + DTT.
Table 1: Activity of Various Toxin Lots

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0 = No Activity  
+ = Low Activity  
++ = High Activity  
? = Not tested

Toxins A and B were purified by the standard methods. The date of purification is reflected by the lot number. The toxins were assayed for actin-cleaving activity by SDS PAGE. The toxins were assayed for casein-cleaving activity by the zymogram method. The toxins were assayed for autodigestion by Western blot analysis. Low activity is the cleavage of 50% of the actin at a single site in the reaction mixture, a faint clearing zone on a casein zymogram, or the amount of autodigestion seen in freshly purified toxin. High activity is the cleavage of 100% of the actin at multiple sites, or a bold clearing band on a casein zymogram. The toxin activity was assayed on two different dates, on 8-11-93 and on 6-20-94.
multiple sites in the absence of DTT (Figure 7). One mole of "old" toxin B was able to cleave approximately 40 moles of actin. The addition of DTT to the reaction mixtures restricted the cleavage of actin to a single site.

Culture filtrates from various strains of *C. difficile* also cleaved actin at a single site as demonstrated by Western blot analysis (Figure 7). The cleavage of actin was not influenced by the addition of dithiothreitol. The cleavage site was not determined by N-terminal sequencing, but was presumed to be located in the N-terminal region because the cleaved product (approx. 35 KDa) reacted with the actin antibody directed towards the C-terminal region and not with the antibody against the N-terminal region. The fragment produced by cleavage with culture filtrate was similar in size to the fragment produced by freshly purified toxin A but migrated slightly faster on an SDS PAGE gel. The ability of culture filtrates to cleave actin did not correlate with the level of toxigenicity of the strain. Culture filtrate from nontoxigenic strain VPI strain 11186 cleaved actin to the same extent as the weakly toxigenic VPI strain 7698 and as the highly toxigenic VPI strain 10463.

**Inhibition of actin-cleaving activity by PCG-4 MAb.** The monoclonal antibody PCG-4 binds to the carbohydrate-binding region of toxin A and inhibits its cytotoxicity. PCG-4 MAb partially reduced the proteolytic activity of purified preparations of toxin A. This neutralization was dose-dependent and required a high antibody:antigen ratio for complete inhibition. If no PCG-4 was added to toxin A, then it was able to cleave approximately 50% of the actin in test reactions. When PCG-4 was added to toxin A at a ratio of 1:50 (wt/wt), the amount of actin cleaved was reduced to approximately 22%. When PCG-4 was added at a wt/wt ratio of 1:12.5, approximately 17% of the actin was cleaved.

**Actin-cleaving activity of recombinant toxins.** The genes for toxin A and toxin B were individually cloned into the pUC19 plasmid and are designated pCDtoxA.03 and
Table 2: Addition of Proteases and Protease Inhibitors to Toxin A

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Various proteases and protease inhibitors were added to two different lots of toxin A in an attempt to alter the actin-cleaving activity. The percentage value represents the approximate amount of actin cleaved in a reaction mixture containing 1mg/ml actin and 0.3mg/ml toxin A.
pCDtoxB (23, 24). The repeating region of toxin B has also been cloned into pUC19 and is designated pCDtoxBP-BRU. The toxin B gene lacking the repeating region has also been cloned into pUC19 and is designated pCDtoxB-R73. The recombinant toxin clones are represented diagrammatically in Fig. 1. Lysates from these four recombinants were made and all contained similar protein concentrations (15 mg/ml to 18 mg/ml). Lysates from each of the four recombinant clones cleaved actin into multiple fragments. The fragments produced by the lysates did not correspond in size, however, to the fragments produced by purified toxins. Lysate from the negative control, pUC19, did not cleave actin. Each lysate was produced and assayed four times for the ability to cleave actin and similar results were found each time. Recombinant toxin A was purified from E. coli lysate by thyroglobulin affinity chromatography and, on two out of four occasions, cleaved actin, producing a fragment of approximately 38,000 daltons.

**Estimation of molecular weight of protease.** Zymogram analysis includes the separation of proteins by polyacrylamide gel electrophoresis in non-reducing, mildly denaturing conditions and the detection of proteolytic activity in the individual protein bands. Since the proteins are partially denatured with SDS, a reasonable estimation of the molecular weight of proteolytically active bands may be obtained. Zymogram analysis showed that "older" purified toxin A digested casein and that the protease activity was due to a 50,000 dalton protein which reacted with PCG-4 MAb. The "older" purified toxin was subjected to non-reducing, denaturing PAGE electrophoresis and the band corresponding to a molecular weight of 50,000 daltons, was excised, eluted, and assayed for actin-cleaving activity. The 50,000 dalton protein was able to cleave actin into multiple fragments.

**Inhibition of actin-cleaving activity.** Reagents that were tested for their ability to inhibit the actin-cleaving activity of the toxins are listed in Table 2. Many protease inhibitors are specific for the type of protease that they will inhibit. Protease inhibitors
specific to the main classes of proteases: serine proteases, metalloproteases, and cysteine proteases were added to the toxins and the affect on actin-cleaving activity was monitored. The chelating agents ortho-phenanthroline and EDTA completely inhibited the proteolytic activity of purified preparations of toxins A and B. No other protease inhibitors inhibited the proteolytic activity of the toxins. These results suggest that the actin-cleaving activity of the purified toxin preparations is due to a metalloprotease. Culture filtrates were not inhibited by ortho-phenanthroline or EDTA but were inhibited by the serine protease inhibitor AEBSF. This suggests that the majority of the actin-cleaving activity present in the culture filtrates is due to a serine protease.

**Treatment of toxin A with proteases and other reagents.** Many proteases require activation before they are able to cleave their substrates. Activation of thesezymogens can sometimes be accomplished by subjecting them to a mild treatment with another protease. Toxin A was treated with several proteases in an attempt to activate the actin-cleaving or casein-cleaving activity (Table 2). At a pH of 7.5, toxins A is resistant to proteolytic digestion by trypsin-like proteases and cathepsins. Trypsin and cathepsins are often used to nick zymogens in order to make them active. When the pH of the toxin preparations was lowered to 5.5 (the toxin was kept soluble at this pH by raising the ionic strength of the solution) it was susceptible to degradation by trypsin and cathepsins B, C, D, and G. Toxin A was cleaved by these proteases at multiple sites. However, none of the proteases that were added to purified toxin A at either pH 7.5 or 5.5 increased the actin-cleaving or casein-cleaving activities of the toxin.

Some detergents alter protein structure and activate certain zymogens. Anionic, cationic, and nonionic detergents were added to toxin A in an attempt to increase the actin-cleaving activity (Table 3). None of the detergents increased the actin-cleaving activity of the toxins after 4 or 60 hours of incubation.

Heavy metal ions are also capable of altering the conformation of certain proteins. Several chloride salts were added to toxin A in an attempt to increase actin-cleaving
### Table 3: Addition of Various Reagents to Toxin A

<table>
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<tr>
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<th>ToxA060194 - DTT</th>
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<th>ToxA050893 - DTT</th>
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Various reagents were added to two different lots of toxin A in an attempt to increase or decrease the actin-cleaving activity. The percentage value represents the approximate amount of actin cleaved in a reaction mixture containing 1mg/ml actin and 0.3mg/ml toxin A.

49
activity or autodigestion (Table 3). Mercuric chloride appeared to increase the autodigestion of purified toxin A to a small extent but did not increase the toxin's ability to cleave actin. None of the other chloride salts increased actin-cleaving activity.

Some proteases require the energy stored in nucleotide triphosphates in order to cleave their substrates. The addition of nucleotide triphosphates to the toxin did not increase the ability of toxin A to cleave actin (Table 3). The addition of the electron donors, NAD and FAD, also had no effect.

**Discussion**

Freshly purified and stored preparations of toxins A and B were homogenous when examined by native polyacrylamide gel electrophoresis, Western analysis, and crossed immunoelectrophoresis. Despite the inability to detect any contamination of the purified toxins by PAGE or crossed immunoelectrophoresis, a protein solution can never be considered absolutely pure. Protein-protein interactions can be very powerful and proteins may co-purify despite rigorous purification procedures. If the concentration of a contaminating protein is low relative to the concentration of the protein of interest, it may escape detection. For example, it was reported that toxin B possessed enolase activity (28) but Fluit et al. later reported that the activity was not due to toxin B, but to the metabolic enzyme, enolase, which co-purified with the toxin (29). Florin and Thelestan reported that toxin B was responsible for the ADP-ribosylation of certain intracellular proteins (30) but later found that it was not the toxin, but a contaminating enzyme, that was responsible for the ADP-ribosylation (31). It has also been found that glutamate dehydrogenase can co-purify with toxin A.

When purified preparations of *Clostridium difficile* toxins A and B were subjected to denaturing PAGE electrophoresis, they broke down into multiple fragments ranging from 250,000 daltons to 50,000 daltons. All of the bands reacted with antibodies specific to the toxins, suggesting that they were not contaminating proteins. When the band
corresponding to the molecular weight of toxin A was excised from the SDS PAGE gel and eluted the protein, it again displayed a multiple banding pattern when subjected to SDS PAGE electrophoresis and analyzed by Western analysis. Denaturing PAGE electrophoresis is an effective method for separating proteins and the denaturing conditions that the toxin samples were subjected to before electrophoresis were harsh. For this reason, the high molecular weight bands that were observed were assumed to contain very pure toxin. I believe therefore, that it is highly unlikely that an aggregation of proteins or a contaminating protease was present in the 300,000 dalton band. If a contaminating protease was present in the purified toxin preparations and was responsible for the degradation of the toxins, it should have migrated to a different position in the SDS PAGE gel than the toxin. Since the protein responsible for the degradation of the toxins was not separated from the toxins by SDS PAGE, I concluded that the toxins were autodigesting. Sometimes, the first clue that a protein is a protease is its ability to autodigest. This was the case for several clostridial toxins which have recently been reported to be Zn metalloproteases (32, 33, 34). The tetanus toxin and the botulism neurotoxin (BoNT) type B cleaves synaptobrevin at a single, specific site (12). The BoNT type E cleaves actin on the C-terminal side of arginine and lysine residues (13). The protease activity of the tetanus toxin and BoNT types B and E went undetected for a long time because they require activation before they are proteolytically active and because the appropriate substrates were not known. In order to become proteolytically active (in vitro), the tetanus toxin and the botulism neurotoxins are nicked by trypsin and reduced with dithiothreitol. The scission by trypsin converts the single chain (150,000 daltons) form of the toxins to a dichain form. The dichain toxins consist of a light chain (50,000 daltons) and a heavy chain (100,000 daltons) connected by a disulfide bridge. The addition of DTT reduces the disulfide bridge, releasing the proteolytically active light chain.

The finding that the active form of BoNT type E cleaves actin, coupled with the finding that the organization of actin is disrupted in tissue culture cells treated with toxins
A and B (9,10), prompted me to investigate the possibility that toxins A and B cleave actin. I assayed ten different preparations of toxin A for actin-cleaving activity. These preparations had been purified sequentially by ammonium sulfate precipitation, anion-exchange chromatography, and isoelectric precipitation (Table 1). All ten of the toxin preparations cleaved actin. I found that freshly purified toxin A (purified toxin A which had been in storage at 4°C for less than six months) that was reduced with DTT, cleaved actin at a single site near the N-terminus, producing a fragment of approximately 38,000 daltons (Figure 6). The 5,000 dalton fragment, corresponding to the small fragment of cleaved actin, was not be seen on an SDS PAGE gel. This suggests that the protease could be an exoprotease, cleaving off one amino acid until it reaches glutamine⁴⁰, rather than an endoprotease which cleaves actin at a single site. I believe, however that the 5,000 dalton peptide is simply too small to be observed on the gel.

The purified preparations of toxin cleaved approximately 50% of the actin within four hours at 37°C, but the remaining 50% was not cleaved even after 72 hours. A very high concentration of fresh toxin A was required to cleave 100% of the actin. The molar ratio of toxin to actin had to be raised to 1:3.5 in order to get 100% of the actin cleaved. It is difficult to explain why 50% of the actin is cleaved relatively quickly, and the remaining 50% seems to be resistant to cleavage. Globular actin monomers contain one molecule of ATP or ADP and one Ca²⁺ or Mg²⁺ ion (35). The actin molecules with ATP bound may be more susceptible to proteolytic cleavage than molecules with ADP bound, or the presence of one of the ions may influence susceptibility to cleavage.

Interestingly, preparations of toxin A that had been stored at 4°C for six months or longer cleaved 100% of the actin in test reactions after only two hours of incubation at 37°C. The molar ratio of toxin to actin required for the cleavage of 100% of the actin was only 1:100. The "older" toxin A also cleaved the actin at multiple sites (Figure 7). The addition of dithiothreitol to mixtures containing one of these older toxin A stocks restricted the cleavage to a single site. Dithiothreitol seems to play an important but paradoxical role in the cleavage of actin. Dithiothreitol stimulates the cleavage of actin
at the histidine\textsuperscript{40} glutamine\textsuperscript{41} site but inhibits the cleavage of actin at other sites. Test samples containing either actin alone or actin reduced with dithiothreitol were incubated at 37°C for 96 hours and analyzed by SDS PAGE. The sample containing actin alone had broken down over the extended incubation whereas actin in the sample containing DTT was still intact. Dithiothreitol apparently stabilizes actin. This could explain why the presence of DTT restricts the cleavage of actin by "older" preparations of toxin A to a single site but does not offer an explanation as to why "fresh" preparations of toxin A will cleave actin only in the presence of DTT.

Two preparations of toxin A purified by thyroglobulin affinity chromatography cleaved actin in a manner similar to toxin A purified by the standard method. The fact that all of the toxin A preparations, regardless of the purification method used, cleaved actin, somewhat reduces the possibility that the actin cleavage was due to a contaminating protease.

Since toxin A is similar in structure and biological activity to toxin B, I expected that if the actin-cleaving activity was due to the toxin and not a contaminating protease, purified toxin B would also cleave actin. I assayed six different preparations of toxin B purified by ammonium sulfate precipitation and ion-exchange chromatography. Purified toxin B which had been stored at 4°C for less than one year did not cleave actin in the presence or absence of DTT. Preparations of purified toxin B that had been stored for one year or longer, cleaved 100% of the actin into multiple fragments (Figure 7).

The age of the purified toxins was apparently a critical factor in the actin-cleaving ability. Since the toxins were found to autodigest, I hypothesized that, during storage, the toxins might be slowly nicking themselves, producing a proteolytically active form. If this were the case, then a fragment of the toxin rather than the entire toxin molecule would be proteolytically active. In order to test this hypothesis, I analyzed the proteolytic activity of the toxin preparations by the zymogram method which allowed me to estimate the molecular weight of the protease. A revised protocol for zymogram analysis, which allows for a fairly accurate estimation of the molecular weight of the protease, was
devised by Garcia-Carreño and Haard (22). In this protocol, the test sample is partially denatured by the addition of SDS with the sample neither reduced nor boiled. Under these conditions, most proteases retain their activity and since the sample is partially denatured, a more accurate estimation of molecular weight may be obtained. The sample is electrophoresed in an SDS PAGE gel which does not contain substrate. After electrophoresis, the gel is impregnated with substrate at 4°C (enzyme activity should be low at this temperature). The gel is then incubated at 37°C (proteolytic activity should be high at this temperature). After incubation, the gel is stained with Coomassie blue. Protease activity appears on the gel as distinct achromatic bands.

In order to obtain an estimate of the molecular weight of the protease in purified toxin preparations, I analyzed the toxins by the modified zymogram method. Zymogram analysis of the proteolytic activity in the purified preparations of both toxins A and B revealed that a single band, with a molecular weight of approximately 50,000 daltons, degraded casein. When I electrophoresed toxin A in an SDS PAGE gel and sliced it horizontally into 1 mm fragments, the 300,000 dalton band did not cleave actin but the 50,000 dalton band did. Toxin A migrates to a position corresponding to a molecular weight of approximately 300,000 daltons, so this experiment showed that the complete toxin was not responsible for the cleavage of actin or casein. The 50,000 dalton band is either a fragment of the toxin or a contaminating protease. Interestingly, this band is approximately equal in molecular weight to the proteolytically active light chain fragments of tetanus and botulism neurotoxins. Western blot analysis showed that the 50,000 dalton band present in toxin A preparations reacted with the monoclonal antibody PCG-4. Even though the 50,000 dalton band reacted with PCG-4 MAb, the possibility that the cleavage of actin was due to a contaminating protease present in the toxin preparations still cannot be ruled out. Since the toxins yield many bands when they are subjected to SDS PAGE, the reaction of the 50,000 dalton band with PCG-4 MAb was expected. Since this band reacted with PCG-4, it must contain a portion of the repeating region of toxin A, but it may contain another protein as well.
Actin is a good substrate for many proteases. I wanted to determine if the site cleaved by the purified toxin preparations was a site common to other proteases. The first nine amino acids at the N-terminus of the 38,000 dalton actin fragment produced by purified toxin A were sequenced by automatic Edman degradation. The cleavage site was subsequently found to be located between His$^{40}$ and Gln$^{41}$ (Figure 6). This site is close to the amino terminus of the intact actin molecule. The N-terminal region of actin is highly susceptible to degradation (36); however, this specific site is not known to be recognized by any other common protease, in fact I could not find any documentation stating that any proteases cleave between a histidine and a glutamine.

Actin is a very highly conserved protein. Actin found in primitive eukaryotes, such as slime molds, shares over 90% homology with the actin found in the most highly evolved mammals. The amino terminal end of actin is the most variable region of the protein, and it is also highly immunogenic. For these two reasons, most antibodies that are available are directed towards the N-terminal region of actin (37). These antibodies can not be used to detect the cleavage of actin by the toxins because the toxins remove the first forty N-terminal amino acids of the protein. Sigma Chemical Co. very recently developed a polyclonal antibody directed towards the C-terminal region of actin. This allowed me to detect actin cleavage by Western blot analysis. Normally the detection of a single cleavage product in a mixture of proteins would be difficult, but with this assay, I could test heterogeneous protein solutions such as culture filtrates for protease activity. Culture filtrate from ten different strains of *C. difficile* ranging in toxigenicity from the highly toxigenic VPI strain 10463 to the nontoxigenic strain 11186 were assayed and all cleaved actin into a single fragment. Thus, *C. difficile* produces a protease, independent of the toxins, that is capable of cleaving actin. However, the protease present in culture filtrate is probably different from the protease present in purified preparations of toxins A and B for the following three reasons. The fragment of actin produced by culture filtrate migrated slightly faster (35,000 daltons) than the fragment produced by purified toxin (38,000 daltons). Dithiothreitol had no affect on the proteolytic activity in the
culture filtrate. Neither nontoxigenic culture filtrate nor culture filtrate from VPI strain 10463 degraded casein in an SDS zymogram.

Determining whether or not the toxin itself is the protease detected in the assays for actin-cleaving activity is a difficult task. The task was approached by trying to remove any possible contaminants from purified preparations of toxin A and by trying to selectively inhibit the proteolytic activity. I added varying amounts of the monoclonal antibody for toxin A, PCG-4 to the purified toxin A preparations, and tested its ability to neutralize the actin-cleaving activity of the toxin. The proteolytic activity of the purified toxin was only partially inhibited by the MAb. This inhibition was dose-dependent but required a very large amount of antibody. This inhibition can not necessarily be considered specific for toxin A; it could well be competitive inhibition since the antibody is a protein and may provide a substrate other than actin for the protease to degrade.

Recombinant toxins A and B expressed by the E. coli DH5-α host cells containing pCDtoxA.03 and pCDtoxB are both cytotoxic. Recombinant toxin A is also enterotoxic. Since the toxins retain their biological activity in the recombinant form, they provide potential tools for the analysis of their mechanisms of action. I hoped to support my argument that toxins A and B are proteases by demonstrating that recombinant toxins A and B cleave actin in a manner similar to the purified preparations of the native toxins. There were several problems inherent with testing recombinant lysates for actin cleaving activity. One problem is that the recombinant lysates contain many E. coli proteins and possibly proteases. Another problem is that the amount of toxin present in recombinant lysate is very low, much less than the amount present in culture filtrate from C. difficile. For this reason, it is very difficult to purify toxins A and B from lysates. I therefore performed Western blot assays for actin-cleaving activity on crude recombinant lysates rather than on purified recombinant toxins. I included, in my study, recombinant toxin A, recombinant toxin B, and toxin B lacking the repeating units. I also included a clone containing pUC 19 alone as well as a recombinant clone, containing only the repeating units of toxin B, as controls. All of the lysates except pUC 19 cleaved actin into multiple
fragments none of which corresponded to the size of the fragments produced by the purified native toxins. Since the lysate from the recombinant containing only the repeating units of toxin B cleaved actin, the proteolytic activity could not be attributed to toxin B. However, since lysate from pUC 19 did not cleave actin, it seems that *E. coli* does not normally produce a protease which cleaves actin. One explanation for the proteolytic activity in the lysate from the recombinant containing toxin B repeating units is that the relatively large insert induced the production of an *E. coli* protease which is usually not expressed. *E. coli* produces certain enzymes in response to stresses such as heat shock (39, 40) and DNA damage (41). I observed a similar phenomenon while studying the hemolytic affects of the recombinant toxins. Any recombinant clone containing a large insert, such as the repeating units of toxin B, would induce the lysis of erythrocytes from several mammals and birds.

In order to overcome the problem of *E. coli* proteases, I purified a small amount of recombinant toxin A by thyroglobulin affinity chromatography. These preparations cleaved 20-25% of the actin in the test samples into a fragment of approximately 38,000 daltons similar to purified native toxin A. The control lysate from pCDtoxBP-BRU was also taken through the purification procedure and did not cleave actin. The experiment was repeated several times in an attempt to cleave a greater proportion of the actin, but the recombinant toxin A lysate failed to cleave actin in a third experiment. Toxin B is more difficult to purify than toxin A, and because it is present in such a low concentration in the recombinant toxin B lysate, I did not attempt to purify recombinant toxin B.

Specific protease inhibitors are available for many different classes of proteases. The actin-cleaving activity and the casein-degrading activity of purified preparations of toxins A and B was completely inhibited by the metalloprotease inhibitors ortho-phenanthroline and EDTA. The toxins were not inhibited by serine protease inhibitors, cysteine protease inhibitors, or an aspartic protease inhibitor. I conclude therefore, that the actin-cleaving activity of purified preparations of toxins A and B is due to a metalloprotease. To investigate the possibility that the proteolytic activity of culture
filtrates from toxigenic and nontoxigenic strains of C. difficile was due to the same protease, I tested the affect of these inhibitors on the activity of the culture filtrates. The serine protease inhibitor 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) is a substitute for the more hazardous chemical, phenylmethyl-sulfonyl fluoride (PMSF). The proteolytic activity in culture filtrate from VPI strain 11186 was inhibited by the serine protease inhibitor AEBSF but not by the chelating agents. The culture filtrate was not inhibited by any of the other protease inhibitors. The ability of the culture filtrate to cleave actin is apparently due to a serine protease rather than the metalloprotease present in toxin preparations.

The inhibition by metal chelators of the cleavage of actin by toxin preparations led me to investigate the possible role of a metal ion in the proteolysis. Springman et al. (39) have reported the existence of a "cysteine switch" mechanism for the activation of certain zinc enzymes. Metalloenzymes that employ this method of activation contain a Zn ion at the active site which is associated with a cysteine residue when the enzyme is in its native conformation. Activation of the enzyme requires the dissociation of the cysteine from the Zn ion. This dissociation may be induced by conformational perturbants, heavy metal compounds, alkylating reagents, disulfides, oxidants, or proteolytic cleavage. When the cysteine dissociates from the Zn atom, a molecule of water is able to move into the active site, and the enzyme begins to autodigest. Toxins A and B do not contain the typical sequence motif present in Zn metalloproteases, HEXXH, but the following characteristics fit the description of certain Zn metalloenzymes capable of autodigestion and operating on the cysteine switch mechanism described by Springman. First, purified preparations of toxins A and B have been shown to contain elevated levels of zinc and iron. Second, the purified toxins are apparently capable of autodigestion and this autodigestion seems to be activated by the conformational perturbant, SDS. Third, purified preparations of toxins A and B are able to cleave actin and casein. Finally, the activity of the toxins seemed to increase over time, suggesting self-activation. I tested the affects of certain chemicals and proteases that Springman (39) had used to disrupt the
cysteine-Zn bond in the Zn metalloprotease, human fibroblast collagenase (Table 2 and Table 3) but none of them increased the actin-cleaving activity of the toxins.

Preliminary data has shown that toxin A binds the triphosphate nucleotides, ATP, GTP, FAD and NAD (Phelps, unpublished). Certain ATP-dependent enzymes bind these high-energy molecules, and require the release of their stored energy in order to carry out their enzymatic function (40). I added ATP, GTP, CTP, UTP, NAD and FAD to toxin A (Table 3), but none of these molecules increased the proteolytic activity.

Conclusions

Many proteins are unstable and will begin to break down over time. This could be an explanation for the multiple banding pattern observed when toxins A and B are subjected to denaturing polyacrylamide gel electrophoresis. That the toxins are simply breaking down due to lack of stability is very unlikely, however, because the toxins are so stable that they can be stored for years at 4°C and retain their biological activity. There is also a possibility that a contaminating protease present in purified preparations of toxins A and B is responsible for degrading the toxins. This explanation is also unlikely because the toxin-degrading protein was not separated from the toxin by SDS PAGE. The most plausible explanation is that toxins A and B undergo autodigestion.

The results obtained from the experiments designed to detect protease activity in the purified toxin preparations indicated that the length of time that the preparations had been stored was critical. The older solutions of purified toxins A and B were able to cleave actin faster and into smaller fragments than freshly purified toxin. Preparations of both toxins which had been in storage for some time also had the ability to degrade casein in the zymogram assay. This increase in proteolytic activity was not accompanied by a change in cytotoxic titer. Several explanations for the increase in proteolytic activity are possible. The toxins may undergo a time-dependent self-activation, or the toxin solutions may become contaminated from an external source as the bottle is opened to
withdraw samples. Elucidating the activation mechanism would have provided clues as to the function of the different regions of the toxin molecule, especially if the proteolytic activity could be attributed to a specific region of the toxin molecule. I was not, however, able to increase the activity of freshly purified toxin A. Increasing the activity of purified preparations of toxin A would have also strengthened the argument that the ability to cleave actin is due to the toxin and not to a contaminating protease, and would have provided a model for the proteolytic activity on a biochemical level.

I was also able to show that culture filtrate from nontoxigenic *C. difficile* VPI strain 11186 cleaves actin at a single site. Since the nontoxigenic strain does not possess the genes for either toxin A or toxin B it seemed that the actin-cleaving activity of the toxin preparations might be due to a contaminating protease. My next step was to answer the question of whether or not the protease present in culture filtrates that cleaved actin was the same as the protease present in purified toxin preparations. As with toxin A, only 50% of the actin in test samples was cleaved by nontoxigenic culture filtrate regardless of the incubation time. However, the fragment produced by the culture filtrate migrated slightly faster in an SDS PAGE gel than the fragment produced by toxin A. Furthermore, the cleavage of actin by culture filtrate was not affected by the addition of dithiothreitol as was the cleavage of actin by toxin A preparations. The culture filtrate also lacked the ability to degrade casein. Perhaps most importantly, the protease present in culture filtrate was inhibited by the serine protease inhibitor AEBSF whereas the protease present in toxin A preparations was inhibited only by metal chelators. I conclude that there is a protease present in purified preparations of the toxins that is not present in nontoxigenic culture filtrate.

Purified preparations of *C. difficile* toxins A and B contain a metalloprotease which is able to cleave actin and casein. The molecular weight of the protease was determined by SDS PAGE to be 50,000 daltons. Therefore the proteolytic activity is either due to a relatively small portion of the toxins or to a contaminating protease. There is strong evidence that the activity is due to the toxins and not to a contaminating
protease but this has not been irrefutably proven. The next step would be to carefully separate the 50,000 dalton protease from the numerous toxin fragments which appear on an SDS PAGE gel containing purified toxin, and have the protein sequenced by Edman degradation. If the N-terminal sequence of the 50,000 dalton protease is not found within the toxin sequence then this is a contaminating protease. If this is the case, the procedure used for toxin purification should be amended in order to remove the contaminant. Furthermore, if the protease is found to be a contaminant, its biological properties should be thoroughly investigated, it could be possible that some of the biological activity originally associated with the toxins is actually due to the contaminant. If the N-terminal sequence is found within the toxin sequence than we would know that the toxins were proteolytic. Knowing the proteolytically active portion of the toxin may allow the investigator to generate the fragment in vitro, so that long periods of storage would not be necessary for full protease activity.

Literature Cited


Section III. Analysis of Aberrant Strains of Clostridium difficile.

Introduction

The discovery of a cytotoxin in fecal samples from patients with antibiotic-associated pseudomembranous colitis (PMC) (1) and the subsequent implication of Clostridium difficile as the source of the toxin (2) have inspired a great deal of investigation into the function and importance of toxin production by C. difficile. In 1980, Taylor (3) and Banno (4) found that C. difficile produces not only one, but two toxins which are separable by anion-exchange chromatography. Shortly after this discovery, the individual toxins were purified to homogeneity, and the characterization of their properties and biological activities began.

Both toxins A and B are cytotoxins. Toxin B is 100 to 1,000 times more cytotoxic than toxin A on most mammalian cell lines. Both toxins are lethal to mice when injected intravenously or intraperitoneally. Toxin A is also able to induce a fluid response when injected into ligated lamb ileal loops, but toxin B lacks this activity. Since purified toxin A can cause colitis and diarrhea in animals, and since a monoclonal antibody against toxin A has been reported to protect mice from acquiring C. difficile induced PMC (5), it has been proposed that toxin A is solely responsible for the symptoms associated with PMC. However, it seems improbable that a bacterial cell would expend such a large amount of energy in the production of toxin B if the toxin does not play a role in the pathology of the disease. It has been suggested that toxin A is responsible for the initial tissue damage in the intestine and that once the initial damage has occurred, toxin B is able to exert its effect (6). Furthermore, since these studies have been conducted with toxins which have been purified and are no longer in their natural
milieu, it is not known whether toxin A and toxin B have different biological effects in the presence of certain enzymes or other pathogenic factors (7) produced by *C. difficile*. A strain of *C. difficile* which produces toxin B but not toxin A or a strain which produces toxin A but not toxin B would be an effective means of studying the biological activity of the toxins in the presence of all of the other substances produced by *C. difficile*.

Strains of *C. difficile* which produce widely varying amounts of both toxin A and toxin B have been well characterized. Strains of *C. difficile* which do not contain the genes for either toxin A or toxin B are also isolated fairly frequently. However, only a single strain has been reported to possess the gene for only one of the two toxins. In 1992, Lyerly et al. (8) described a strain of *C. difficile*, VPI strain 8864, which produced toxin B but did not contain the gene for toxin A. The strain was able to induce diarrhea and death in the hamster model, which was somewhat surprising since toxin A is generally considered more important biologically than toxin B. The literature offers two possible explanations of why a tox A-/tox B+ strain of *C. difficile* would be enterotoxigenic and pathogenic. 1) Lyerly et al. reported that toxin B produced by VPI strain 8864 induces a greater fluid response in ileal loops than toxin B from the highly toxigenic strain VPI 10463 which suggests that VPI strain 8864 produces an aberrant form of toxin B which is both highly cytotoxic and enterotoxic. 2) Borrelli et al. (9), however, did not find that purified toxin B from VPI strain 8864 induced a greater fluid response in ileal loops, the explanation for the pathogenicity of VPI strain 8864 suggested in their report is that *C. difficile* may produce other pathogenic factors which enhance the activity of toxin B, enabling the organism to elicit the symptoms of colitis. Regardless of the source of pathogenicity, a strain of *C. difficile* which produced "normal" toxin B (normal as indicated by tests for biological activity as well as Southern probe analysis) but not toxin A would further our knowledge of the biological activity of toxin B in its natural state. Likewise a strain which produced "normal" toxin A but not toxin B may reveal that certain factors, produced by *C. difficile*, affect its activity in the intestine.

Two aberrant strains of *Clostridium difficile*, CF2 #5340 and CF2 #5362, as well
as the control strain Y3 #5231, were sent to our lab from Dr. Stuart Johnson at the Medical Science Building, Chicago, IL. Preliminary studies in Dr. Johnson's laboratory, including restriction endonuclease analysis typing and oligonucleotide probe analysis indicated that strain Y3 #5231 contained the gene for toxin A as well as the gene for toxin B whereas CF2 #5340 and CF2 #5362 contained the gene for toxin A but lacked the gene for toxin B. The purpose of my work was to confirm their finding that the two CF2 strains are toxin A-negative/toxin B-positive and to quantify the production of the toxin or toxins.

**Materials and Methods**

**Stock cultures.** Stock cultures of strains CF2#5340, CF2#5362, and Y3#5231, were provided by Stuart Johnson, M.D. (Medical Science Bldg. Chicago, IL). These stocks were used to inoculate 5 ml chopped meat tubes (10). The tubes were inoculated anaerobically using an anaerobic transfer device (10).

**Production of culture filtrate.** Dialysis flasks containing 2 liters of Brain Heart Infusion broth (Difco Laboratories Detroit, MI) were prepared as described by Sterne and Wentzel (11) and inoculated from stock cultures. The flasks were incubated for 72 hours at 37°C. The contents of the dialysis bags were centrifuged and filtered through a 0.45 micron filter to remove cells.

**Antibodies and Antisera.** Goat antiserum against *Clostridium difficile* VPI strain 10463 was produced according to Ehrich et al. (12). Affinity purified polyclonal goat antibody against toxin A and affinity purified polyclonal goat antibody against toxin B were prepared as described by Lyerly et al. (13). Monoclonal mouse antibody PCG-4 against toxin A was prepared as described by Lyerly et al. (14). Anti-goat IgG whole molecule peroxidase conjugate and anti-mouse IgG peroxidase conjugate were purchased
from Sigma Chemical Co. St. Louis MO.

**Denaturing PAGE.** Culture filtrates from different strains were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis according to the method of Laemmli (15). Reagents for SDS PAGE were purchased from Sigma Chemical Co. Test samples of culture filtrate were denatured with 2.5% sodium dodecyl sulfate and reduced with 5% β-mercaptoethanol and heated to 100°C for five minutes. Samples were subjected to electrophoresis in 7.5% separating/4% stacking SDS PAGE gels. Gels were stained with Coomassie Blue R-250 and destained with 7% glacial acetic acid and 5% methanol.

**Crossed Immunelectrophoresis.** Crossed immunoelectrophoresis was performed in 1.2% low electroendosmotic agarose (Sigma Chemical Co.) in 0.0125 Tris-tricine buffer, pH 8.6, containing goat antisera against C. difficile VPI strain 10463 in the second dimension. The basic method of Axelsen (16) was followed.

**Western blot analysis.** Culture filtrates were tested for the presence of toxins A and B by Western blot analysis. Test samples of culture filtrate were subjected to denaturing polyacrylamide electrophoresis in 7.5% separating/4% stacking SDS PAGE gels. Proteins were transferred from the gel onto a nitrocellulose membrane. The transfer took place in 0.025M Tris-0.192 M glycine buffer, pH 8.3 containing 20% methanol and was complete after four hours at 50 V. Following electrotransfer, the membrane was rinsed in 50 mM Tris-Cl containing .15 M NaCl (TBS). The membrane was blocked in 3% gelatin for one hour. The primary antibody, affinity purified polyclonal A or B antibody or PCG-4 monoclonal antibody was added to a 1% solution of gelatin (1:2500 antibody:gelatin solution) and this mixture was left on the membrane for four hours. The membrane was washed three times for five minutes each time in TBS. The secondary antibody (anti-goat IgG peroxidase conjugate for blots using polyclonal antibody or anti-
mouse IgG peroxidase conjugate for blots using monoclonal antibody) was added to 1% gelatin solution and left on the membrane for one hour. The membrane was rinsed two times for ten minutes each time in deionized distilled water. The substrate, Opti-Mist horseradish peroxidase reagent (TSI, Boston MA), was added to the membrane. The substrate was left on the blot for five minutes while a blue color developed. The blot was rinsed with ddH2O and allowed to air-dry.

**Detection of toxin A by ELISA.** Culture filtrates were tested for toxin A by the Tox-A test (Techlab Blacksburg, VA) according to manufacturer's instructions.

**Cytotoxicity assay.** Culture filtrates were assayed for cytotoxic activity as previously described by Ehrich et al. (12). Ten-fold dilutions of culture filtrate were made in microtiter wells containing a confluent layer of Chinese hamster ovary K1 cells and 180 μl of tissue culture media. Plates were incubated at 37°C in an atmosphere containing 5% CO2. Cells were observed for rounding after 4 hours and 18 hours. The cytotoxic titer was determined as the highest dilution which caused rounding of 100% of the cells.

**Neutralization of cytotoxicity.** Neutralization of cytotoxicity was determined following the procedure previously described by Ehrich et al. (12). Ten-fold dilutions of culture filtrate were made using TBS. Twenty microliters of either PBS, affinity purified toxin B antibody, or affinity purified toxin A antibody were added to wells of a 96-well microtiter plate. Twenty microliters of each of the culture filtrate dilutions were added to the microtiter wells. The plate was incubated at 25°C for one hour. Antibody-toxin mixtures from the microtiter wells were assayed for cytotoxicity as described above.

**Hemagglutination assay.** Hemagglutination assays were conducted as previously described by Clark et al. (17). Rabbit blood was purchased from The Brown Laboratory
(Topeka, KS). A 2% suspension of rabbit erythrocytes was made by mixing 0.2 ml of rabbit blood with 4.8 ml of 50mM Tris, pH 7.5 containing .15 M NaCl (TBS). The red blood cells were removed from solution by centrifugation at 2,000 rpm. The erythrocyte pellet was washed and suspended in TBS.

Serial two-fold dilutions of culture filtrate were made in a 96 V-shaped well microtiter plate from Dynatech Laboratories (Chantilly, VA.). Using TBS as the diluent, 50 µl of each dilution was made. The plate was allowed to equilibrate and 50 µl of the 2% erythrocyte suspension was added to each well. The plate was kept at 4°C and observed for hemagglutination after 4 hours and 16 hours. Wells containing erythrocytes spread out over the sides of the well were recorded as positive for hemagglutinating activity. Wells containing a tight pellet of erythrocytes at the bottom of the well were considered negative.

**Isolation of genomic DNA.** Genomic DNA was isolated from C. difficile strains according to Johnson (18). This protocol is a modified version of the Marmur method (19). The solution was warmed to 55°C in a Gilford 2400 spectrophotometer and the concentration of DNA was measured at 260 nm. If the concentration of DNA in the sample was not 0.5 mg/ml or higher, the procedure was repeated.

**Southern Transfer and hybridization.** XbaI/HindIII, Sau3A, and EcoRI/HincII restriction digests of genomic DNA were electrophoresed in 0.7% agarose gels in Tris-acetate buffer containing 10mM EDTA at 15 V for 16 hours. The DNA fragments were transferred onto a nitrocellulose filter according to the method described by Southern (20). The nitrocellulose membrane was baked at 80°C for four hours to permanently bind the DNA. Probes were made as described by Lyerly et al. (8). The membrane was prehybridized and probed using the ECL (enhanced chemiluminescence) kit for direct nucleic acid labelling and detection from Amersham International plc (Buckinghamshire, England).
Results

Denaturing PAGE analysis of culture filtrates. Culture filtrate from a nontoxigenic strain of Clostridium difficile, VPI strain 11186, the highly toxigenic VPI strain 10463, and strains Y3 #5231, CF2 #5340, and CF2 #5362 were analyzed by denaturing polyacrylamide gel electrophoresis (Figure 1). In the lane containing culture filtrate from VPI strain 10463, the toxins were seen as prominent high molecular weight bands. The largest protein band present in culture filtrate from the negative control, VPI strain 11186, was only about 100,000 daltons, much smaller than the toxins. The largest protein band which was observed in the lanes containing filtrate from strains Y3 #5231, CF2 #5340, and CF2 #5362, was only about 150,000 daltons. This suggests that culture filtrates from these strains do not contain large amounts of toxin A or toxin B.

Immunodetection of toxins A and B. Crossed immunoelectrophoresis indicated that culture filtrates from VPI strain 10463, strain Y3 #5231, CF2 #5340, and CF2 #5362 all reacted extensively with goat antiserum against VPI strain 10463. There were several marked differences in the protein arc patterns of culture filtrate from VPI strain 10463 and the culture filtrates from the other strains, including the apparent lack of a toxin A peak (Figure 2). The patterns of protein arcs of the two aberrant strains were very similar. When polyclonal antibodies specific for toxins A and B were used in the second dimension, I found that culture filtrates from Y3 #5231 and the two aberrant strains did not contain enough toxin A or toxin B to form visible peaks.

In Western blots of all four culture filtrates, polyclonal antibodies for toxin A and toxin B detected multiple bands (Figure 3 and Figure 4). The positive control VPI strain 10463 reacted extensively with the polyclonal antibodies for toxin A and B revealing so many bands ranging from 300,000 to 50,000 daltons that the entire lane appeared almost as a smear. The control strain Y3 #5231 and the aberrant strains did not react as extensively with the polyclonal antibodies as VPI strain 10463 but again multiple bands
**Figure 1**: Denaturing polyacrylamide gel electrophoresis analysis of culture filtrates. SDS PAGE analysis of *C. difficile* culture filtrates. Brain heart infusion broth dialysis cultures were inoculated with a control strain or an aberrant strain. Cultures were grown at 37°C for 72 hours. Cells were removed by centrifugation and the supernatant was passed through a 0.45 micron filter. **Lane 1**, high molecular weight standards from Bio-Rad: myosin 194,000, β-galactosidase, 116,000, bovine serum albumin, 85,000, ovalbumin, 49,000. **Lane 2**, highly toxigenic positive control, VPI strain 10463. **Lane 3**, nontoxigenic negative control, VPI strain 11186. **Lane 4**, weakly toxigenic control strain Y3 #5231. **Lane 5**, aberrant strain CF2 #5340. **Lane 6**, aberrant strain CF2 #5362.
Figure 2: Crossed immunoelectrophoresis of culture filtrates. Crossed immunoelectrophoresis of culture filtrates from control strains and from aberrant strains of *C. difficile*. Wells were loaded with 10 µl of culture filtrate. Goat antiserum against *C. difficile* VPI strain 10463 is used in the second dimension. **Plate 1**, positive control, VPI strain 10463. **Plate 2**, positive control, strain Y3 #5231. **Plate 3**, aberrant strain CF2 #5340. **Plate 4**, aberrant strain CF2 #5362. The arrow in Plate 1 points to the speculated toxin A peak. Culture filtrate from VPI strain 10463 is the only one that contains a toxin A peak.
Figure 3: Western analysis of culture filtrates using a polyclonal antibody for toxin A. Western blot of culture filtrates from control strains and aberrant strains of C. difficile. Culture filtrates were electrophoresed in a 4% stacking/7.5% separating SDS PAGE gel. The gel was blotted onto nitrocellulose and analyzed by Western blot using a polyclonal antibody specific for toxin A. This blot shows that culture filtrate from all of the strains except the negative control strain react with a polyclonal antibody for toxin A. Lane 1, highly toxigenic positive control, VPI strain 10463. Lane 2, non toxigenic negative control, VPI strain 11186. Lane 3, weakly toxigenic control strain # 5231. Lane 4, aberrant strain CF2 #5340. Lane 5, aberrant strain CF2 # 5362.
Figure 4: Western analysis of culture filtrates using a polyclonal antibody for toxin B. Western blot of culture filtrates from control strains and aberrant strains of *C. difficile*. Culturefiltrates were electrophoresed in a 4% stacking/7.5% separating SDS PAGE gel. The gel was blotted onto nitrocellulose and analyzed by Western blot using a polyclonal antibody specific for toxin B. This blot shows that culture filtrate from all of the strains except the negative control, react with a polyclonal antibody for toxin B. Lane 1, highly toxigenic positive control, VPI strain 10463. Lane 2, nontoxigenic negative control, VPI strain 11186. Lane 3, weakly toxigenic control strain # 5231. Lane 4, aberrant strain CF2 #5340. Lane 5, aberrant strain CF2 # 5362.
were observed, the largest of which was approximately 300,000 daltons. The band patterns of strain CF2 #5340 and strain CF2 #5362 were very similar. Some cross-reactivity between the polyclonal antibody for toxin B and culture filtrate from the nontoxigenic control VPI strain 11186 occurred as well. Culture filtrate from VPI strain 11186 and from the two CF2 strains did not react with the monoclonal antibody to toxin A, PCG-4 (Figure 5). The positive control, VPI strain 10463 reacted extensively with PCG-4 and a single high molecular weight band from Y3 #5231 also reacted.

The Tox-A test is a highly sensitive assay for the detection of toxin A. The test is an enzyme-linked immunosorbant assay (ELISA) which employs both the monoclonal antibody PCG-4 and polyclonal antibodies against toxin A. The results of the Tox-A test should correspond to the results of Western analysis using the PCG-4 MAb but the test may detect even smaller quantities of the toxin than the Western. The Tox-A test showed a strong positive result for the presence of toxin A in culture filtrates from the positive controls, VPI strain 10463 and strain Y3 #5231. The Tox-A test showed a negative response for culture filtrates from the negative control, VPI strain 11186, and from the aberrant strains CF2 #5340 and CF2 #5362 indicating the absence of toxin A.

Cytotoxicity. Culture filtrates from the highly toxigenic positive control, VPI strain 10463, the weakly toxigenic control strain Y3 #5231, and the aberrant strains CF2 #5340, CF2 #5362, were assayed for cytotoxic activity. After four hours at 37\(^{\circ}\)C, VPI strain 10463 had a cytotoxic titer of \(10^5\). This titer had increased to \(10^6\) by 18 hours. After four hours Y3 #5231 had a cytotoxic titer of \(10^2\). After 18 hours Y3 #5231 had a cytotoxic titer of \(10^3\), still only 0.1 % of the cytotoxic titer of VPI strain 10463. Strains CF2 #5340 and CF #5362 displayed cytotoxic titers of \(10^1\) after for hours and after 18 hours had a cytotoxic titer of \(10^4\), 1 % of the cytotoxicity displayed by VPI strain 10463.

The addition of affinity purified polyclonal antibody against toxin B to culture filtrate from VPI strain 10463 resulted in 99.9% inhibition of cytotoxic activity. The affinity purified polyclonal antibodies against toxin A had no affect on the cytotoxicity.
Figure 5: Western analysis of culture filtrates using the monoclonal antibody for toxin A, PCG-4. Western blot of culture filtrates from control strains and aberrant strains of *C. difficile*. Culture filtrates were electrophoresed in a 4% stacking/ 7.5% separating SDS PAGE gel. The gel was blotted onto nitrocellulose and analyzed by Western blot using the monoclonal antibody specific for toxin A, PCG-4. This blot shows that the positive controls: VPI strain 10463 and Y3 #5231 react with PCG-4 whereas the aberrant strains do not. **Lane 1**, high molecular weight standards from Bio-Rad: myosin 194,000, B-galactosidase 116,000, bovine serum albumin 85,000, ovalbumin 49,000. **Lane 2**, highly toxigenic positive control, VPI strain 10463. **Lane 3**, nontoxigenic negative control, VPI strain 11186. **Lane 4**, weakly toxigenic control strain # 5231. **Lane 5**, aberrant strain CF2 #5340. **Lane 6**, aberrant strain CF2 # 5362.
of VPI strain 10463. Goat antiserum against culture filtrate from VPI strain 10463 resulted in a 100,000-fold decrease in the cytotoxicity of VPI strain 10463. Specific polyclonal antibodies against toxin A and toxin B did not have any inhibitory affect on Y3 #5231, CF2 #5340, or CF2 #5362 but the goat antiserum inhibited 100% of the cytotoxicity of these strains.

**Hemagglutinating activity.** Hemagglutination assays showed that VPI 10463 had a hemagglutinating titer of 2⁴ whereas strains Y3 #5231, CF2 #5340, and CF2 #5362 lacked any hemagglutinating activity. However, when culture filtrates from strains CF2 #5340 and CF2 #5362 were concentrated 25 times using a Minicon concentrator from Amicon (Beverly, MA.) they had a hemagglutinating titer of 2².

**Southern hybridization.** Four probes directed towards toxin B and three probes directed towards toxin A were made as described by Lyerly et al. (8). The probes are shown diagrammatically in Figure 6. The results of Southern hybrid analysis of genomic DNA from strain Y3 #5231, CF2 #5340, CF2 #5362 and VPI strain 10463 are shown in Table #1. All four of the strains hybridized with probes for both toxins A and B.

**Discussion**

A denaturing PAGE profile of culture filtrates from strains Y3 #5231, CF2 #5340, and CF2 #5362 seemed to indicate that none of the strains produced toxin A or toxin B. Similar to the nontoxigenic control strain, no high molecular weight proteins present in these culture filtrates were detected by Coomassie blue staining. Since restriction enzyme analysis in Dr. Stuart Johnson's lab had already shown the presence of toxin A in all three strains (and toxin B in strain Y3 #5231) I assumed that the toxins were not absent, but produced at levels below the detection limit of Coomassie blue staining. Further
Figure 6: Probes used in Southern hybridization. A partial restriction endonuclease map showing the approximate locations of XbaI, HindIII, HincII, and Sau3A sites in the genes for toxins A and B. Two open reading frames are shown, one between the toxigenes, and the other after the toxin A gene. The functions of the open reading frames are not known. This map also shows the approximate locations of probes used to detect toxins A and B in Southern hybrid analysis.
Table 1: Southern Hybrid Analysis

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Toxin B Probes</th>
<th>Toxin A Probes</th>
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<tbody>
<tr>
<td></td>
<td>J66</td>
<td>H39</td>
</tr>
<tr>
<td>XbaI/HindIII</td>
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<td></td>
</tr>
<tr>
<td>10463</td>
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</tr>
<tr>
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<tr>
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<td>0.5</td>
</tr>
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<td>5362</td>
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<td>0.5</td>
</tr>
<tr>
<td>Sau3A</td>
<td></td>
<td></td>
</tr>
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</tr>
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<td></td>
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<tr>
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DNA from *C. difficile* strains Y3#5231, CF2#5340, CF2#5362, and VPI strain10463 (positive control) was digested with restriction enzymes and analyzed by Southern hybridization using the probes shown in Figure 6. This table shows the approximate sizes of the fragments (in kilobases) that were detected with the probes.
experimentation was done using more sensitive methods for the detection of the toxins.

Crossed immunoelectrophoresis is an effective method for comparing a large number of antigens in complex mixtures. Antigenic cross-reactivity patterns can be used to assess the relatedness of different species, or strains of the same species. Culture filtrates from VPI strain 10463, and strains Y3 #5231, CF2 #5340, and CF2 #5362 were analyzed by crossed immunoelectrophoresis using antiserum against VPI strain 10463. All of the strains reacted extensively with the antiserum. The two aberrant strains produced very similar arc patterns. Culture filtrates were also analyzed by crossed immunoelectrophoresis using a polyclonal antibody for toxin A. Toxin A was only detected in the positive control, VPI strain 10463. Crossed immunoelectrophoresis using a polyclonal antibody specific for toxin B, revealed a light toxin B peak in culture filtrate from VPI strain 10463 but not in the other strains. Toxin B does not immunoprecipitate as well as toxin A does and therefore produces a very light peak. These results suggest that either the toxins are expressed at very low levels by these strains or that they are not expressed at all.

Western blot analysis is a more sensitive assay for the presence of a particular protein than the Coomassie stain and crossed immunoelectrophoresis. At least 30 ng of protein must be present in a band such that it can be visualized by Coomassie stain, whereas Western blot analysis using antibodies conjugated to peroxidase can detect proteins in picogram quantities. Culture filtrate from the positive controls, VPI strain 10463 and Y3 #5231, as well as the aberrant strains, CF2 #5340, and CF2 #5362, all reacted with affinity purified polyclonal antibodies specific for toxins A and B. All four strains yielded multiple banding patterns when probed with both antibodies, but many more bands were observed in the lane containing culture filtrate from VPI strain 10463. A protein band of approximately 300,000 daltons was detected by the polyclonal antibody for toxin A and the polyclonal antibody for toxin B in Western blots of all four culture filtrates. Western analysis suggests that the two aberrant strains are very similar in their toxin production and that not only is toxin A present in the aberrant strains but that toxin
B is present as well. However, these results were complicated by the fact that nontoxigenic control VPI strain 11186 cross-reacted with the polyclonal antibody for toxin B but it did not react with the polyclonal antibody for toxin A.

The polyclonal antibodies for toxin A and toxin B were affinity purified from antiserum against VPI strain 10463, but the possibility exists that some contaminating antibodies that are not specific for the toxins co-purified. This would explain why the polyclonal antibody for toxin B reacted with culture filtrate from the nontoxigenic VPI strain 11186. This presents the possibility that proteins other than toxin B were detected by the polyclonal antibody in the other four strains. The same possibility exists for nonspecific reactions between the polyclonal antibody for toxin A and the four culture filtrates. It is more likely, however, that the polyclonal antibodies for the toxins were reacting specifically with the toxins in the four strains, because the antibody reacted with proteins of the appropriate molecular weight (approximately 300,000 daltons). In order to confirm the presence of toxin A in culture filtrates from Y3 #5231, CF2 #5340, and CF2 #5362, they were analyzed using the monoclonal antibody PCG-4 which is specific for a site in the repeating region of toxin A. A multiple banding pattern was detected in the lane containing culture filtrate from VPI strain 10463; this is thought to be the result of autodigestion of the toxin A molecule. Monoclonal antibody PCG-4 reacted with a single 300,000 dalton protein band in culture filtrate from Y3 #5231 and did not react with the nontoxigenic control VPI strain 11186, or the two aberrant strains. The Tox-A test, a sensitive ELISA based on the monoclonal antibody PCG-4 also was negative with VPI 11186, and the two aberrant strains. A monoclonal antibody for toxin B was not available, and therefore could not be used to support the evidence from analysis with the polyclonal toxin B antibody which indicated the presence of toxin B in culture filtrates from strains Y3 #5231, CF2 #5340, and CF2 #5362.

It was somewhat surprising that the two CF2 strains reacted with the polyclonal antibody for toxin A but not with the monoclonal antibody PCG-4. It could be that the two strains do not actually produce toxin A and the polyclonal antibody was reacting non-
specifically with proteins other than the toxins. It is also possible that toxin A is produced in such low quantities by the aberrant strains that it is not detected by the MAb PCG-4. I believe that it is most likely, however, that the CF2 strains do produce a form of toxin A and that the site which is recognized by MAb PCG-4 is altered in such a way that the antibody no longer recognizes it.

I attempted to further support the evidence that the control strain Y3 #5231, and the aberrant strains, CF2 #5340, and CF2 #5362 do produce both toxins A and B by analyzing their biological activity. If the polyclonal antibodies for toxin A and toxin B did react specifically with the toxins in culture filtrate from the strains CF2 #5340 and CF2 #5362, then the antibodies should be able to neutralize the cytotoxic activity of the culture filtrates. The cytotoxic titers of culture filtrate from strain Y3 #5231 and from the two CF2 strains were considerably lower than the titer of culture filtrate from VPI strain 10463. The cytotoxic titer of VPI 10463 was not affected by the polyclonal antibody for toxin A but was reduced considerably by the polyclonal antibody for toxin B. These results are to be expected because toxin B is 1,000 times more cytotoxic than toxin A, so that even if toxin A were neutralized, a significant decrease in the overall cytotoxic titer would not be observed. The cytotoxic titers of Y3 #5231, CF2 #5340, and CF2 #5362 also were not reduced by the polyclonal antibody for toxin A but they were also not reduced by the polyclonal antibody for toxin B. The cytotoxicity of all four strains was completely inhibited by goat antiserum prepared against culture filtrate from VPI strain 10463. The reason that the goat antiserum was more effective at neutralizing cytotoxicity than the purified polyclonal antibodies against the toxins is not known. As suggested in the introduction, perhaps C. difficile produces other factors which play a role in toxicity and pathogenicity. In other words, the crude antiserum may contain antibodies against other toxigenic factors present in the culture filtrate.

Results from experiments designed to detect toxin A in culture filtrate from the aberrant strains were conflicting. I conducted a hemagglutination assay in an attempt to reach a more conclusive answer about the production of toxin A. The hemagglutination
assay is based on the fact that toxin A binds to the carbohydrate Galα1-3Galβ1-4GlcNAc on the surface of rabbit red blood cells. The toxin contains more than one carbohydrate binding site and is therefore able to cross-link the erythrocytes and cause them to agglutinate. Since this binding is specific, red blood cells have been used to purify toxin A from culture filtrate. Culture filtrate from VPI strain 10463 was found to have a hemagglutinating titer of $2^4$ whereas culture filtrates from strains Y3 #5231, CF2 #5340, and CF2 #5362 did not agglutinate the red blood cells. When the culture filtrates from the control strain Y3 #5231 and from the two aberrant strains were concentrated 25 fold, however, they had a low hemagglutinating titer. This suggests that the were expressing toxin A but at very low levels. The concentrated culture filtrates from the CF2 strains were subjected to the Tox-A test but were again negative. This supports the idea that toxin A is produced by the aberrant strains in a form which is not recognized by the monoclonal antibody PCG-4.

There are many different methods for the detection and quantification of certain proteins present in complex protein mixtures. The data generated from assays using antibodies, however, may be somewhat confusing because slight mutations can mask a given protein and because expression levels may vary. A somewhat more decisive approach is to look for the presence of the gene encoding for the protein of interest. If the sequence of a gene is known then multiple probes can be generated. A major advantage of using probes is that their exact target is known. Antibodies can be made towards a protein, but since they are produced in vivo, the sites to which they will be directed are unpredictable and antibodies against proteins other than the protein of interest may be present. I seemed to encounter some of the problems inherent to the use of antibodies when I was searching for toxins A and B in culture filtrates from the two aberrant strains, therefore analysis of the aberrant strains on the genetic level was a particularly important aspect of the characterization of the strains. The genomes of the control strain Y3 #5231 and the aberrant strains CF2 #5340 and CF2 #5362 were examined for the presence of the toxin genes by Southern hybrid analysis. Probes were
made from the recombinant toxin clones. The probes were chosen so that any portion of the toxin genes present in the genome would be detected. Genomic DNA from strain Y3 #5231 reacted with probes for both of the toxin genes, and the gene fragments detected by the probes were the same size as the fragments detected in genomic DNA from VPI strain 10463. Genomic DNA from strains CF2 #5340 and CF2 #5362 also reacted with probes for both of the toxin genes, but while the sizes of the gene fragments detected in the two CF2 strains were the similar to each other, they differed from the fragments detected in VPI strain 10463.

Conclusions

The results from the studies of toxin A production in strain Y3 #5321 indicate that toxin A is produced, but that either the level of toxin expression very low or that the toxin produced is aberrant and not as reactive with toxin A antibodies as normal toxin A. It seems that if Y3 #5231 produces toxin B, it does so at a very low level. This is indicated by the fact that cytotoxicity is so low and that the polyclonal antibody for toxin B does not reduce the cytotoxicity titer. Southern hybrid analysis confirmed that strain Y3 #5231 contains the genes for toxin A and for toxin B and that the genes are very similar to, if not the same as, the genes in VPI strain 10463. Since Southern analysis indicates that the toxin genes are normal, low level expression of the toxins is the only reasonable explanation for the low cytotoxic and hemagglutinating titers of the culture filtrate.

The results from the studies of toxin A production in strains CF2 #5340, and CF2 #5362 are conflicting. The polyclonal antibody for toxin A reacted with the culture filtrate from these two strains but the monoclonal antibody, PCG-4, did not. The culture filtrate from these two strains also had a lower level of hemagglutinating activity. These results suggest that the two CF2 strains do produce toxin A to some extent. The MAb PCG-4 is a very sensitive antibody, so the finding that it does not react with the CF2
strains suggests that the toxin A produced by these two strains lacks the epitope recognized by PCG-4. Since enough toxin A is produced by these strains to exhibit some hemagglutinating activity, it is possible that the low level of cytotoxicity is due to toxin A and that toxin B is not produced at all. It is also possible that an aberrant form of toxin B which is much less cytotoxic than normal toxin B, is produced. Southern hybrid analysis of the CF2 strains indicated that the strains do contain the genes for both toxin A and toxin B. Southern analysis also indicates that the toxin genes present in these strains are aberrant forms of the genes present in VPI strain 10463. This is not surprising considering the results from the protein analysis. The deviation of the toxin genes present in these strains from the toxin genes present in VPI strain 10463 is probably responsible for the conflicting results in the immunodetection assays. Low level expression of the toxins is probably responsible for the low cytotoxic titer of these strains. Strains CF2 #5340 and CF2 #5362 had displayed similar protein profiles, had similar cytotoxic and hemagglutinating titers, and appeared identical when analyzed with the toxin gene probes. I conclude that strains CF2 #5340 and CF2 #5362 are isolates of the same strain, and that they are aberrant for toxins A and B, although they are not tox A+/tox B- as was indicated by restriction enzyme analysis in Dr. Johnson's laboratory. This strain contains the genes for both toxin A and toxin B and is therefore not particularly useful in the study of the toxins.

**Literature Cited**


19. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from

CURRICULUM VITAE

Kimberly Kay Jefferson

EDUCATION:

<table>
<thead>
<tr>
<th>Institution</th>
<th>Degree or Title</th>
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<tr>
<td>Virginia Tech, Blacksburg, VA</td>
<td>Master of Science</td>
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<td>Biochemistry &amp; Anaerobic Microbiology</td>
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- Plant Biology
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- Zoology
- Soil Microbiology + Lab
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- Ecology
- Biochemistry
- Comparative Studies of Nucleic Acids
- Food Microbiology
- Molecular Biology of Cell

EXPERIENCE:

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" " Rainforest Action Network  1990-1991
" " Phi Sigma: National Biological Honor Society  1990-1994
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National  American Society for Microbiology  1993-1995
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Emphasis in protein purification and analysis including: FPLC, affinity chromatography, anion-exchange chromatography, capillary electrophoresis, SDS and native PAGE, zymogram analysis, Western blot analysis, crossed immunoelectrophoresis, spectrophotometric protein assay, expression of recombinant proteins in E. coli, ELISA, and protease assay.

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