Use Of An In-Vitro Polarized Cell Culture Model To Study The Translocation Of *Clostridium difficile* Toxins

By

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A Thesis
Submitted to the Faculty of Graduate Studies,
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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CDAD	*Clostridium difficile*-associated diarrhea
CPE	Cytopathic effect
CPU	Cytopathic unit
CROPS	Clostridial repetitive oligopeptide sequence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HFF	Human foreskin fibroblasts
LAL	Limulus amebocyte lysate
LCT	Large clostridial toxins
LPS	Lipopolysaccharide
ORF	Open reading frame
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PFGE	Pulsed field gel electrophoresis
PMC	Pseudomembranous colitis
RD_{50}	Decrease in electrical resistance by 50%
REA	Restriction enzyme analysis

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ABSTRACT

Clostridium difficile is the etiologic agent of antibiotic-associated diarrhea; the most common form of nosocomial infectious diarrhea. In cases of severe infection, patients may suffer from hypovolemic shock, toxic megacolon, and secondary sepsis leading to death. Presently, the basis for these complications are not known. *C. difficile* produces two large molecular weight protein exotoxins; toxins A and B. These toxins are believed to be main virulence factors of *C. difficile* responsible for inciting disease symptoms.

Although toxins A and B have been shown to affect cytoskeletal structure, it is not known if they are able to translocate across the gut mucosa to contribute to systemic symptoms. It is also not known if these toxins can facilitate the passage of foreign molecules, such as lipopolysaccharide, from the gut mucosa into the systemic circulation.

Patients treated for *C. difficile* infections will often suffer from multiple relapses. The basis for recurring relapses are not well understood, but may be influenced by humoral or mucosal antibody responses to the pathogen and its toxins. To address the problems of multiple relapses, several toxin inhibitory agents such as Cholestyramine and Synsorb CD have been produced and marketed. The efficacy of these agents in preventing disease is not well characterized or understood.

In this study, a polarized tissue culture model employing Caco-2 cells grown on Transwell inserts was established to study the translocation of purified *Clostridium difficile* toxins A and B. *Clostridium difficile* toxins were ^{125}I labeled and inoculated onto confluent polarized Caco-2 cell monolayers to study translocation dynamics. The ability of toxins A and B to enhance translocation of lipopolysaccharide was also assessed. Electrical resistance

measurements were utilized to monitor monolayer confluence and tight junction integrity. Sera from patients afflicted with *C. difficile*-associated diarrhea were analyzed for circulating cytotoxin, as well as, serum neutralizing antibodies. Synsorb CD and Cholestyramine were also examined for their ability to inhibit cytotoxic effects on Caco-2 cells and Human foreskin fibroblasts.

Toxin A produced a 50% reduction in electrical resistance in 3.0 hrs whereas the same concentration of toxin B required at least 7 hrs to achieve the same effect. These results suggest that toxin A is more effective in disrupting epithelial monolayers than toxin B. Both toxins A and B were able to translocate across confluent monolayers of Caco-2 cells, however, the combination of toxin A and toxin B together was synergistic with respect to enhancing the translocation of toxin B. The addition of toxin A resulted in a two-fold increase in the amount of toxin B able to translocate across the Caco-2 monolayer, however, no increased translocation of toxin A was observed. These findings suggest a model of pathogenesis in which *C. difficile* toxin A enhances the translocation of toxin B across the gut mucosa into the submucosa.

Although studies were initiated to ascertain whether toxins A and B could promote translocation of lipopolysaccharide, definitive conclusions could not be made on the basis of our experimental data. Analysis of serum samples from patients with *C. difficile*-associated diarrhea did not reveal cytotoxin in the systemic circulation. Patients with *C. difficile*-associated diarrhea also did not appear to have significantly greater titres of toxin B serum neutralizing antibodies when compared to control patients.

Inhibition studies utilizing Synsorb CD and Cholestyramine failed to show effective neutralization of toxin A by these inhibitors. Neither inhibitor was effective in preventing electrical resistance drops in Caco-2 cell monolayers treated with toxin A. Titration assays on Human foreskin fibroblasts also failed to show reduced cytopathic tires of toxin A despite incubation with either inhibitory compound. These findings suggest that the inhibitory agents may not be as efficacious as previously reported.

Literature Review

1.) Historical Perspective

Clostridium difficile was first isolated from the stools of healthy newborns in 1935 by the researchers, Hall and O'toole (67). In their initial description of *C. difficile*, the organism was referred to as *Bacillus difficilus;* the difficult *Bacillus*. The nomenclature of *Bacillus difficilus* was partly derived from the difficulties encountered in the cultivation and isolation process. Since its initial discovery, the nomenclature of *Bacillus difficilus* has been changed to *C. difficile* to reflect a more descriptive and taxonomically appropriate name.

The toxigenic nature of *C. difficile* was revealed in 1935, however, because the organism was isolated from healthy infants, it was not considered a significant pathogen. As a consequence of this belief, little attention was devoted to studying the organism or its toxins until the late 1970's. In fact, it was not until the late 1960's that the fortuitous association of *C. difficile* with disease was made (104). At this time, an impetus was put forth to create new drugs capable of killing anaerobic bacteria. Clindamycin, a drug developed by the Upjohn company, was found to be especially effective against anaerobes. Unfortunately, patients treated with Clindamycin often developed pseudomembranous colitis (PMC); a disease characterized by severe inflammation of the colonic mucosa, sometimes leading to death. This was a perplexing problem for researchers at the time, and soon the high mortality rates associated with 'Clindamycin colitis' sparked great curiosity and interest into the disease. In their initial investigations, researchers found high levels of cytotoxic activity associated with stools of patients afflicted with PMC. At the outset, it was believed that these findings were attributable to mycoplasma or viral agents. When tests consistently

excluded these pathogens, suspicions of a toxin-producing bacterium began to arise.

In studying the cytotoxic activity of the stools, researchers found that gas gangrene antiserum was able to abrogate the effects of the toxins. As gas gangrene antiserum was made from of a mixture of different antiseras raised from crude toxin preparations derived from several clostridial species, investigators began to test antiseras individually. It was hoped that these studies would reveal a single antisera responsible for the neutralization effects. In their studies, they found that *Clostridium sordellii* antisera was able to neutralize the cytotoxic effects. This finding, undoubtedly, led investigators to believe that *C. sordellii* was the causative agent of PMC. Unfortunately, *C. sordellii* could almost never be isolated from patients with PMC. Eventually, researchers found high numbers of *C. difficile* organisms in stools of patients with PMC. Moreover, *C. difficile* also produced a cytotoxin which was neutralized by *C. sordellii* antiserum (12, 59).

It is now known that the toxins of *C. difficile* and *C. sordellii* are very similar. Once the discoveries were reported, researchers began to document evidence to implicate *C. difficile* in the pathogenesis of the PMC. Major breakthroughs in the process occurred with the establishment of suitable animal models for studying *C. difficile* pathogenesis. In the late 1970's researchers found that hamsters treated with antibiotics would subsequently develop diarrhea and fatal enterocolitis with high numbers of toxigenic *C. difficile* in their stools (12, 13, 134, 135). The toxin isolated from the stools of hamsters was also very similar to the toxin found in patients suffering from *C. difficile*-associated disease. These findings further implicated *C. difficile* in the promotion of PMC. One of the most important discoveries to arise from the extensive research in that era was the association between PMC and antibiotic

usage. This was indeed a novel finding because it represented a "new" disease created by the antibiotic treatment given for another disease.

Since its initial discovery, *Clostridium difficile* has arisen from a relatively obscure and benign existence to become one of the most important nosocomial pathogens of our time (136, 151). In an era which has seen steady increases in the prescription and use of broad spectrum antibiotics, one of the adverse side-effects has been an increased predisposition of hospitalized patients to infection by *C. difficile*. Compounding the problem is the ability of *C. difficile*'s to produce highly resistant endospores. These factors have combined in recent years to make *C. difficile* one of the most formidable and expensive nosocomial pathogens. Today, *C. difficile* continues to be a major financial burden for many healthcare institutions. The costs associated with implementing patient isolation precautions continue to escalate (178). Despite extensive research into the pathogenicity and virulence factors of *C. difficile*, the organism continues to confound scientists. As one of the most enigmatic pathogens, *C. difficile* has indeed raised more questions than it has provided answers. In order to fully understand and appreciate the scope of this challenging pathogen, much research has still to be accomplished.

2.) Epidemiology

C. difficile is listed in Section 13 of Bergey's Manual of Determinative Bacteriology; a section which encompasses the gram positive, endospore-forming bacteria. As a member of the genus of *Clostridium*, *C. difficile* is an obligate anaerobe that produces oval subterminal spores, and is motile by peritrichous flagella. Surface colonies on blood agar are circular, occasionally rhizoid, flat or convex, opaque, grayish or whitish, and have a matt to glossy surface (24). Optimal growth temperature for *C. difficile* is 37 ^{0}C. *C. difficile* strains will produce a green fluorescence under ultraviolet light after 48 hours incubation on brucella agar supplemented with hemin and vitamin K_1 (61). Some strains may produce chains consisting of two to six cells aligned end to end. *C. difficile* is hydrophilic, and has been shown to possess plasmids, some of which are antibiotic resistance plasmids (47, 137).

C. difficile has been discovered in a variety of hosts including both birds and mammals (18). Despite its ubiquitous presence, transmission from animal reservoirs to humans has not been considered an important aspect of pathogenesis (49). The major reservoirs for *C. difficile* appear to be in young animals and humans. Asymptomatic colonization of *C. difficile* is found in ~3-4% of healthy adults (80), but may be as high as 15-75% in neonates (98, 133). The precise reasons for the high rates of asymptomatic carriage in infants are unclear (139), however, several theories have been proposed. These theories include maternal neutralising antibodies in human milk (92), immaturity of inflammatory response, masking of toxin receptors, immature receptors, and lack of receptors (141).

C. difficile can produce highly resistant endospores which allow for prolonged survival in the environment. This is an important aspect of pathogenesis as nosocomial transmission of the organism becomes a significant threat. Already, nosocomial acquisition and transmission of *C. difficile* has been well documented (51, 80, 90, 109), and outbreaks of *C. difficile*-associated diarrhea (CDAD) have occurred on many clinical services (15, 111, 84, 160, 14). In a study performed by McFarland et al., 21% of 428 inpatients became colonized by *C. difficile* during their hospital stay (109).

C. difficile is the most frequently isolated nosocomial gastrointestinal pathogen. It is purported to be responsible for 10 to 25% of all cases of antibiotic-associated diarrhea, for 60% of antibiotic-associated colitis, and for almost all cases of pseudomembranous colitis (11, 153). The major route of transmission appears to be via the fecal-oral route, however, mechanisms of nosocomial acquisition or transmission are not completely understood. The environment (90, 118), cross-infection between patients (35, 118), and personnel carriage by hospital employees have all been cited as plausible sources of infection.

The majority of healthy adults are resistant to infection by *C. difficile*. The colonization barrier afforded by the indigenous microflora found within the gut serves as an excellent defense mechanism against development of *C. difficile* disease. Under normal conditions, *C. difficile* is unable to compete successfully with the normal adult microbial flora. It is only when the normal bacteria flora is compromised, that toxigenic *C. difficile* organisms are allowed to proliferate and cause disease (5, 10). *C. difficile*-associated diseases occurs at all ages, but it is most frequent in the elderly and immunocompromised (49). Other risk factors include patients with cancer or burns, and patients undergoing

surgery.

The major predisposing factor for *C. difficile* infection is broad spectrum antibiotic use since disruption of the normal microflora paves the way for colonization and proliferation by toxigenic *C. difficile*. The majority of illnesses are associated with antibiotic usage, and it is rare to find incidences of disease where illness was not exacerbated by use of antimicrobics (49). Once toxigenic strains of *C. difficile* are able to overgrow the bowel, the resultant toxin production leads to illness. The symptoms of *C. difficile*-induced disease often occur after 5 to 10 days of antimicrobial therapy, although documented symptoms have been demonstrated to occur as early as the first or second day of therapy, or as late as 2 to 10 weeks after the offending antimicrobial agent has been discontinued (60, 157).

Despite proliferation of *C. difficile* in the bowel, studies have shown that the organism is non-invasive, and systemic spread of the organism in blood has not been reported (49). Toxigenic strains of *C. difficile* are known to cause various clinical symptoms ranging from asymptomatic colonization through to life-threatening pseudomembranous colitis (11). The severity of *C. difficile* disease can be influenced by several different host factors including colonization resistance afforded by the indigenous microflora (17) and expression of toxin-binding sites at colonic cell surfaces (95).

C. difficile disease is characterized by acute inflammation of the colonic mucosa. Pseudomembranous colitis is the severe form of the disease and is characterized by the presence of pseudomembranes, or whitish plaques along the mucosal wall of the colon (56). The histologic features of pseudomembranous colitis induced by *C. difficile* toxins are divided into three types (128). Type I, the earliest lesion, is characterized by patchy

epithelial necrosis accompanied by an exudation of fibrin and neutrophils into the colonic lumen. The type II lesion has a more prominent exudate that erupts as a volcano or summit lesion from a focus of epithelial ulceration; the surrounding mucosa remains intact. The type III lesion is characterized by more diffuse epithelial necrosis and ulceration overlaid by a pseudomembrane consisting of mucin, fibrin, leukocytes, and cellular debris. Pseudomembranous colitis usually affects the epithelium and superficial lamina propria of the distal colon. The presence of pseudomembranes is diagnostic for *C. difficile* because the organism causes essentially all cases of pseudomembranous colitis (104).

3.) Virulence Factors

Virulence of *C. difficile* is contingent on the ability of the organism to produce two chromosomally encoded protein exotoxins (79); toxin A and toxin B (104, 105, 106, 156). The initial discovery of *C. difficile* toxins were made in 1935 when broth cultures of the organism proved to be lethal when injected into rabbits and guinea pigs (104). Initially, the toxins were erroneously believed to be neurotoxins because they caused convulsions in animals (104). In addition to toxin production, *C. difficile* also produces a collagenase which may help in the breakdown of intestinal mucosa (19, 149). Some strains of *C. difficile* are also known to possess a capsule that confers protection from phagocytosis (37).

The genes for toxin A (46, 78, 115, 130, 145, 174) and toxin B (8, 79, 172) have been cloned and sequenced. The gene for toxin A encodes for 2,710 amino acids, while that for toxin B encodes for 2,366 (171). Amino acid analysis reveals that toxins A and B share 49% identity, and 63% similarity when conserved substitutions are considered (171). This has led scientists to speculate that the toxins may have arisen as a result of gene duplication. The

toxigenic element of *C. difficile* is 19.6 kb long, and consists of five open reading frames (68). The genes for toxin A and B lie within 1.4 kb of each other, with the gene for toxin B situated upstream (5') to the gene for toxin A (113). Regulation of toxin production is believed to be controlled by *txe*R gene located upstream (5') from the toxin B gene (113) (Fig. 1).

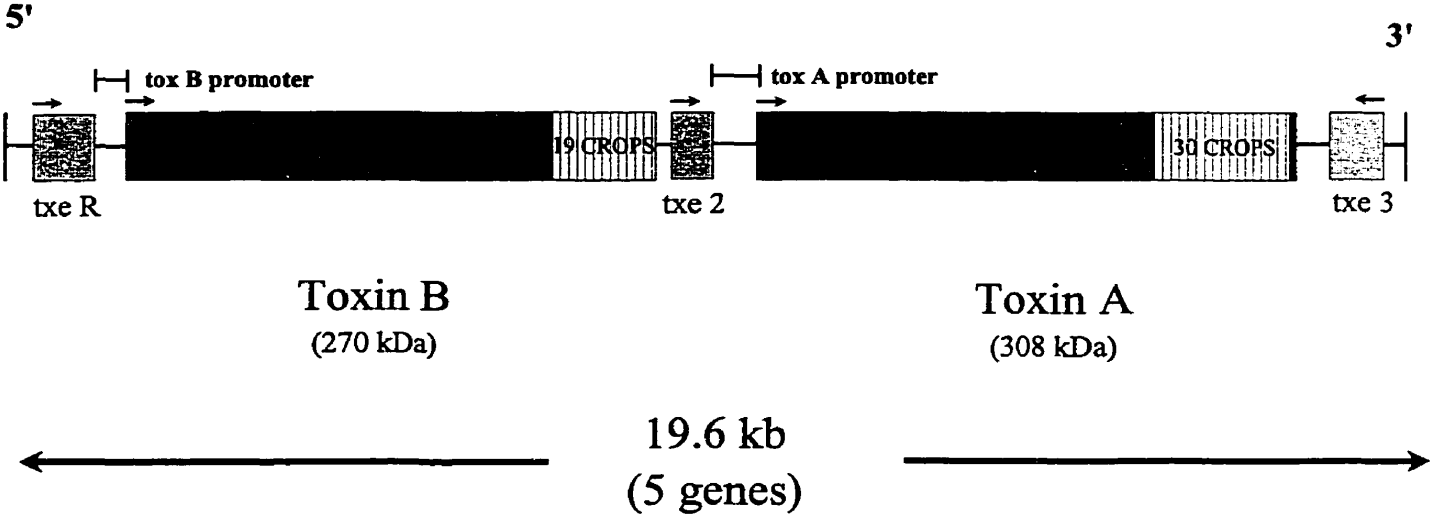


Figure 1. Pathogenicity Locus of *Clostridium difficile* toxins A and B.

Schematic diagram of the toxigenic element of *C. difficile* strain VPI 10463. The pathogenicity locus consists of 19.6 kb and five open reading frames. Regulation of toxin production is regulated by the *txe* R gene located 5' to toxin B gene. The hatched boxes represent the repeating units of the toxins (CROPS). Arrows indicate the direction of transcription. (Diagram adapted from Moncreif et al., 1997)

Toxins A and B of *C. difficile* belong to a family of large clostridial toxins (LCT) (16). They are among the largest bacterial toxins known to exist today (113). These toxins are characterized by three domains: A C-terminal domain involved in receptor binding, a middle hydrophobic domain involved in membrane translocation, and a N-terminal domain associated with biological activity (73). Despite their large molecular size, the LCTs act within eukaryotic cells. Electron microscopy and biochemical studies have revealed that *C. difficile* toxins are endocytosed (72) via coated pits (175). The precise sites of translocation after internalization are, however, unknown.

Toxigenic and non-toxigenic strains of *C. difficile* are known to exist, and have been described (181). Even within toxigenic strains, there are variations in the amounts of toxin produced (181). Some researchers report a range of 6 logs in the amount of toxin produced by *C. difficile* (104). The majority of clinically significant infections have been caused by strains of *C. difficile* capable of producing both toxins A and B. Strains deficient in toxin A production have, however, been reported (103). One strain called Serogroup F, which has deletions in the toxin A gene (103), lacks toxin A production and has been found only in neonates (42). Although cytotoxic activity has been detected in the stool samples of patients infected with this organism, most carriers are asymptomatic, and thus far, no clinically significant disease has been associated with this strain. Furthermore, germfree mice inoculated with this strain also do not develop diarrhea (42).

C. difficile toxins can be harvested by cultivating the organism in a dialysis sac bathed in brain heart infusion broth. The resultant diffusion of complex nutrients into the sac promotes slow growth of the organism and results in optimal toxin production (154).

Although antibiotics have been shown to promote colonization of toxigenic *C. difficile* in the intestine, they have not been shown to promote toxin production (7). *C. difficile* toxins are usually purified by ammonium sulfate precipitation followed by ion-exchange chromatography. The designations A and B refer to the elution profiles of the toxins on anion-exchange columns. Toxin A has less affinity for the column and elutes before toxin B.

Toxin A is a single polypeptide with an estimated molecular weight of about 308 kDa. It is considered to be the primary virulence factor of *C. difficile*. Toxin A is a UDP-glucosyl-transferase that glycosylates the cellular protein A, a GTP-binding protein involved in the regulation of microfilament organization (83, 121). The substrate for this reaction is UDP-Glucose, and mass spectroscopy has confirmed the addition of a 162 Da molecule, equivalent to a monohexose, on modified Rho proteins (83). Researchers have also shown that UDP-glucose deficient cell lines are less susceptible to the glucosytransferase acitivity of *C. difficile* toxins (29).

The effects of toxin A on the intestinal tract include disruption of intracellular tight junctions, fluid influx, diarrhea, inflammation, and neutrophil recruitment (9, 86, 114). Toxin A is also lethal when injected into animals (2), cytotoxic, and capable of inducing hemagglutination of rabbit red blood cells (57). The binding of toxin A to epithelial cells within the intestine is believed to lead to their destruction, however, internalization of toxin A by endocytosis is deemed necessary for this event to occur. The subsequent destruction of the protective epithelial cells is believed to mediate fluid secretion, and ultimately development of diarrhea.

A prominent feature of toxin A is its carboxy-terminal domain composed of clostridial repetitive oligopeptide (CROP) sequences (170, 173). There are approximately 30 CROP sequences in toxin A, with each individual CROP sequence consisting of 20 or 50 amino acids repeated 14-30 times (46, 74, 173). Toxin A is believed to bind via its CROP sequences to a trisaccharide receptor Galα1-3Galβ1-4GlcNac expressed on rabbit red cells and brush border membranes of hamster intestine (95, 138, 142). Binding of toxin A to brush border membranes is optimal at 4°C and decreases with increasing temperature (95). Monoclonal antibodies directed against the C terminus of toxin A have been reported to neutralize the enterotoxicity of toxin A in vitro (33). The trisaccharide receptor found on hamsters is, however, not present on humans. Human intestinal epithelial cells do, however, possess the carbohydrate Lewis X blood group antigen Fucα1-3Galβ1-4GlcNac. This trisaccharide sequence is conformationally similar to the hamster receptor, and there is evidence that this molecule is the toxin A receptor in man (165). In addition to receptor-mediated endocytosis, *C. difficile* toxins may also be taken up nonspecifically as toxins lacking CROP sequences (173), or cellular receptors (164) have been reported to exert biological activity.

Toxin A is inactivated by freezing and thawing, and is also sensitive to SDS and oxidizing agents. Despite having cytotoxic activity, and sequence similarities, both toxins A and B are immunologically distinct (57).

Toxin B is a single polypeptide with an estimated molecular weight of about 270 kDa. Similar to toxin A, toxin B is also a glucosyltransferase that glycosylates Rho proteins at residue Threonine-37 (82). However, unlike toxin A, there are only 19 CROP sequences in toxin B (46, 74, 171). The reduced number of CROP sequences seen in toxin B have led investigators to speculate that toxin B may be allowed to get into the systemic circulation to incite additional symptoms. The catalytic domain of toxin B involved in glucosytransferase activity has been localized to the N-termianal 546 amino acids (73). Toxin B is less stable than toxin A and is more sensitive to pH extremes and proteases(57). Similar to toxin A, toxin B is also lethal when injected into animals (2). Toxin B is also sensitive to SDS and oxidizing agents. Although research into *C. difficile* toxins to date has been focused primarily on toxin A, toxin B is a much more potent cytotoxin. The cytotoxicity of toxin B is believed to be a 1000 fold greater than that of toxin A. Consequently, much of the research regarding the cytotoxic activities of the toxins has been centered on toxin B (125). The effects of toxin B on intestinal cells are unclear, however, toxin B has been shown to decrease protein synthesis in hamster cecal explants (126). Toxin B is also capable of disrupting the microfilament system, and as a result, can cause polarization of the nucleus.

4.) Epidemiological Typing of *C. difficile* Strains

In order to assess outbreak strains of *C. difficile,* and to distinguish between potentially pathogenic and nonpathogenic strains, it is important to have available accurate and reliable typing schemes. Currently, several methods are available for identification and analysis of *C. difficile* strains:

i) Antibiotic Sensitivity Testing.

Antibiograms are profiles of an organisms differential susceptibilities to different concentrations and classes of antibiotics. Since differing strains of an organism can possess distinct susceptibility patterns, these differences in profiles have been exploited to distinguish amongst *C. difficile* organisms. However, because it is not impossible for two different strains of an organism to possess the same antibiogram, this method has somewhat limited utility as an epidemiological marker. The major limitation of this methodology for differentiation between strains of *C. difficile* is that *C. difficile* organisms have only a narrow range of antimicrobial susceptibilities. Results of in vitro testing reveal that *C. difficile* organisms are either highly sensitive, or highly resistant to many antibiotics (155).

ii) Bacteriophage and Bacteriocin Susceptibility

Bacteriophage and bacteriocin testing of *C. difficile* has also been used as an epidemiological marker. The technique was first developed for use on *C. difficile* by Sell et al. in 1983 (150). Since then, it has been used successfully to discriminate between different strains of *C. difficile* based on inhibition of growth, and differential susceptibility to different bacteriophages (107). However, the utility of this method is hampered by the instability of the bacteriocins, and the lack of suitable bacteriophages. The use of these epidemiological

markers is also very cumbersome and requires expertise in dealing with bacteriocins and bacteriophages. Furthermore, none of these techniques is sufficient alone in being able to discriminate amongst different strains of *C. difficile*.

iii) Immunologic Markers.

Methods that use immunologic markers as a means of distinguishing between different strains of *C. difficile* include crossed immunoelectrophoresis (127, 185) and immunobloting (117, 127). These methods have been used to identify and trace epidemic strains, but again, are unreliable as sole typing schemes. Serotyping is another method that is employed, and is based on slide agglutination of formol-treated cells with various rabbit antisera (40). This method has been applied extensively in epidemiological studies (161), however, variations in antisera used by different laboratories have made the interpretation of results difficult. If serotyping is to be used as a widespread tool for epidemiological analysis, standardization in protocol for reagent use is necessary.

iv) Genetic Markers

Plasmid analysis was first applied by Wust et al. in 1982 (185), and has since received widespread use by others (117). This method may be useful in identifying strains harbouring specific plasmid patterns but has limited application since only 30%-60% of strains of *C. difficile* possess plasmids (185). Restriction enzyme analysis (REA) methodologies, such as pulsed field gel electrophoresis (PFGE) have also been applied . This technique is more useful and definitive (43, 96, 184), but is also more labour intensive, and requires trained personnel. More recently, oligonucleotide probes specific for *C. difficile* 16S rRNA have been used to discriminate between stains of *C. difficile* and other clostridial

species (179). Whether this technique will be able to differentiate within the species of *C. difficile* is unknown. DNA probes which target specific proteins (38, 182, 183), toxins (78, 180), or antibiotic resistance genes (183) have also be used as epidemiological markers.

5.) Humoral Immunity

It has been proposed that human antibody responses to *C. difficile* and its toxins may play a role in protecting against severe, prolonged, or recurrent *C. difficile* diarrhea (85). It is believed that individuals with low anti-*C. difficile* antibody levels are prone to severe or relapsing *C. difficile* diarrhea, while asymptomatic carriers may have high anti-*C. difficile* antibody levels (3, 4, 81, 85, 99, 116). Indeed, animal studies have indicated that active and passive immunizations against *C. difficile* are protective against subsequent antibiotic challenge (91, 101). Although serum IgG antibodies to *C. difficile* toxins A and B have been reported to be as high as 60% in Americans above the age of 2, the protective roles of antibodies against *C. difficile* remain controversial (88, 169). Some studies report a significant increase in specific IgG and IgA antibodies against toxins A and B in convalescent-phase sera of patients with *C. difficile* colitis. This response seemed to correlate with reduced severity of disease, and also with reduced number of relapses (4). Furthermore, children with low levels of anti-toxin IgG seemed to be predisposed to chronic relapsing *C. difficile* colitis (99). Others, however, have reported a lack of correlation between immunity and clinical response. In a study which looked at systemic and mucosal antibody responses to toxin A, researchers found that individual serum responses to toxin A varied greatly, and that natural infection with *C. difficile* was unlikely to induce a protective serum antibody response (81).

It has been proposed that protective factors in milk may block toxin A activity in humans (39,92). Protection of hamsters from lethal intragastric doses of toxin A by pre-incubation with human milk has been demonstrated (141). Interaction of immunoglobulin and non-immunoglobulin fractions of milk with toxin A to prevent its binding to intestinal BBM receptors have also been shown (141). Already, it has been shown that milk can block the pathogenic effects of *Vibrio cholerae* toxin and *Escherichia coli* enterotoxins, without preventing the colonization of the intestinal tract by these organisms (34, 63, 97). In a similar fashion, these findings may suggest that human milk may block the toxin activity of *C. difficile* while allowing the organism to persist in the infant colon.

6.) Diagnostic Testing

In order to alleviate patient suffering and reduce severity of disease, it is important to have proper intervention plans made available in a timely fashion. These factors are influenced by rapid, reliable, and accurate diagnosis of CDAD. The currently available diagnostic tests include colonoscopy, tissue culture cytotoxin assay, stool cultures for toxigenic *C. difficile*, latex agglutination, counter-immunoelectrophoresis, enzyme-linked immunosorbent assay (ELISA), and molecular diagnostics such PCR (112). Colonoscopy is rapid and specific, but lacks sensitivity. The procedure is also invasive and expensive to perform making it impractical for many situations.

i) Tissue Culture Cytotoxin Assay

The tissue culture cytotoxin assay was originally described in 1977, and is now considered to be the "gold standard" test in the diagnosis of CDAD (28). In this test, stool which has been filtered and diluted, is inoculated onto cultured mammalian cells in the presence or absence of specific antiserum and examined for its ability to induce cytopathic effects. The presence of visible cell rounding in cultured cells is an indication for presence of toxin B. The major advantage of this test is its excellent record for diagnostic accuracy in terms of sensitivity and specificity. The sensitivity of the assay is reported to be 94-100%, while the specificity is reported at 99% (30). The disadvantage of the cytotoxin assay is the requirement for tissue culture facilities, and the length of time required for test results (24-48 hours). Concerns regarding false-positive and false-negative reactions have also been raised with the cytotoxin assay. There is speculation, however, that the majority of false positive reactions are due to testing of stools without clinical indications of CDAD, while the false-negative reactions may be attributed to inappropriately large dilutions of specimen, (10) or instability of toxin in stool. Although the cytotoxin assay detects only toxin B, *C. difficile* strains that produce toxin A invariably produce toxin B. To date, there has been no reported case of a *C. difficile* strain with a toxin A+/toxin B- genotype. Thus, detection of toxin B should accurately reflect the presence of toxin A.

ii) ELISA Assays

Diagnostic tests which are based on antigen detection, such as that of the ELISA, are also widespread. The rapid availability of results (2.5 to 3.5 h) and ease of automation make the ELISA technology a very attractive alternative. Several investigators (6, 20, 41, 45, 112),

report the sensitivities of the ELISA assay to approach or equal that of the cytotoxin assays. Sensitivities of ELISA assays are listed between 87% to 98%, while specificities are listed at 99-100% (30). ELISA assays which target toxin A, or toxins A and B, are now presently available through many commercial sources. The major concern with ELISA technology is in the potential for reagent variability amongst different manufacturers.

iii) Culturing

Culturing is considered to be the most sensitive assay for *C. difficile* (112). A differential and selective medium developed by George et al. containing cycloserine-cefoxitin-fructose and egg yolk (CCFA medium) has proven to be beneficial for the isolation of *C. difficile* from clinical samples (61). However, because culturing cannot discern toxigenic strains from non-toxigenic strains, a subsequent cytotoxin assay must be performed to determine if the isolate is toxigenic. This makes the procedure very time consuming and labour-intensive. The advantage of the culturing technique is that it allows the organism to be available for epidemiological analysis; this may be beneficial in assessing outbreaks.

iv) Latex Agglutination

The latex agglutination test, although rapid and easy to perform, is unreliable as a sole diagnostic test for *C. difficile*. The test targets the enzyme glutamate dehydrogenase, an enzyme found in many species of bacteria. Consequently, many latex agglutination tests will result in false-positives (87, 94). The sensitivity of the latex agglutination reaction is reported to be around 48-59%, while the specificity is reported at 95-96% (30). Although the latex agglutination test may be beneficial as an initial screening tool for *C. difficile*, current recommendations are that it should not be used as the sole diagnostic test.

v) PCR Technology

In the quest for more rapid, specific, and cost-effective means of diagnosing *C. difficile*, researchers have also implemented PCR technology (66). The advantage of PCR procedure is that it allows for sensitive detection of a small fragment of DNA sequence in a complex mixture of other molecules. Certainly, with the decreased cost of thermostable DNA polymerase, PCR has been made a more cost-effective alternative to traditional diagnostic procedures. Labs utilizing this methodology must be cognizant of the potential for PCR inhibitors to exist in stool specimens. Furthermore, it is imperative that primers selected be specific only for toxigenic strains of *C. difficile*.

vi) Criteria for *C. difficile* Testing

Asymptomatic carriage of toxigenic strains of *C. difficile* makes it critical that diagnostic testing be done only for symptomatic patients. Testing of stool samples from patients without clinical indications of CDAD is not only an unnecessary cost, but may also complicate patient care if unnecessary antibiotic treatment is given. Testing for CDAD should not be based on length of hospitalization stay, but rather, on the presence of clinically significant diarrhea (94) (e.g. 4 or more bowel movements per day for 3 or more days) with a history of antibiotic therapy. Testing for CDAD may also be indicated in patients who present with acute abdominal syndrome with little or no diarrhea (49). Clinicians ordering cytotoxin tests on patients less than one year old should be aware of the high asymptomatic carriage rate within the neonatal population. Although there are rare instances where toxin B in children less than one year old is significant (131), up to 50% of neonates may be colonized with toxigenic strains without clinical symptoms (11, 44, 48). It has also been

reported that 35% of patients with cystic fibrosis can also be asymptomatic carriers of toxigenic *C. difficile* (123). In order to invoke correct diagnosis, and therefore treatment of *C. difficile* infections, it is necessary that Clinicians to be aware of the guidelines which stipulate patient testing.

7.) Treatment Options

C. difficile-associated disease is a paradoxical illness in that the disease state is both incited and cured with the use of antibiotics. Many intervention plans are currently available for the management of *C. difficile* infections. However, in selecting the appropriate intervention plan, the patients specific needs must be addressed. It should be noted that not all patients with CDAD need to be treated with antibiotics. When patients have mild or moderate symptoms, it is sometimes sufficient in about 25% of instances to discontinue treatment with the precipitating antibiotic and to give supportive therapy with fluids and electrolytes (49). In instances where antibiotic treatment is necessary because of other underlying illnesses, the recommendation is to discontinue use of the inciting agent and to substitute it with a drug that is more specific for the infection, and less likely to cause recurrence of CDAD.

Presently, two antibiotics are recommended for the treatment of CDAD; vancomycin and metronidazole (49). Current attempts at reducing the development of vancomycin resistant organisms have led to guidlines to reduce the use of vancomycin therapy (26). As such, many centres are reducing the use of vancomycin for initial therapy of CDAD. Vancomycin, when given orally, is poorly absorbed from the gastrointestinal tract. This reduces drug toxicity in patients, and also allows for greater concentrations of drug to be

achieved within the bowel. Metronidazole is also highly effective against *C. difficile* infections, however, seriously ill patients have failed to respond to this drug (132). As metronidazole is readily absorbed by the small intestine, questions have been raised regarding the ability to maintain adequate levels of drug within colonic lumen. The minimal inhibitory concentrations of vancomycin or metronidazole necessary for treatment of *C. difficile* infections are usually around 5 mg/L or less (49). Although most patients respond to metronidazole or vancomycin, approximately 20% of patients relapse 2 to 8 weeks after discontinuation of antibiotic therapy (50). While most of these patients respond to a second course of therapy, up to 30% of these patients will experience multiple relapses (22, 77). To address this dilemma, several different approaches have been attempted, including pulse doses of vancomycin, slowly tapering doses of vancomycin (159), and combination therapy with vancomycin and rifampin (22) or cholestryramine (158).

As restoration of the normal microflora is requisite for cessation of *C. difficile*-associated disease, many efforts have been made to restore the normal colonic microbial flora. Several approaches have been attempted, each with varying degrees of success. The administration of lactobacillus GG (64), or *Saccharomyces boulardii* plus metronidazole or vancomycin (110) have been attempted. Researchers speculate that the success of therapeutic treatment with *Saccharomyces boulardii* may be attributable to a serine protease that the organism excretes (23). Using in-vitro experiments, they were able to demonstrate serine protease inhibition of toxin A binding to intestinal receptors, and decreased ileal secretion in rats. Finally, rectal installation of stool (147) or mixed broth cultures of fecal flora (166), however unpleasant, have also been attempted.

Patients with *C. difficile* infections often suffer from multiple relapses. The occurrence of relapses are thought to result from either failure to eradicate the organism, or from re-infection from environmental or human sources (50). It is unlikely that relapses are due to resistance to antimicrobials, however, *C. difficile* has been found to possess multiple antibiotic resistance genes (137). Already, clinical isolates of *C. difficile* resistant to vancomycin and metronidazole have been reported (47, 52). This is a grave concern for clinicians as it raises serious questions regarding the efficacy of these antimicrobial agents for treatment of future illnesses. To combat this trend, the Centers for Disease Control and Prevention have recommended limiting the use of oral vancomycin for treatment of *C. difficile* infections. Relapses in cases of CDAD treated with antibiotics may be influenced by the fact that antibiotics used for treatment of *C. difficile* are predominately broad-spectrum and nonselective. Because broad-spectrum antibiotics are known to disrupt the normal gut microflora, this may invariably predispose the patient to CDAD relapse (119).

As an alternative to antibiotic therapy, passive immunotherapy for CDAD has also been attempted. The medical advantages anticipated in this approach include greater specificity, fewer relapses, and relief from selective pressure of antibiotic resistance in normal flora (26). Avian antibodies made against recombinant epitopes of toxin A and toxin B have been shown to be effective against preventing CDAD in the hamster model (93). The antibodies with the greatest efficacy were found to be derived from the epitopes near the carboxy-terminal regions of the toxins. These regions possess a series of repetitive amino acid domains believed to be involved in intestinal cell-binding (173). Hamsters treated with these recombinant antibodies did not relapse, and were also refractory to reinfection with *C.*

difficile. However, a mixture of antibodies to both toxins A and B were required for complete neutralization. Treatment with antibodies to epitopes on toxin A were effective in preventing death in hamsters, but were ineffective in preventing diarrhea.

Antiperistaltic agents are not recommended for treatment of CDAD. Although antidiarrheal agents may provide symptomatic relief for the patient, they do so at the expense of toxin excretion from the bowel. The resultant accumulation of toxic fluid within the lumen of the intestine may adversely promote damage to the colon. Cases of patients becoming worse from treatment with antiperistaltic agents have been documented (120).

8.) Disease Prevention

The most ideal situation for managing *C. difficile* infections would be to devise a treatment plan which would prevent the colonization by toxigenic strains of *C. difficile*. Since this not a realistic possibility, much research has been devoted to devising methods which can neutralize toxin activity. As binding of toxin A to its oligosaccharide receptor on intestinal epithelium represents the first stage of development of CDAD, one approach to treatment has been to try to neutralize the ability of toxin A to bind to its receptor. Oligosaccharide sequences attached to inert supports have been used to adsorb toxin A (71). If the levels of toxin A activity can be reduced by the use of immobilized oligosaccharides, then these compounds could be used as a potential therapy for CDAD. This form of therapy is, however, limited in that it cannot affect the levels of toxin B within the intestine. The benefit of this therapy is that the material is specific for toxin A, is nontoxic, and resistant to mechanical and chemical decomposition. The use of this therapy also negates the need for antibiotics, thus allowing for the re-establishment of the normal intestinal microflora.

Similar treatment regimens have also been initiated with cholestyramine resins which are believed to bind to toxin molecules nonspecifically.

Passive immunotherapy and vaccinations (143, 162) have also been proposed for treatment of *C. difficile* infections. Most studies have yielded incomplete or controversial results, but some studies have shown promise. One study examined the ability of bovine colostral antibodies obtained from gestating Holstein cows immunized with *C. difficile* toxoids to neutralize the biological effects of *C. difficile* toxins A and B (89). The bovine immunoglobulin concentrate contained high levels of bovine immunoglobulin G specific for *C. difficile* toxins A and B, and was found to be able to neutralize the cytotoxic effects of purified toxin A and B on cultured human fibroblasts. The anti-*C. difficile* bovine IgG also blocked the binding of toxin A to rabbit ileal brush border membranes in-vitro and inhibited the enterotoxic effects of *C. difficile* toxins on the rat ileum in-vivo. This study shows promise for passive immunization against *C. difficile* toxins as a potential therapeutic treatment in the management of *C. difficile*-induced infections.

9.) Models for Studying *C. difficile* Pathogenesis

In order to understand the mechanisms behind *C. difficile*-associated diseases, it is important to be able to establish suitable models for research and investigative purposes. Ideally, humans would be the preferred subjects for these investigations. However, due to ethical and technical constraints, much of the work must be done with animal and in vitro models. Fortunately, the parallel between human disease and animal disease is remarkable. For these reasons, much of what is known about the pathogenesis of *C. difficile* has been derived from studies utilizing animal models. Presently, there are several models available:

A) In vivo Models

i) Hamster Model

C. difficile is able to cause antibiotic-associated disease in a number of animal species, including hamsters, guinea pigs, and rabbits (53, 104). The susceptibility of the animals to *C. difficile* infection has enabled many researchers to use them as suitable models for studying *C. difficile* pathogenesis. Indeed, much of the current knowledge about the etiology of pseudomembranous colitis in humans has relied on the use of animal models (94). Among the animal models, the hamster model is the most well-known and most utilized (12, 13, 19, 21, 27, 53, 54, 62, 91, 93, 101, 102, 129, 134, 142, 162). Similar to human infections, the hamster disease can also be incited with a variety of antibiotics (27, 53, 102, 129). If toxigenic strains of *C. difficile* are present in the environment, the hamster usually develops diarrhea, and may die of fatal enterocolitis. Unlike the disease in humans which is localized to the distal colon, the hamster disease is localized in the proximal colon (cecum), with some involvement of the ileum. Pathological examinations of ceca of hamsters with *C. difficile* infections will sometimes reveal inflammatory debris consisting of erythrocytes, inflammatory cells, bacteria, and sloughed mucosal cells characteristic of PMC in humans. Despite the differences in the location of infection, the hamster model has been well accepted as a model for studying *C. difficile* pathogenesis. Interestingly, the asymptomatic carriage of toxigenic *C. difficile* in human infants is also seen in infant hamsters (140).

ii) Germfree Animals

In addition to the hamster model, studies on *C. difficile* pathogenicity have also been performed on germfree mice and rats (32, 36, 122, 168). Unlike the hamsters, the germfree animals cannot develop CDAD. The advantage of using gnotobiotic animals is that they are able to be mono-infected with *C. difficile*. Furthermore, these germfree animals are able to develop intestinal pathology in the colon similar to PMC in humans. In this regard, it has been suggested that this animal model may be more suitable for studying PMC. However, these models are very expensive to implement, and the fact that no other organisms are involved in the infection make it a very unlikely representation of true PMC in humans.

iii) Ligated Intestinal Loop Assay

Among the more well known in vivo assays are the ligated intestinal loop assay, and the oral inoculation assay. For the ligated intestinal loop assay, small intestinal (jejunal or ileal) or colonic ligated intestinal segments are inoculated with toxin preparations or bacterial cultures (163). Following inoculation, the loops are monitored for presence or absence of secretion. The presence of secretion helps establish the importance of the toxin, or the bacterial culture, to the pathogenesis of the disease.

iv) Oral Inoculation Assay

The oral inoculation assay is also useful for identifying the pathogenic roles of toxins. In this assay, the animals are inoculated with toxins orally and examined for changes in intestinal fluid secretion and development of pathology. This model has been used successfully to demonstrate that *E. coli* Sta is able to stimulate fluid secretion in suckling mice (75).

B) In vitro Models

i) Ussing chambers

In vitro assays are also common tools for studying the pathogenesis of organisms and their toxins. The elimination of costs associated with purchasing and caring for animals has made this approach much more desirable for research. Among the in vitro assays, the Ussing Chambers and tissue culture assays are most common. The Ussing chamber model has been used extensively to identify specific changes in active ion transport induced by enteric bacterial toxins (55, 146, 167). In this approach, native intestinal epithelium or polarized monolayers of cultured intestinal cells are mounted between Lucite chambers under conditions of ionic, osmotic, and electrical equilibrium. The ability of an enteric toxin to stimulate anion secretion and/or inhibit NaCl absorption, both potentially contributing to net intestinal secretion, is then monitored (148).

ii) Cultured Cell Lines

Another experimental approach used to identify activities of enteric toxins is with the use of intestinal cell lines. In this approach, the activity of the toxin is assessed by monitoring morphological or cytotoxic responses in cells.

It is now possible for researchers to grow polarized monolayers of various cell lines on membrane filters. This approach has allowed researchers to mimic the in vivo state as cells grow on membrane filters will differentiate and exhibit polarization (69, 70) similar to the in vivo state. The human colonic carcinoma cell line, Caco-2, has been used extensively for in vitro studies. This cell line has been shown to exhibit spontaneous in-vitro enterocytic differentiation characteristic of mature enterocytes (124). Post-confluent cultures of Caco-2

cells were shown to be polarized by electron microscopy studies, and also exhibited both tight junction and brush border microvilli formation. These characteristics have made Caco-2 cells an excellent cell line for in-vitro studies of epithelium transport.

Cells grown on membrane inserts also more closely approximate their in vivo state than those grown on plastic surfaces. The insert model also allows for access to both apical (top) and basolateral (bottom) sides of epithelial cells; an amenity not otherwise possible using conventional tissue culture plastic. The ability to assess the cell monolayers from both apical and basolateral domains allows for the study of: transport across the cell monolayer, permeability of the cell monolayer, and electrophysiology of the cell monolayers including transepithelial resistance. Transepithelial electrical resistance measurements have been used extensively to monitor monolayer confluence. This is especially important in experiments where intact monolayers are required. Electrical resistance measurements are also an easy way to measure monolayer permeability after exposure to potentially toxic agents.

Some inserts feature thin, tissue culture treated, microscopically transparent polyester membranes that facilitate cell spreading and attachment. These clear inserts provide excellent cell visability under phase contrast microscopy, and also allow for assessment of cell viability and monolayer formation. The inserts usually have both membrane surface and inside wall tissue culture treated to ensure uniform cell attachment. A cell monolayer grown on these inserts is integral across the membrane, and is also firmly attached to the polystyrene side walls. The polystyrene membrane filter with monolayers attached may be also be removed by careful cutting with a scalpel. This feature is especially important in attachment studies.

10.) Experimental Hypothesis

Toxin A and/or toxin B of *C. difficile* are known to damage the integrity of the intestinal bowel wall. Our hypothesis is that even in early stages, when the mucosa is still intact, that the tight junction damage elicited by toxin A and/or B facilitates the translocation of either toxins, or toxic factors, from the lumen of the gut into the deeper tissues and/or systemic circulation. Based on our hypothesis, our objectives were to assess polarized epithelial cell cultures as an in vitro model for studying toxin translocation dynamics. If translocation of toxin across intact cell monolayers is feasible, we would assess various treatment options to determine if inhibition of translocation can be achieved. The examination of toxin translocation dynamics is an important aspect of pathogenesis to consider since understanding the basis of systemic symptoms is necessary for implementing appropriate prevention measures. Similarly, if toxins are able to get into systemic circulation, it is important to determine if any toxin molecules are able to persist in the blood of patients with PMC. Finally, if patients are exposed these toxins systemically, do they develop humoral immune responses to the toxin, and is this humoral immune response beneficial to the outcome of the infection.

MATERIALS AND METHODS

1. Cell Culture

Caco-2 (ATCC HTB 37, human colon adenocarcinoma) and T84 (ATCC CCL 248, human colon carcinoma) cell lines were maintained in antibiotic-free RPMI 1640 (ICN Biomedicals Inc., Costa Mesa, California) medium supplemented with 20% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 1mM Na pyruvate (ICN Biomedicals Inc.) and 2 mM L-glutamine (ICN Biomedicals Inc.). Human Foreskin Fibroblasts (HFF), a nontransformed cell line derived from pooled foreskins from children, was maintained in antibiotic-free RPMI 1640 (ICN Biomedicals Inc) medium supplemented with 10% FBS (Gibco BRL), 1mM Na pyruvate (ICN Biomedicals Inc.) and 2 mM L-glutamine (ICN Biomedicals Inc.). The tissue culture cells were incubated at 35°C in 5% (vol/vol) CO_2 in a water-saturated incubator. Cells were passaged biweekly using a 0.05% trypsin solution in 0.53 mM EDTA · 4Na (1X) to disperse the monolayer followed by a 1:2 split into fresh medium.

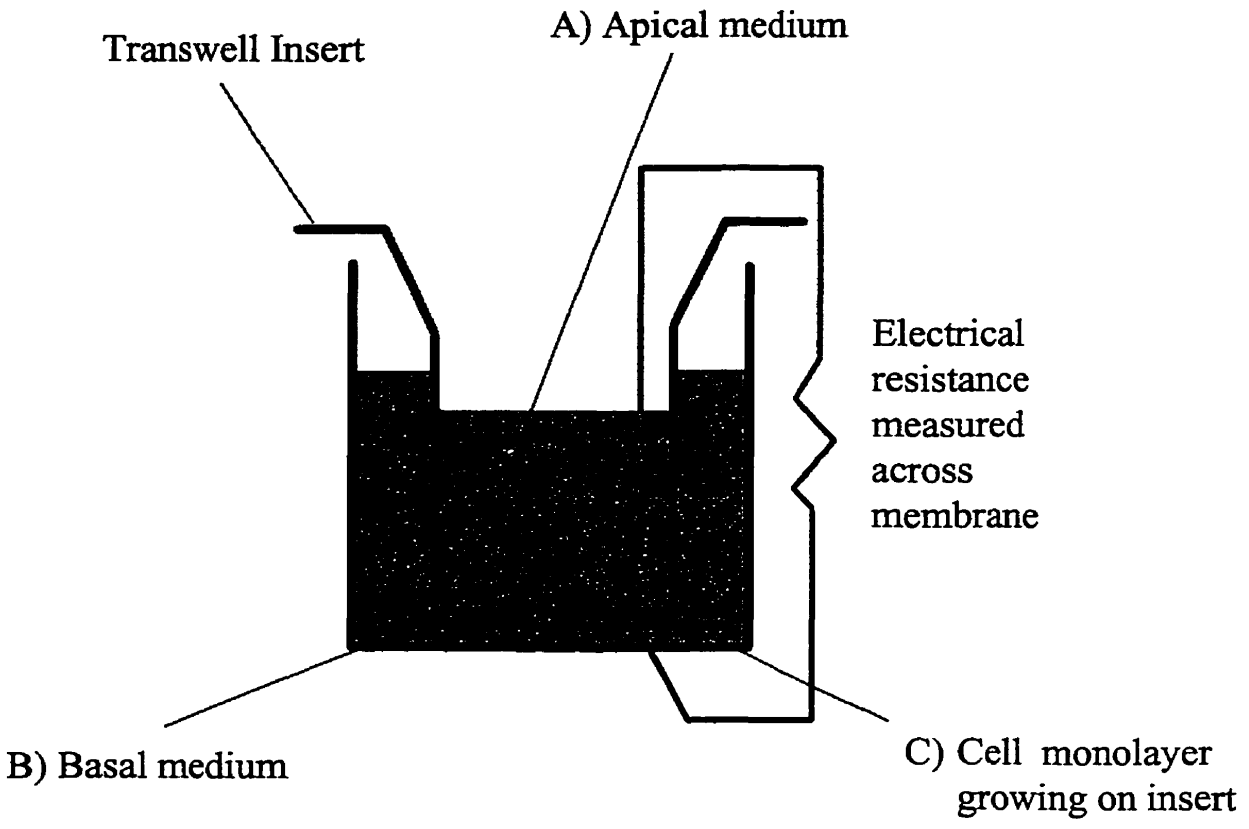
2. Polarized Cell Monolayers

Caco-2 and T84 cells were seeded onto Transwell® inserts with 0.4 μm pores (Corning Costar, Cambridge, Massachusetts) by adding 0.5 mL of a cell suspension containing 1.0 X 10^5 cells/mL. Tissue culture medium consisting of RPMI 1640 (ICN Biomedicals) supplemented with 20% FBS (Gibco BRL), 1mM Na pyruvate (ICN Biomedicals Inc.), 2 mM L-glutamine (ICN Biomedicals Inc.), and 100 IU/mL of Penicillin-Streptomycin (ICN Biomedicals Inc.) was added (1.5 mL) to the basal side of the insert in each cluster plate. After 24 hours incubation at 35°C, dead and non-adherent cells were

removed from the apical side of the insert and replaced with fresh tissue culture medium. Tissue culture medium was replaced three times weekly until cells reached confluence. Confluence of polarized cell monolayers was monitored by performing electrical resistance measurements using MILLICELL®-ERS probes (Millipore, Bedford, Massachusetts) (Fig. 2.). MILLICELL®-ERS probes were cleaned by rinsing with distilled water and 70% ethanol followed by an equilibration step in sterile tissue culture medium prior to performing electrical resistance measurements. All experiments were carried out on confluent monolayers of Caco-2 cells as determined by incubating until high stable resistance readings (400 ohm · cm^2 or greater) were established. This usually required 4 - 7 days of incubation post-inoculation. Each test condition was assayed by taking triplicate readings at each time interval. Three Transwell inserts were used for each test case giving a total of nine replicate readings.

3. Purified *C. difficile* toxins A and B

Highly purified *Clostridium difficile* toxin A (Lot: 0197005, Cat. # T3001) and toxin B (Lot: 0197010, Cat. #T3002) were generously provided by Dr. David Lyerly (TechLab Inc., Blacksburg, Virginia). Toxins A and B were purified to homogeneity to concentrations of 0.46 mg/ml and 0.21 mg/ml respectively. In addition, mouse monoclonal antibody to *C. difficile* toxin A (Lot: 0896008, Cat. #T1003) (Ascites) and affinity purified goat antibody to *C. difficile* toxin B (Lot: 1097019) were also provided by Dr. David Lyerly. Due to potential inactivation of toxins from freezing and thawing, preparations were stored at 4°C as per manufacturer's instructions.



Procedure: Step I Take samples of media from Apical and Basal sides of insert.

Step 2 Wash and cut out cell monolayer growing on insert

Step 3 Determine CPM

Figure 2. Polarized Tissue Culture Insert Model Seeded with Caco-2 Cells.

This schematic diagram of the polarized tissue culture insert model indicates where in the Transwell apparatus the apical (A) and basal (B) fluid samples, as well as the monolayer sample (C) from the porous insert were obtained. When ^{125}I-labeled components were tested, radioactivity (cpm) was measured in the test samples (A,B,C). When translocation of CPE was evaluated, basal (B) samples were tested in the HFF CPE assay as described in the Materials and Methods.

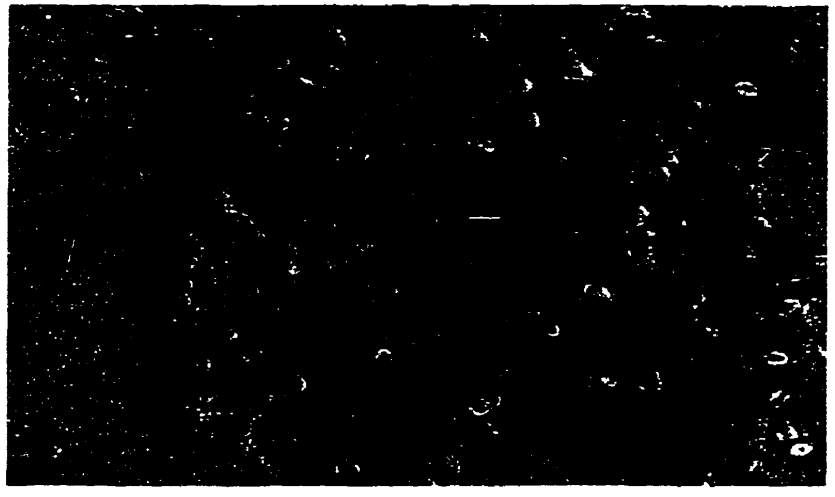
4. Cytotoxicity Assays

HFF cell monolayers used for cytotoxin assays were prepared as follows: HFF cells (100 µl) suspended at a concentration of 1.0 X 10^5 cells/mL in RPMI 1640 (ICN Biomedicals) supplemented with 10% FBS (Gibco BRL), 1mM Na pyruvate (ICN Biomedicals Inc.), 2 mM L-glutamine (ICN Biomedicals Inc.), and 100 IU/mL of Penicillin-Streptomycin (ICN Biomedicals Inc.) were inoculated onto 96 well Cell Wells (Corning glass works, Corning, New York) and allowed to grow to confluence. Once confluent, HFF monolayers were maintained by performing weekly tissue culture medium changes. For assessment of cytotoxicity in test samples, 50 µl of tissue culture medium was sampled from the basal side of the Transwell insert at various time intervals and inoculated onto HFF monolayers. The inoculated HFF monolayers were incubated for 48 hours at 35°C and examined for cytopathic effect (CPE). Wells with >50% of the cells exhibiting 'rounding up' CPE were scored as positive. Specificity of CPE produced by purified toxins were confirmed by neutralizing cytotoxic effects with polyvalent anti-*C. difficile* toxin B serum (Bartels Inc., Issaquah, Washington) (Fig. 3). Negative and positive controls were included for each HFF CPE assay performed.

5. Determination of CPE Titre for toxins A and B

Highly purified *Clostridium difficile* toxins A (Lot: 0197005) and toxin B (Lot: 0197010) were serially diluted 1:2 onto HFF cell monolayers (prepared as previously described). After 48 hours incubation at 35°C, cell monolayers were examined for CPE. CPE titres for toxins A and B were determined by scoring wells with the greatest dilution of toxin still capable of inducing >50% cell rounding.

Control



Toxin Treated

Figure 3. Phase Contrast Microscopy of Control and Toxin Treated HFF Cells.

Phase contrast microscope pictures of control and toxin treated Human foreskin fibroblasts after 48 hours incubation. Non-treated cells appear as smooth fibroblasts, while toxin treated cells appear rounded.

6. Assessment of Cytotoxicity in Patient Sera

Presence of circulating toxin B in sera of control patients and patients afflicted with *C. difficile* disease was assessed by inoculating patient sera (fresh or frozen) diluted 1:4 onto HFF cell monolayers (prepared as previously described). After 48 hours incubation at 35°C, cell monolayers were examined for cytopathic effect (CPE). Presence of circulating toxin B in sera was interpreted if >50% of the cells exhibited specific 'rounding up' CPE that could be neutralized by polyvalent anti-*C. difficile* toxin B serum (Bartels Inc.).

7. Assessment of toxin B Neutralizing Antibodies in Patient Sera

Neutralizing antibodies to toxin B were assessed by incubating 30 μL of patient sera with 30 μL of purified toxin B (the concentration of toxin B used was 5 CPE units in Microtiter® plates (Dynatech Labs, Chantilly, Virginia). Following 30 min. incubation, the mixture (50 μL) was transferred onto HFF cell monolayers. Patients were considered to be positive for neutralizing antibodies to toxin B if no CPE was seen after 48 hours of incubation. Commercial antitoxin (Bartels) that neutralized the same concentration of toxin B served as a negative control.

8. ELISA Assay for Detection of toxin B Antibodies

Microtiter® plates (Dynatech Labs) were coated overnight with 50 μL of purified toxin B (0.005 μg/ μL in carbonate buffer) (Tech Labs) at 4 °C in a moist chamber. Plates were blocked overnight with 75 μL of 5% skim milk containing 1% (v/v) rabbit pre-immune serum (Cedarlane Laboratories, Hornby, Ontario). Patient sera (diluted 1:50 in 1% BSA-PBS) was added (50 μL) and incubated at 35 °C for 1 hour. Goat Anti-toxin B (diluted 1:200 in BSA-PBS) was included (50 μL) as the positive control, and 1% BSA-PBS was used for

the blank. Plates were washed 4X using 75 µL PBS-Tween 20. Conjugate detection was performed by adding 50 µL conjugate (1:1000 dilution of polyvalent Goat anti-human IgG, IgM, IgA (Cedarlane Laboratories) in 1% (w/v) skim milk) and incubating for 1 hour. Swine anti-goat (50 µL) (1:1000 dilution) was used for detection of the positive control. Plates were washed 4X using 75 µL PBS-Tween 20. Color development was performed using 50 µL ABTS (2,2'-Azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium) (Sigma Chemical Co., St. Louis Missouri) with 20 min. incubation in the dark at room temp. The reaction was stopped by adding 50 µL of 10% SDS. The absorbance was read using an Emax microplate reader (Molecular Devices, Menlo Park, California) at 405 nm. An absorbance reading ≥3 times the background absorbance was considered positive. (The average background Abs. was ≤0.50, therefore an Abs. of ≥0.150 was considered positive).

9. Radiolabeling of Test Proteins

Purified test protein (50 µg) was radiolabeled with carrier free ^{125}I (Mandel, Geulph, Ontario) in a 2.5 mL polypropylene Cryo-lock® vial (Sin-Can, Calgary, Alberta) containing 100 µL of Phosphate buffered saline (PBS) pH 7.5 using IODO-BEADS® according to manufacturers specifications (Pierce chemical co., Rockford, Illinois). The radiolabeling reaction was allowed to occur for 5 min. at room temp. Radiolabeled proteins were separated from unbound ^{125}I by using a 30 cm x 3.5 mm sizing column packed with Sephadex G100 (Pharmacia, Uppsala, Sweden). The radiolabeled sample was loaded onto the column, and PBS (pH 7.5) was used to elute the sample. Fractions (0.5 mL) were collected into 2.5 mL polypropylene Cryo-lock® vials (Sin-Can). The progression of the first radioactive peak through the column was followed using a Ludlum 44-21 Geiger counter (Sweetwater,

Texas). To quantitate efficiency of the radiolabeling reaction, trichloroacetic acid (TCA) precipitations were performed on fractions from the radioactive peak with the greatest activity. TCA precipitations were performed by adding 100 µL of a 100% TCA (Fisher, Fair Lawn, New Jersey) solution to 5 µl of labeled protein and 900 µl of a 1% BSA solution in an eppendorf tube. A TCA (-) tube containing 5 µl of labeled protein and 1 mL of 1% BSA was used as a negative control. Eppendorf tubes were held on ice for 10 min. and then centrifuged at maximum rpm using a Micro-centrifuge (Fisher). Supernatant (100 µl) was removed from each tube, placed in a 4 mL scintillation vial (Fisher) and counted for one minute using a Gamma 5500 counter (Fullerton, California). The efficiency of this counter was 75%. All TCA precipitations performed were greater than 75% as previously reported (39). Fractions reporting the highest radioactive counts and TCA precipitabilities (usually greater than 80%) were then tested for biological activity. Biological activity was assessed by using a HFF cytotoxin assay and by measuring electrical resistance changes in Caco-2 cells. The titre of biological activity of the radiolabeled proteins was essentially unchanged.

10. Translocation of *C. difficile* Toxins

Medium from Transwell inserts showing high stable resistance measurements were removed and replaced with fresh tissue culture medium (0.5 mL) containing unlabeled and/or radiolabeled proteins in different test combinations. Tissue culture medium (1.5 mL) from the basal side of the insert was also replaced (no labeled proteins added). Resistance measurements were recorded at various time intervals to monitor monolayer integrity. Translocation of labeled proteins during the time course of the experiment was assessed by sampling tissue culture media from the basal side of the insert and inoculating onto HFF

monlayers (as previously described). In addition, partitioning of labeled proteins was determined by performing radioactive counts per minute (cpm) per microlitre on samples obtained from the apical and basal sides of the insert. Binding of toxin to the tissue culture monolayer was assessed by washing the monolayer three times with fresh tissue culture medium and then cutting the rigid insert (with the monolayer attached) out of the Transwell apparatus. All samples were counted for one minute using a Gamma 5500 (Fullerton, California). The efficiency of the counter was 75%.

11. Translocation of *E. coli* Lipopolysaccharide

To assess whether *C. difficile* toxins can aid in the translocation of bacterial endotoxins, varying nanogram amounts of purified *E. coli* lipopolysaccharide (LPS) (Sigma Chemical Co), were inoculated onto confluent Transwell inserts containing 0.5 mL of *C. difficile* toxins A and B (toxins were diluted to a concentration of 0.250 μg /mL) in RPMI supplemented with 20% FBS. *E. coli* LPS inoculated onto empty Transwell inserts was used as a positive control. Electrical resistance measurements were monitored at various time intervals to assess monolayer integrity. Translocation of lipopolysaccharide during the time course of the experiment was assessed by sampling tissue culture medium (50 μL) from the basal side of the insert and performing endotoxin assays.

12. Endotoxin Assay for Quantitation of Gram-negative Lipopolysaccharide

Endotoxin assays were performed using the Limulus Amoebocyte Lysate (LAL) Pyrochrome Chromogenic Test kit (Associates of Cape Cod, Falmouth, Massachusetts) according to manufacturers instructions. All assays were performed utilizing the endpoint method. Briefly, a standard curve of varying endotoxin units per mL (EU/mL)was created

using the control standard endotoxin (CSE) powder provided (2 EU of powder). CSE powder was reconstituted in 2 mL of LAL reagent water (LRW) (Associates of Cape Cod) and diluted 1:2 to give endotoxin concentrations ranging from 1.0 EU/mL to 0.31 EU/mL. Samples (50 μL) to be tested for endotoxin were inoculated onto pyrogen-free microtiter Pyroplates (Associates of Cape Cod). After addition of samples, 50 μL of pyrochrome reagent (reconstituted using 3.2 mL of Pyrochrome reconstitution buffer) was added to each well and incubated for 30 min. to allow for color development. Color development was stopped with addition of 25 μL of 50% acetic acid. Absorbance was read at 405 nm using an Emax microplate reader (Molecular Devices). The concentration of EU/mL for unknown samples was determined from the standard curve given by the Emax software.

13. Toxin A Inhibition Assays Using Synsorb CD and Cholestyramine

Toxin A (1 mL) made to a concentration of 0.250 μg/mL in either PBS (pH 7.5) or RPMI 1640 (ICN Biomedicals Inc.) medium supplemented with 20% FBS (Gibco BRL, Grand Island, NY), 1mM Na pyruvate (ICN Biomedicals Inc.) and 2 mM L-glutamine (ICN Biomedicals Inc.) was incubated with varying concentrations of Synsorb CD (Synsorb Biotech, Calgary, Alberta) or Cholestyramine (Bristol Laboratories, Montreal, Canada) in a 1.5 mL eppendorf tube. Untreated toxin A in PBS or RPMI served as a positive control while PBS or RPMI served as a negative control. After 1 hour incubation at 35 °C, 500 μL of supernatant from each test case was transferred to confluent Caco-2 cell monolayer growing on Transwell inserts to monitor electrical resistance changes. Samples (100 μL) were also inoculated onto HFF cell monolayers and titrated to determine if there were any reductions in CPE titre as a result of Synsorb CD or Cholestyramine binding to toxin A.

RESULTS

Section One:

Titration of Purified Toxins A and B Used in Translocation Experiments.

To assess the purity and potency of purified toxins A and B, CPE assays were performed on confluent HFF cell monolayers using serial 1:2 dilutions. The cytopathic titre for purified toxin B (0.21 mg/mL) was 5.24 X 10^7 cytopathic units/mL, whereas the cytopathic titre for toxin A (0.46 mg/mL) was 6.4 X 10^4 cytopathic units/mL. These results are in agreement with literature which state that toxin B is a far more potent cytotoxin than toxin A (125). However, in contrast to literature which reports that toxin B is a 1000 fold more potent than toxin A, our experiments show that toxin B is only ~800 times more potent than toxin A.

Section Two:

Establishment of a Polarized Cell Culture Model.

In order to assess the translocation dynamics of *Clostridium difficile* toxins A and B, it was first necessary to be able to establish a polarized cell culture model. To accomplish this task, we grew polarized monolayers of Caco-2 and T84 cells on clear Transwell inserts. Caco-2 and T84 cell lines were chosen because they were carcinoma cell lines derived from the colon. As *C. difficile* toxins A and B target intestinal epithelial cells, these cell lines were ideal for our study purposes. Both Caco-2 and T84 cells attached very well to the tissue culture treated polyester insert membrane, and within a weeks time became confluent (Fig. 4). The confluency and integrity of the monolayers was also assessed quantitatively by taking electrical resistance measurements (Fig 5).

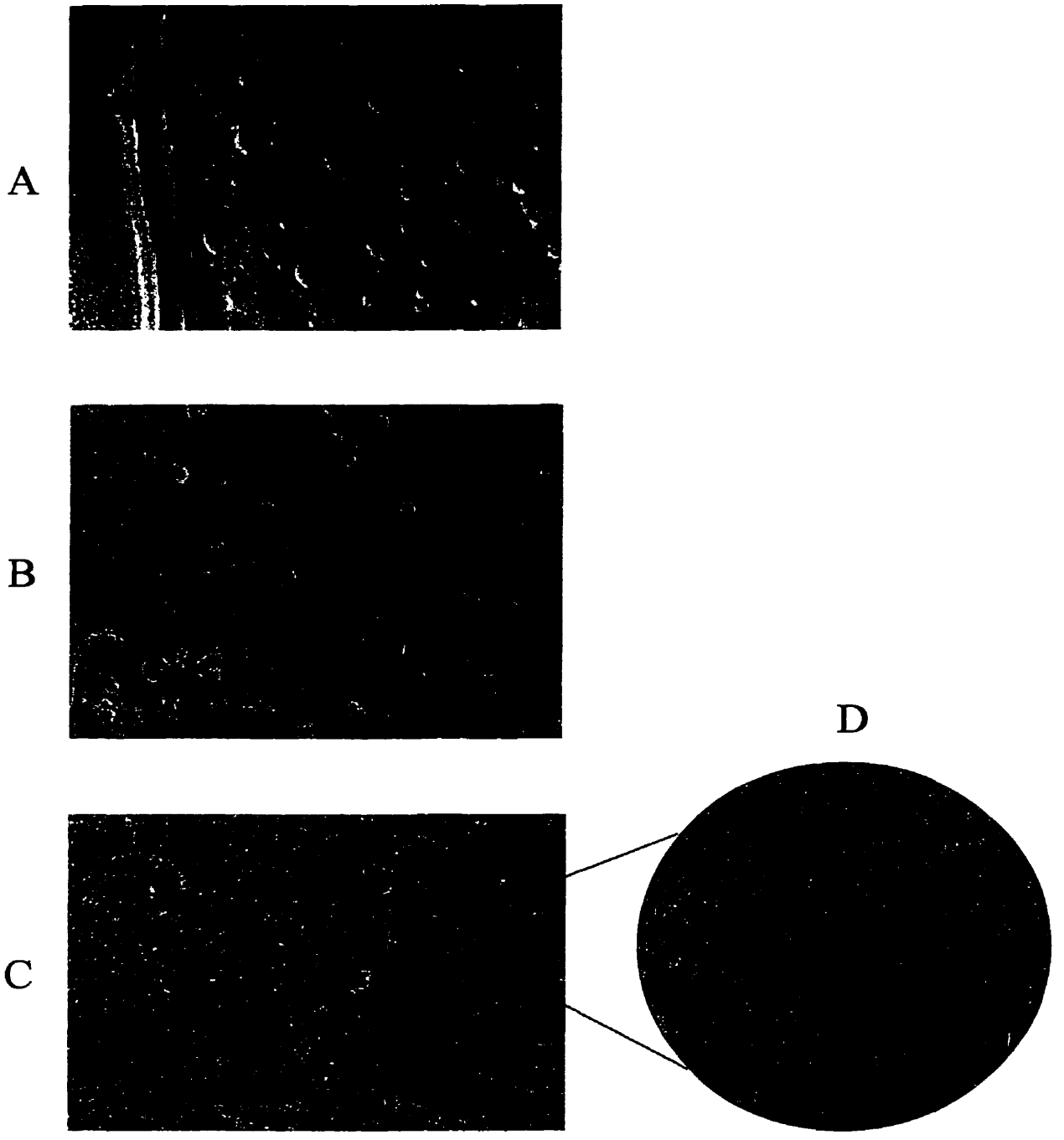


Figure 4. Phase Contrast Microscopy of Caco-2 Cells Growing on Transwell Insert.

Phase contrast microscope pictures of Caco-2 cells growing on Transwell inserts at A) Day 1, B) Day 3, and C) Day 6. At Day 1, cells are attached and begin to spread. By Day 6, cells are visibly confluent with tight junctions evident (D).

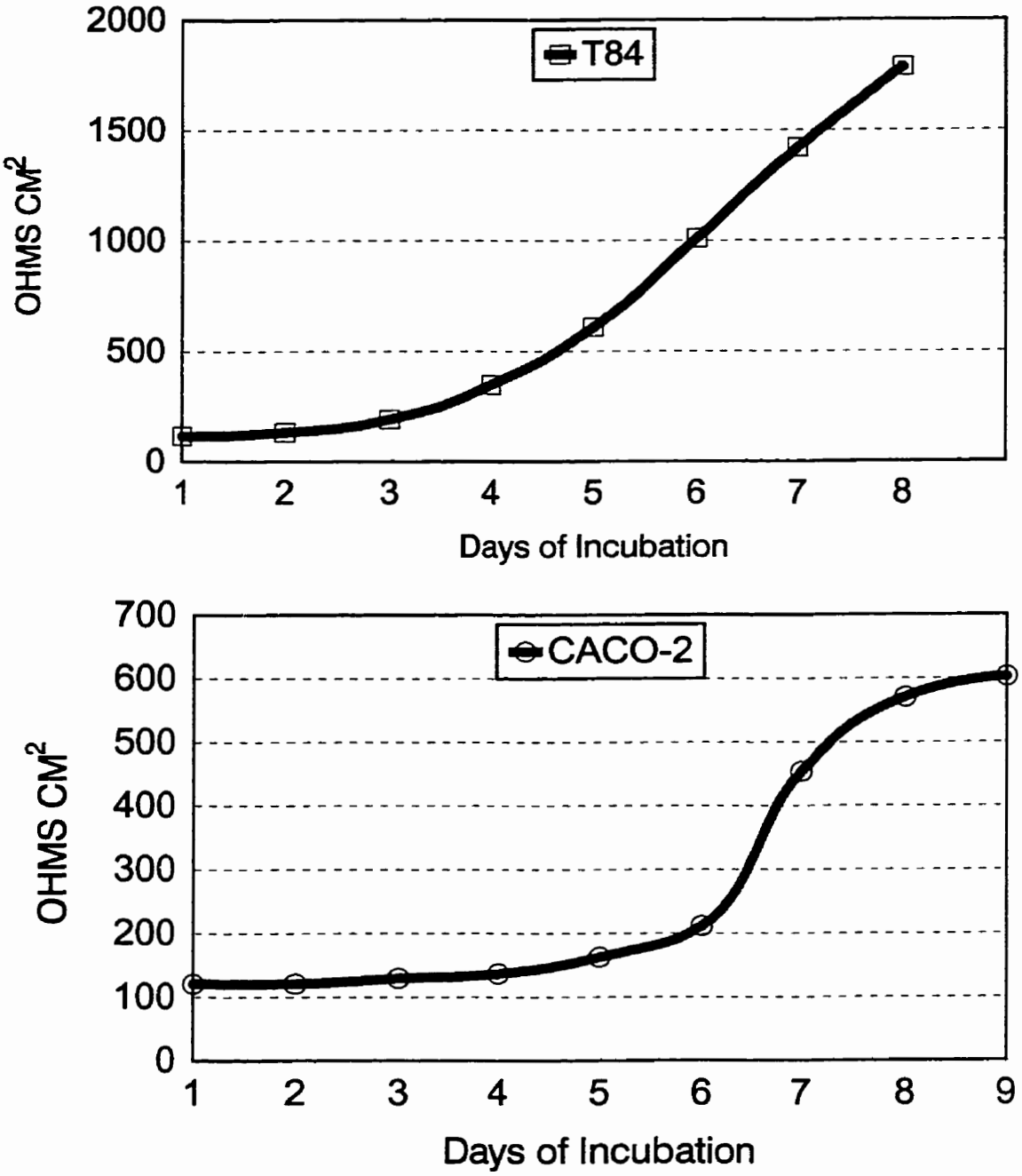


Figure 5. **Electrical Resistance Measurements of T84 and Caco-2 cells Growing on Transwell Inserts.**

Caco-2 and T84 cells were inoculated onto Transwell inserts at a concentration of 1.0 X 10^5 cells/mL. Dead and non-adherent cells were removed the next day and electrical resistance measurements were monitored daily using the Millicell ERS system. Electrical resistance plateaus for Caco-2 cells occurred after one week of incubation. No electrical resistance plateaus were seen with the T84 cell line. Standard errors for electrical resistance measurements were less than 5%, therefore error bars were not included.

High electrical resistance measurements signify greater monolayer confluence. The electrical resistance measurements for Caco-2 cells increased steadily each day, and plateaued after a weeks incubation. Unlike the Caco–2 cells, we could not observe an electrical resistance plateau for T84 cells. The precise reasons for this are unclear, but it appeared under phase contrast microscopy that the T84 monolayers may have grown on top of one another. For this reasons, it was decided that we would use polarized Caco-2 cell monolayers for translocation experiments.

Section Three:

Effect of *C. difficile* toxins A and B on Confluent Polarized Caco-2 Cell Monolayers.

Once the polarized Caco-2 cell model was established, studies were initiated to ascertain: a) whether the toxins could effect the integrity of the monolayer, and b) whether toxins A and/or B could translocate across the polarized monolayer. For these studies, three test conditions were utilized: i) Treatment with toxin A, ii) Treatment with toxin B, and iii) Treatment with both toxins A and B (Fig. 6). As no previous study had ever reported concentrations of toxins found within the bowels of patients with PMC, it was hard to ascertain an appropriate concentration of toxin to use. Consequently, trial concentration of toxins (0.125 μg/mL to 1.00 μg/mL) used in preliminary experiments were derived from previous studies which also assessed the effects of *C. difficile* toxins on electrical resistance in intestinal epithelial cells (70). The final concentration of toxins A and B chosen for translocation experiments was 0.250 μg/mL. To examine for translocation of *C. difficile* toxins, samples (50 μl) from the basal side of the insert were taken at various time intervals and inoculated onto HFF cell monolayers.

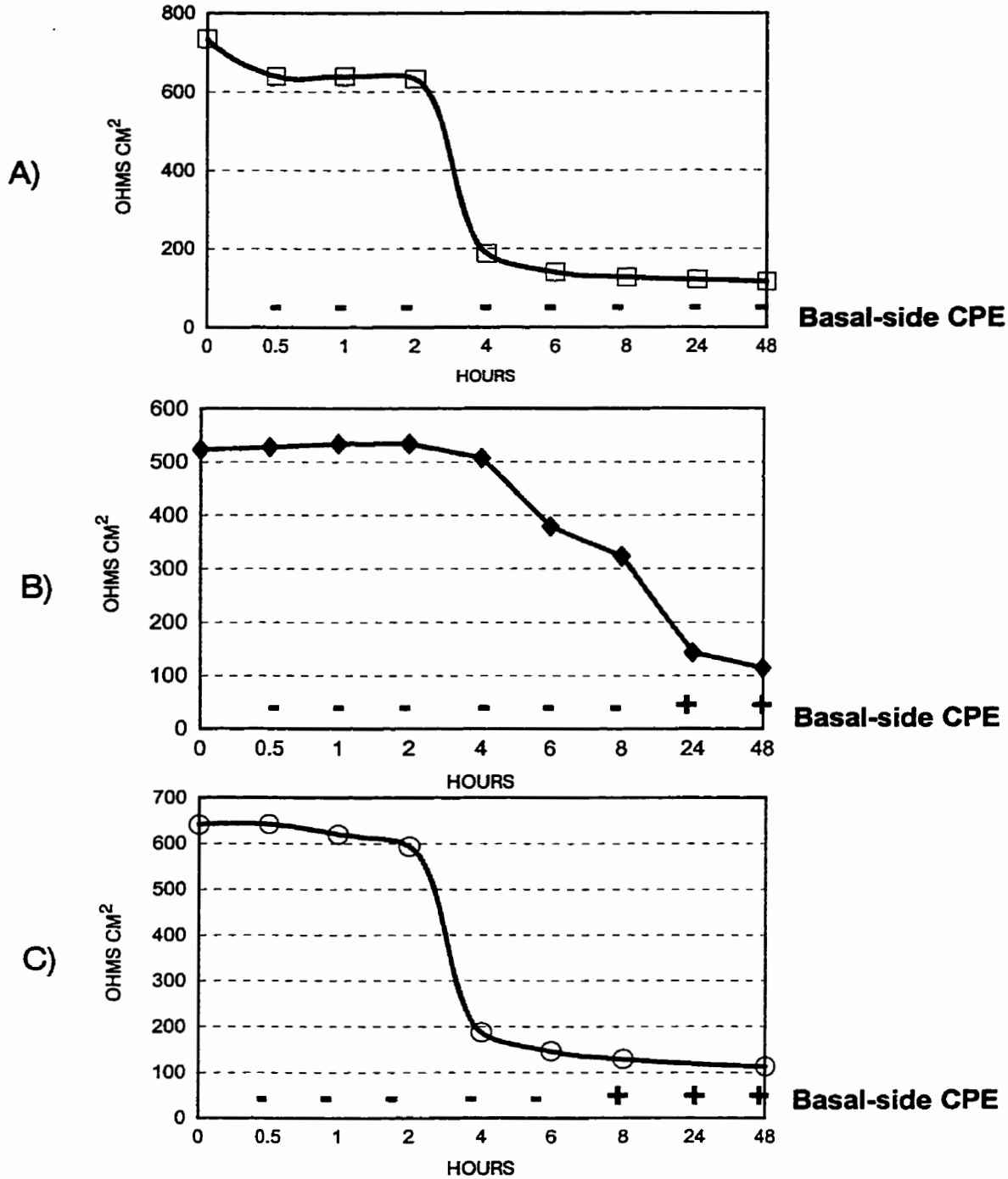


Figure 6. Effect of *Clostridium difficile* toxins on Electrical Resistance Measurements and Translocation of CPE in Caco-2 cells.

Transwell inserts were inoculated with purified toxin A (A), toxin B (B), or toxin A and toxin B together (C). Samples were taken from the basal side and tested for CPE using HFF cell monolayers as described in the Materials and Methods. Samples that were negative (-) or positive (+) for CPE have been shown at the bottom of the graph corresponding to the time of the sample collection. Standard errors for electrical resistance measurements were less than 5%, therefore error bars were not included.

Treatment of confluent Caco-2 cell monolayers with purified toxin A showed dramatic effects on transepithelial resistance measurements. As previously reported with T84 cells (70), toxin A was also very effective at reducing transepithelial resistance in Caco-2 cells. The time required for electrical resistance drops to decrease by 50% (RD_{50}) was only ~2.5 hours. This decrease in the transepithelial resistance is due to a compromise in the integrity of the Caco-2 cell monolayer. As is clearly illustrated in Fig. 7, cells treated with toxin A are rounded and also lose their ability to maintain tight junctions. Test samples obtained from the basal side of the insert failed to produce any CPE on HFF cells. From these results we were unable to conclude that toxin A was able to translocate.

Treatment of confluent Caco-2 cell monolayers with purified toxin B showed results similar to those of treatment with toxin A. However, in contrast to treatment with toxin A, the electrical resistance drops produced by toxin B were not as dramatic. The RD_{50} for treatment of Caco-2 cell monolayers with toxin B was ~8 hours. These results are in agreement with previously published data which state that toxin B is less effective at promoting electrical resistance changes (69). Interestingly, test samples obtained at 24 hours from the basal side of the insert showed that a cytotoxic factor was able to translocate across the monolayer. These results suggested that toxin B may have been able to translocate the intact monolayer.

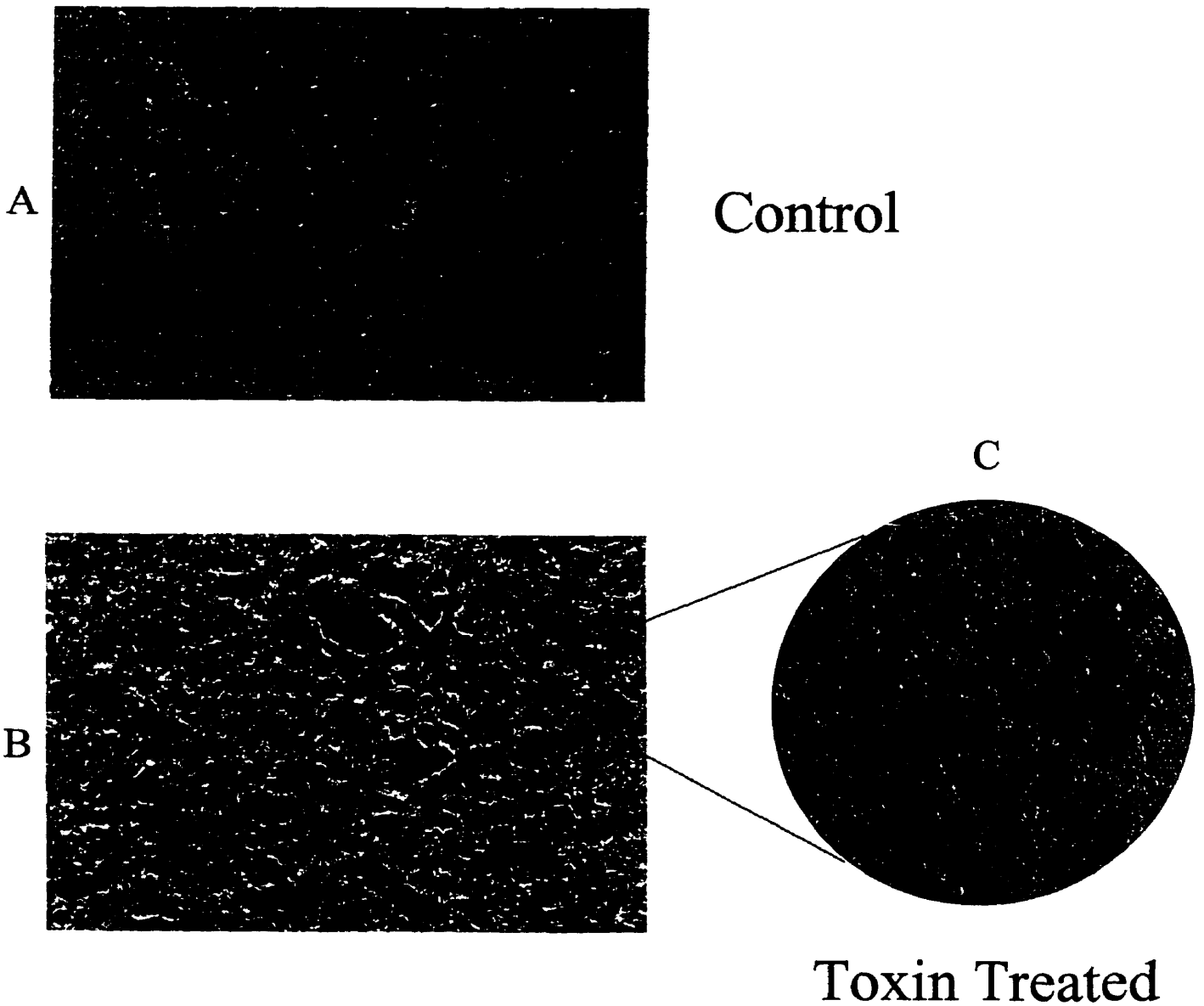


Figure 7. Phase Contrast Microscopy of Control and Toxin Treated Caco-2 Cells.

Phase contrast microscope pictures of A) control and B) toxin treated Caco-2 cells 48 hours post incubation. Control cells are confluent with distinct tight junctions visible. Toxin treated cells(C) are rounded and appear detached from neighbouring cells.

To assess the in vivo state where the intestinal epithelial cells were likely to be exposed to both toxins, treatment of confluent Caco-2 cell monolayers with purified toxins A and B were initiated. Results of this test condition showed data similar to those of treatment of Caco-2 cell monolayers with toxin A alone (See Fig. 6). The electrical resistance drops produced by toxins A and B were not any greater than those produced by toxin A alone. The RD_{50} for treatment of Caco-2 cell monolayers with both toxins A and B was ~2.5 hours. These result suggest that toxin A may play the major role in disrupting the epithelial tight junctions. Surprisingly, test samples obtained at 8 hours from the basal side of the insert showed that a cytotoxic factor was able to translocate across the monolayer. The combination of toxin A and toxin B together seemed to have expedited the time required for translocation of a toxic factor. These results suggest that a synergistic effect may have been seen with respect to promoting the translocation of a cytotoxic factor. However, as neither toxins were labeled, we could not definitively conclude which toxin(s) had translocated.

Section Four:

Assessment of Monoclonal and Polyclonal Antibodies Raised Against toxins A and B.

In the previous experiment we showed that treatment of intact Caco-2 cell monolayers with both toxins A and B allowed the passage of a cytotoxic factor. Since it was not possible to conclude which toxin(s) had translocated, it was necessary to perform further experiments to elucidate this problem. To accomplish this task, we assessed the abilities of monoclonal antibodies to toxin A, and polyclonal antibodies to toxin B to neutralize the cytotoxic and/or enterotoxic effects of the toxins.

If polyclonal antibodies to toxin B are specific only for toxin B, then we could treat the media obtained from the basal side of the insert with the polyclonal antibody to toxin B. If subsequent incubation revealed CPE on the HFF monolayers, then we could conclude that toxin A was able to translocate. Similarly, if monoclonal antibodies to toxin A are specific only for toxin A, then we could treat the media obtained from the basal side of the insert with the monoclonal antibody to toxin A. If subsequent incubation revealed CPE on the HFF monolayers, then we could also conclude that toxin B was able to translocate. Unfortunately, we found that the polyclonal antibody to toxin B also cross reacted with toxin A. Both toxins were neutralized by the polyclonal antibody to toxin B, however, toxin B was neutralized more efficiently as witnessed by titration assays (Data not shown). The cross reactivities of the polyclonal antibody to both toxins A and B may have been due to the structural similarities between the two toxins.

Interestingly, the monoclonal antibody to toxin A did not neutralize either toxins ability to induce CPE on HFF cells. The monoclonal antibody to toxin A was, however,

effective in reducing the effects of toxin A on transepithelial resistance. When toxin A was incubated with its monoclonal antibody, we found that the RD_{50} for toxin A had increased from ~2.5 hours to about ~7.0 hours (Data not shown). This RD_{50} was similar to treatment of Caco-2 cell monolayers with toxin B alone. The results of these experiments show that toxin A may have two separate enzymatic domains. One domain which is responsible for inducing CPE, and another domain which is responsible for inducing changes in cytoskeletal structure.

As monoclonal and polyclonal antibodies were unable to separate the effects of either toxins, it was decided that ^{125}I radiolabeling would be used to sort out the problem of toxin translocation dynamics (see Section 9).

Section Five:

Assessment of the Ability of toxins A and B to Facilitate the Translocation of Gram Negative Lipopolysaccharide.

As patients with severe cases of PMC may suffer from systemic shock, it was important to ascertain whether translocation of lipopolysaccharides could be facilitated by the addition of toxins A and B. If *C. difficile* toxins A and B are found to facilitate the translocation of Gram negative lipopolysaccharide, this information would be novel in understanding the model of *C. difficile* pathogenesis. To test our hypothesis, inserts were inoculated with varying concentrations of *E. coli* LPS in the presence of both toxins A and B (Fig. 8). Samples were obtained from the basal side of the insert at various times and analyzed for LPS content using a commercial LAL assay.

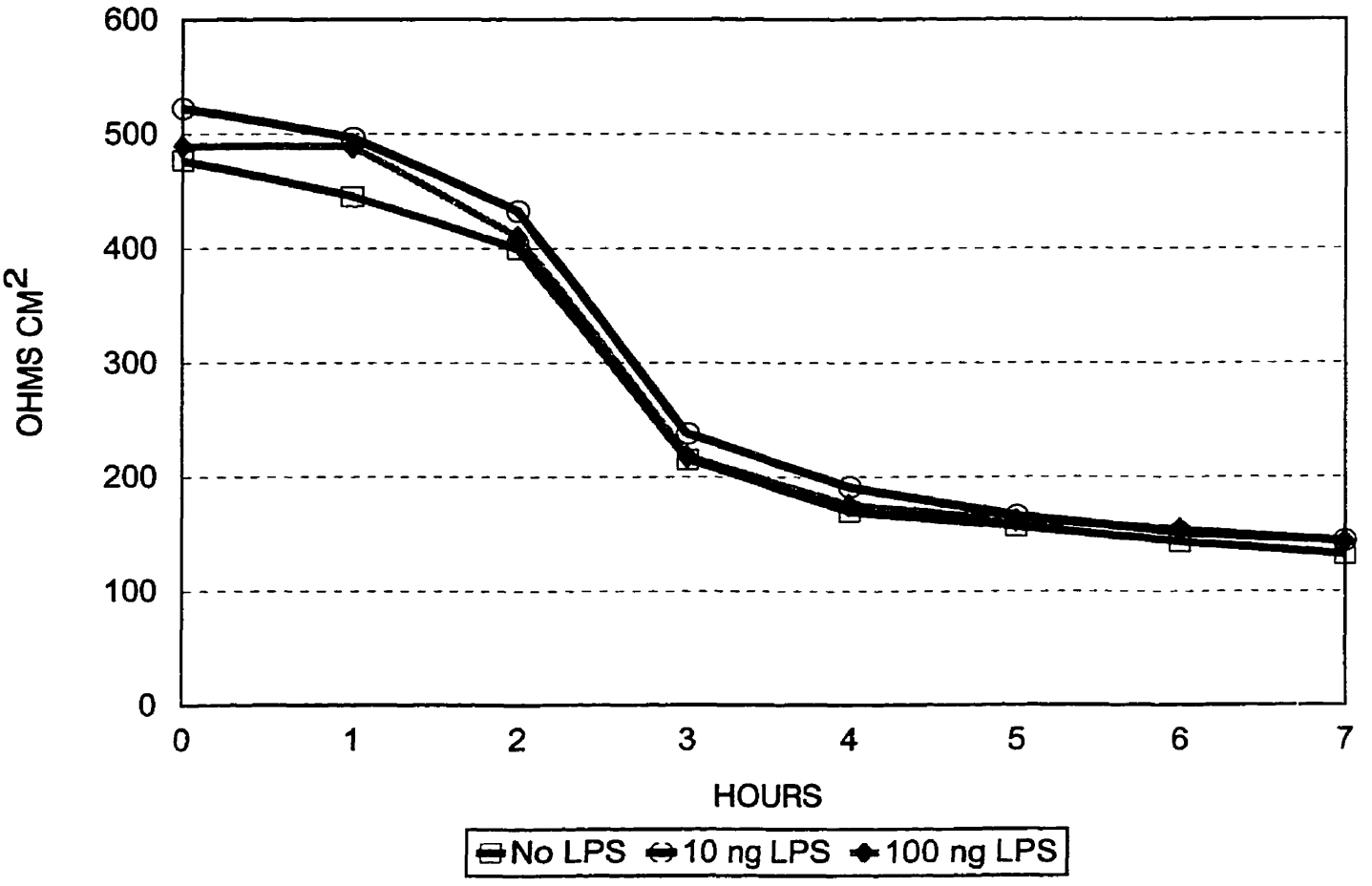


Figure 8. Electrical Resistance Measurements for Caco-2 cells treated with Toxins A and B and Varying Amounts of *E. coli* Lipopolysaccharide.

Transwell inserts were inoculated with either 0 ng, 10 ng, or 100 ng of *E. coli* LPS in the presence of 0.5 mL of toxins A and B (0.250μg/mL). Samples of media were obtained from the basal side of the insert and assayed for LPS content using a commercial LAL assay. The concentration of LPS did not seem to affect the electrical resistance measurements induced by toxins A and B. Standard errors for electrical resistance measurements were less than 5%, therefore error bars were not included. No LPS was detected on the basal side of the insert by the methods we employed.

The addition of *E. coli* LPS did not appear to affect the ability of toxins A and B to induce electrical resistance changes in Caco-2 cell monolayers. From this data, it does not appear that *E. coli* LPS is able to obstruct toxin enzymatic activity either by binding to toxin molecules, or to toxin receptors. Results of LAL assays performed on basal tissue culture media samples failed to reveal appreciable amounts of LPS, however, samples from the apical side of the insert revealed presence of large amounts of LPS. These results imply that no translocation of LPS was evident. However, positive control inserts, which had no Caco-2 cell monolayers growing, also failed to reveal significant amounts of LPS on the basal side. The possible reasons for these aberrant findings are addressed in the Discussion section.

Section Six:

Assessment of Cytotoxic Activity in Patient Sera.

As preliminary data indicated that toxins were able to translocate across intact Caco-2 cell monolayers, we wanted to ascertain whether there could be circulating toxin molecules in sera of patients with CDAD. The presence of toxin molecules in the systemic circulation may explain why some patients suffer from systemic failure. From a study which looked at prolonged excretion of *C. difficile* in hospitalized patients (1), we were able to obtain serum samples from 74 cytotoxin positive patients and 72 cytotoxin negative control patients. Analysis of the serum samples failed to reveal any cytotoxic activity associated with both test and control groups. As all serum samples had been previously frozen, we could not conclude that toxins may have been inactivated by freezing (see Section Seven). Furthermore, it is also possible that factors (such as antibodies or proteases) present in the serum may have inactivated the toxins. To rule out the possible inactivation of toxin by freezing and thawing, fresh serum samples were also obtained from three patients suffering from PMC. Analysis of their serum samples also failed to reveal any cytotoxic activity associated the serum. Due to our limited sample size, we could not definitively conclude that patients with CDAD did not have circulating cytotoxin in their serum. It is possible that the cytotoxin may have been present, but in such minute amounts, that it was not detectable by the methods we employed.

Section Seven:

Inactivation of toxin B by Freezing and Thawing.

As we could not detect cytotoxic activity in sera of patients with CDAD, we wanted to determine if absence of cytotoxic activity was due to inactivation of toxin from storage. To mimic the presence of toxin in sera, normal control sera was inoculated with either 5 or 100 cytopathic units (CPU) of toxin B. This toxin/sera mixture was then repeatedly frozen and thawed and tested against the same mixture kept at 4°C. The effect of freezing and thawing on the 5 CPU of toxin B was assessed daily for one week. Results from HFF cell titrations showed that there was a two-fold decrease in the cytopathic titre for the frozen toxin/sera mixture when compared to the non-frozen toxin/sera mixture. The effect of freezing and thawing on the 100 CPU of toxin B was assessed daily for one month. Results from HFF cell titrations showed that there was also a two-fold decrease in the cytopathic titre for the frozen toxin/sera. These results indicate that the absence of cytotoxic activity in sera of patients with CDAD may have been influenced by inactivation of toxin due to freezing and thawing.

Section Eight:

Assessment of toxin B Antibodies in Patient Sera.

As *C. difficile* toxins act to disrupt the epithelial tight junctions, it may be possible for toxins to gain access to deeper tissues to induce a humoral immune response. To assess for the prevalence of the toxin B serum neutralizing antibodies, sera from 74 cytotoxin positive (CP), and 72 cytotoxin negative (CN) patients were tested using an ELISA and toxin B neutralization assay.

Of the 74 CP patients examined, eleven (14.9%) were found to contain antibodies capable of neutralizing the cytopathic effects of purified toxin B (Table 1). Ten of 72 (13.9%) CN control patients were also found to have neutralizing ability (Table 1). In the ELISA assay, seven of 74 (9.5%) CP patients and three of 72 (4.2%) CN patients were positive for antibodies to toxin B (Table 1). Of the eleven CP patients capable of neutralizing toxin B, seven were also positive for toxin B antibodies using ELISA. However, only three of ten CN controls capable of neutralizing toxin B were found to be positive for toxin B antibodies using ELISA. In view of these findings, it appears that although CN patients may have neutralizing antibodies to toxin B, these antibodies may not be specific for toxin B. Nine patients in the CP group experienced relapses in diarrhea. It is of interest to note that none of these patients had neutralizing antibodies to toxin B, or were ELISA positive for antibodies to toxin B.

As previous studies have indicated a correlation between human antibody response and clinical course of infection (152, 176), we wanted to determine if patients with CDAD would develop a humoral immune response to toxin B. To accomplish this task, serum samples from CP patients were analyzed for toxin B antibodies at week one, and week three (or week four) (Table 2). Results from our studies did not indicate a significant development in humoral immune response to toxin B in the study population we examined. Of the 74 CP patients examined, only two patients developed toxin B serum neutralizing antibodies over the course of their illness. Similarly, four CP patients were shown to have developed toxin B antibodies using the ELISA assay.

	C. difficile Patients	Diarrhea Controls
ELISA Positive for Antibodies to Toxin B	**7/74 (9.5%)**	**3/72 (4.2%)**
Serum Neutralization of Toxin B	**11/74 (14.9%)**	**10/71 (13.9%)**

Table 1: Assessment of Humoral Antibodies to toxin B in Cytotoxin Positive and Cytotoxin Negative Patients

Humoral antibodies to toxin B were assessed in 74 cytotoxin positive and 72 cytotoxin negative control patients using ELISA, and a cytotoxin neutralization assay (AS described in Materials and Methods. Cytotoxin positive patients did not seem to have a significantly greater prevalence of toxin B antibodies than control patients. Neutralization of toxin B in CN patients may have been non-specific as only three patients were positive for toxin B antibodies using ELISA.

	1st SERUM SAMPLE	2nd SERUM SAMPLE
ELISA Positive for Antibodies to Toxin B	**7/74 (9.5%)**	**11/74 (14.9%)**
Serum Neutralization of Toxin B	**11/74 (14.9%)**	**13/74 (17.6%)**

Table 2: Assessment of Humoral Immune Response to toxin B in Cytotoxin Positive Patients.

To determine if cytotoxin positive patients develop humoral immune responses to toxin B over the course of their infection, serum samples were analyzed for antibodies to toxin B at week one and week three (or week four). No significant development in antibodies to toxin B could be observed in the population we examined.

Section Nine:

Radiolabeling of *C. difficile* toxins to Elucidate Translocation Dynamics.

Preliminary results from our experiments which treated Caco-2 monolayers with *C. difficile* toxins indicated that toxin A was much more efficient at eliciting tight-junction changes compared to the same concentration of toxin B (Fig. 6). Samples of tissue culture media from the basal side of these Transwell inserts were inoculated onto confluent HFF cells to examine for translocation of a toxic component using a conventional HFF cell CPE assay. Translocation of a CPE-causing component was not detected when only toxin A was tested, but was detected when toxin B alone was tested. The time required for translocation of a CPE-causing component was shortened when both toxin A and toxin B were combined (Fig. 6). Although toxin B is 1000-fold more effective as a cytotoxin than toxin A, it was not possible to conclude that only toxin B had enhanced translocation when both toxins were inoculated together onto the apical side of the Transwell insert (Fig. 6). Attempts at utilizing monoclonal and polyclonal antibodies targeted against toxin A and toxin B were unsuccessful in identifying which toxin(s) had translocated.

To investigate further, we employed a more sensitive method involving ^{125}I radiolabeling of toxin A and toxin B. Radiolabeled BSA served as the control protein in translocation experiments. The monolayer confluence and tight junction integrity of Caco-2 cells grown on Transwell inserts was tested by inoculating inserts with ^{125}I-BSA. Confluent epithelial cell monolayers have defined tight junctions and regulated cellular trafficking. Under such conditions, cells will not allow unregulated passage of large molecular weight proteins. Treatment of inserts with ^{125}I-BSA resulted in no change in transepithelial

resistance at 10 or 24 hours (data not shown). As Fig. 9 shows, there was also no significant change in the distribution of ^{125}I-BSA on the transwell insert over a 24 hour period. These results suggest that monolayer integrity was maintained, and that over the 24 hour test period, proteins such as BSA (66,430 Da) are unable to translocate efficiently. Fig. 10 shows treatment of inserts with unlabeled toxins A and B in conjunction with ^{125}I-BSA. In contrast to treatment with ^{125}I-BSA alone, there was a significant drop in the transepithelial resistance measurements of the Caco-2 cells when toxin A and toxin B were included (Fig. 10). The time required for a drop in transepithelial resistance to 50% of the original value (RD_{50}) was ~3.0 hours. Analysis of the distribution of ^{125}I-BSA in the insert model shows a gradual increase in the amount of radiolabeled BSA appearing on the basal side over the 10 hr test period. The results indicate a compromise in the integrity of the Caco-2 cell monolayer elicited by toxin A and B, followed by non-specific passage of labeled BSA from the apical to the basal side of the insert.

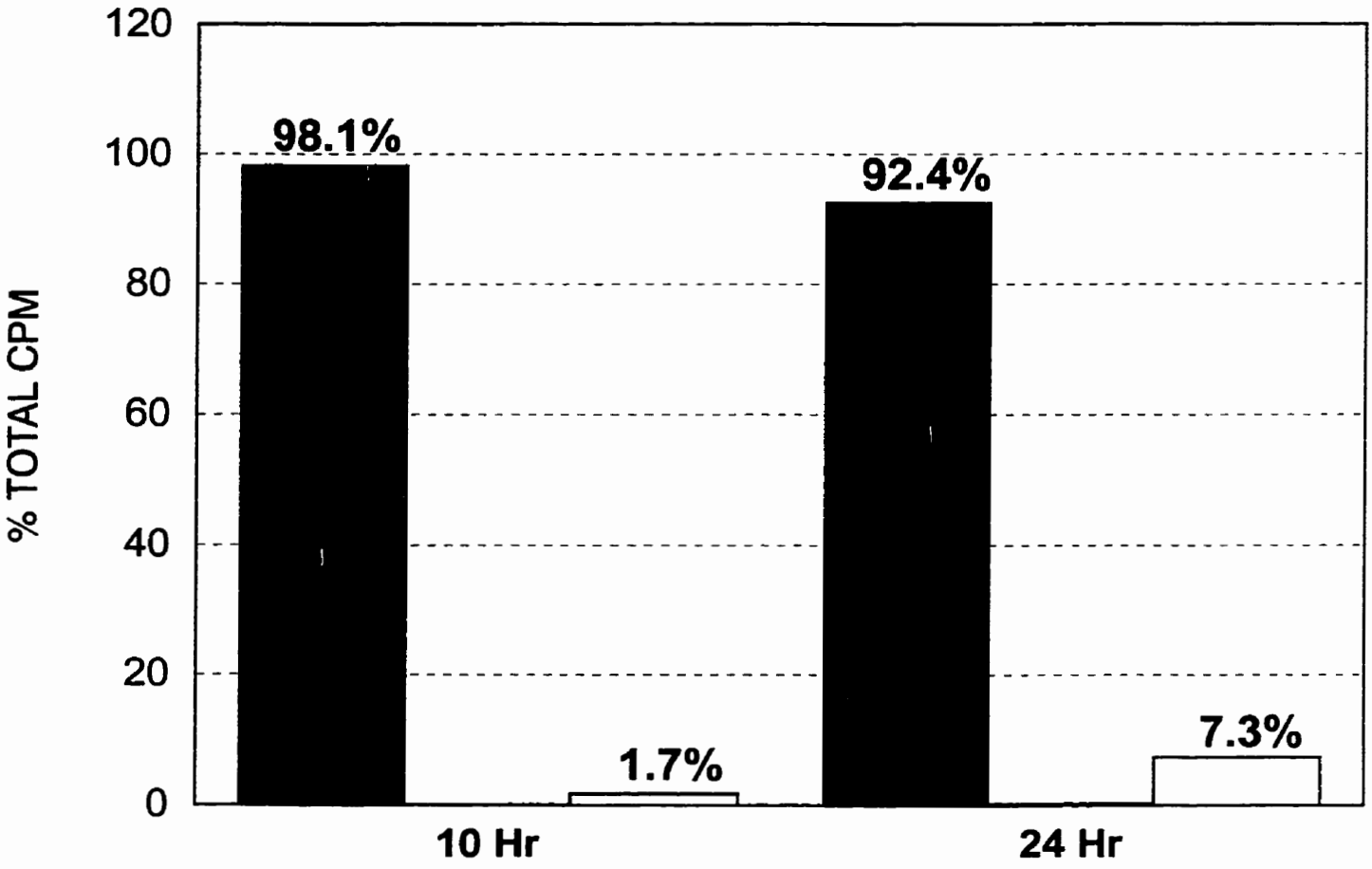


Figure 9. Non-specific Translocation of ^{125}I-BSA Across Caco-2 Cells in Transwell Insert.

Transwell inserts were inoculated with 500 µl of ^{125}I-BSA (0.250 µg /ml, 2.74 x 10^4 cpm/ml). Samples from apical (■), basal (□), and insert (▨) were tested at 10 and 24 hours post-inoculation to determine the percent of the total input cpm. The electrical resistance measurements at 10 and 24 hrs confirmed that tight junction integrity was maintained (data not shown). The total amount of radioactivity inoculated onto the insert in the form of radiolabeled BSA was 1.37 x 10^4 cpm.

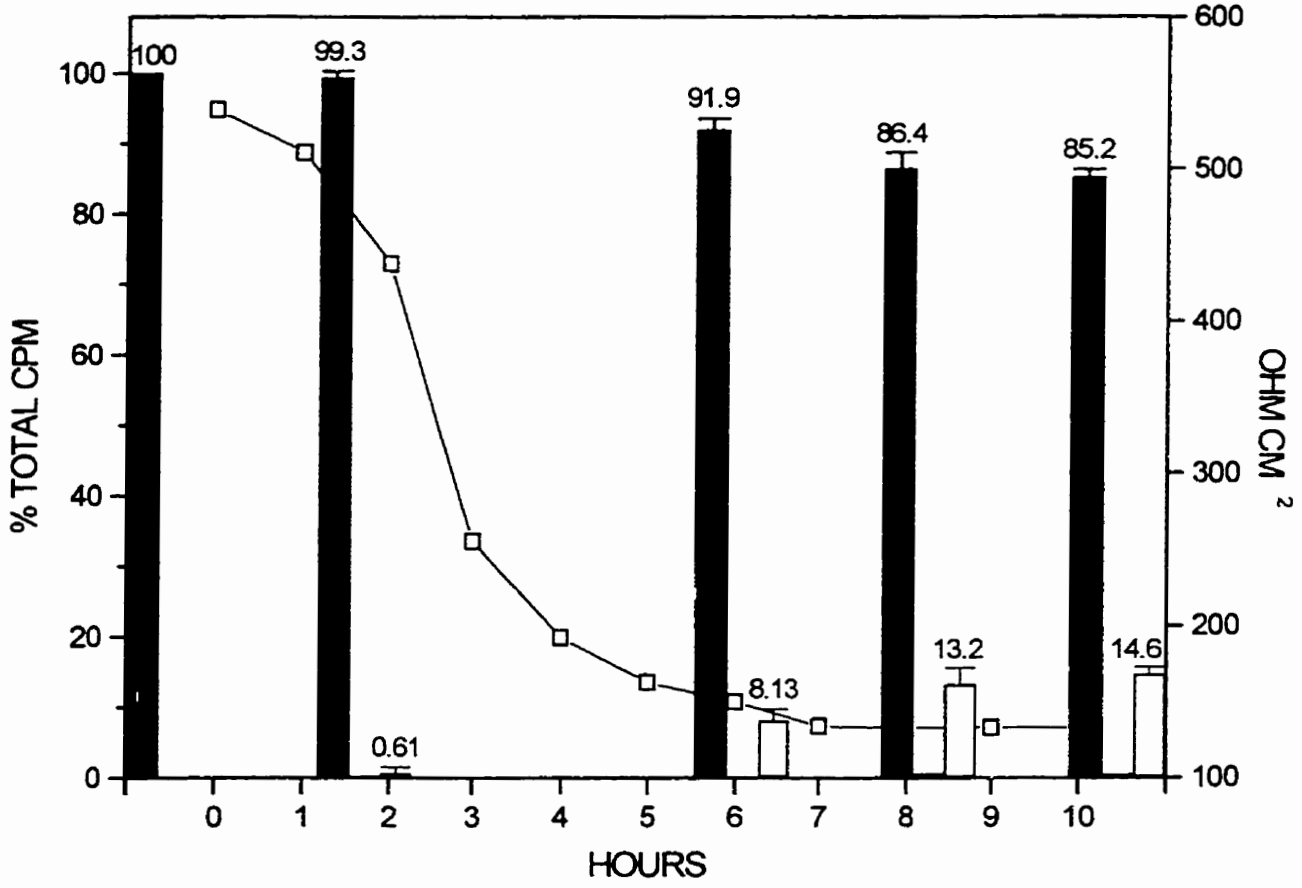


Figure 10. Translocation of ^{125}I-BSA in the Presence of Toxin A and Toxin B.

Confluent Caco-2 cell monolayers on Transwell inserts were inoculated with 500 µl of a mixture of both toxins A and Toxin B (both toxins at 0.250 µg /ml) and 100 µl ^{125}I-BSA (0.250 µg /ml). Samples from apical (■), basal (□), and insert (▨) were tested for percent of the total input cpm. The figure represents the average of 3 separate experiments. An electrical resistance curve is included to indicate the level of tight junction integrity. The total amount of radioactivity inoculated onto the insert in the form of radiolabeled BSA was 2.8 x 10^3 cpm.

In the next series of experiments, inserts were also treated with ^{125}I-toxin A (Fig. 11) and examined for translocation and/or binding of the radiolabeled toxin A to epithelial cells. Similar to unlabeled preparations, ^{125}I-toxin A alone was sufficient to cause an Rd_{50} after ~3 hours. Analysis of the distribution of ^{125}I-toxin A showed a marked increase in the amount of labeled toxin A appearing on the basal side of the insert at each time point tested when compared to treatment with ^{125}I-BSA alone. The amount of ^{125} I-toxin A appearing on the basal side of the insert was approximately two-fold greater than that of ^{125}I-BSA at each time point tested. The results suggest that the translocation of toxin A may occur by a specific mechanism, and that the process may be directly regulated by eukaryotic cells. There are at least two plausible mechanisms by which toxin A translocation across an intact Caco-2 monolayer may occur: a) toxin A is internalized, transported through the cell, and then expelled from the basal side of the monolayer, or b) toxin A is able to pass through tight junction openings of Caco-2 cells created by *C. difficile* toxins. Binding of toxin A to receptors on Caco-2 cell monolayers grown on Transwell inserts was also assessed by cutting the insert out and performing counts. Results indicated that the amount of toxin A bound to Caco-2 cell monolayers was at background levels. The finding that no appreciable amounts of radiolabeled toxin A was found to be associated with Caco-2 cells was unexpected as previously published data indicated that toxin A was internalized by eukaryotic cells.

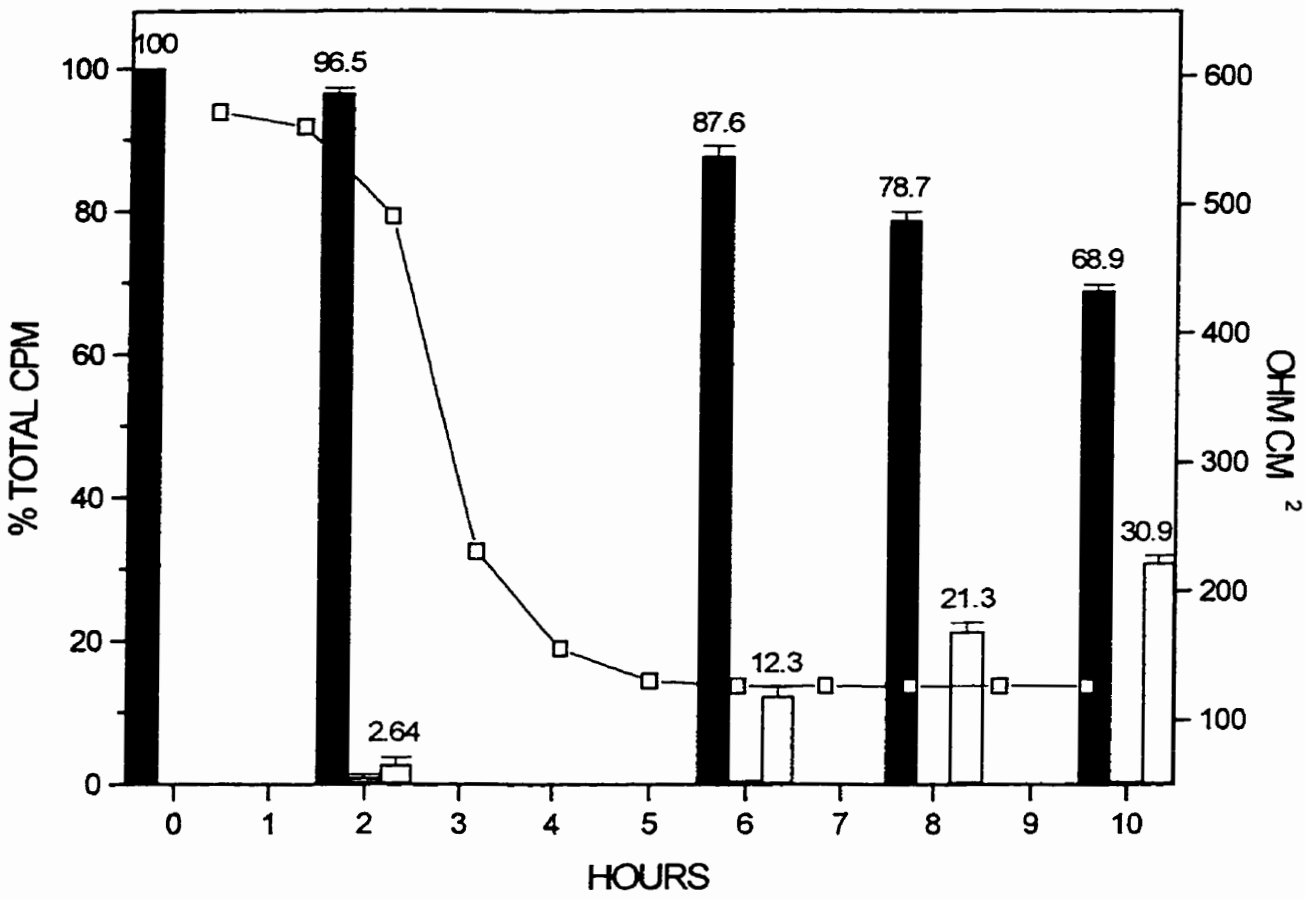


Figure 11. Translocation of ^{125}I-Toxin A.

Confluent Caco-2 monolayers on Transwell Inserts were inoculated with 500 µl of ^{125}I-toxin A (0.250 µg /ml). Samples from apical (■), basal (□), and insert (▨) were tested for percent of the total input cpm. Each data point represents the average of 3 separate experiments. An electrical resistance curve is included to indicate the level of tight junction integrity. The total amount of radioactivity inoculated onto the insert in the form of ^{125}I-Toxin A was 9.2 x 10^3 CPM.

To determine if the addition of toxin B would have a synergistic effect on the translocation of toxin A, inserts were treated with unlabeled toxin B and ^{125}I-toxin A (Fig. 12). The addition of toxin B did not appear to increase the rate at which transepithelial resistance drops occurred. These findings suggest that toxin A plays the main role in the disruption of epithelial tight junctions, and that the limiting factor for tight junction modulation may be the amount of toxin A present. The distribution of radiolabeled toxin A in this test condition also paralleled that of treatment of inserts with toxin A alone. The addition of toxin B did not seem to influence or augment the amount of toxin A capable of translocating across an intact Caco-2 monolayer (Fig 12).

Toxin B is a potent cytotoxin capable of causing rearrangements in cytoskeletal structure. To examine if toxin B is able to modulate tight junctions and translocate across an intact Caco-2 cell monolayer, inserts were inoculated with ^{125}I-toxin B (Fig.13). In contrast to treatment with toxin A alone, the RD_{50} for toxin B was 7 hours. This corresponded to a two-fold increase in the time required to elicit the same tight junction effects when compared to toxin A. When analyzing the distributions of ^{125}I-toxin B and ^{125}I-toxin A in experiments which tested the toxins individually, it was found that toxin B translocation was about 5% lower at each time frame tested. This may be attributable to the decreased ability of toxin B to affect tight junctions thereby limiting the amount of toxin B capable of translocating through holes created in the tight junctions.

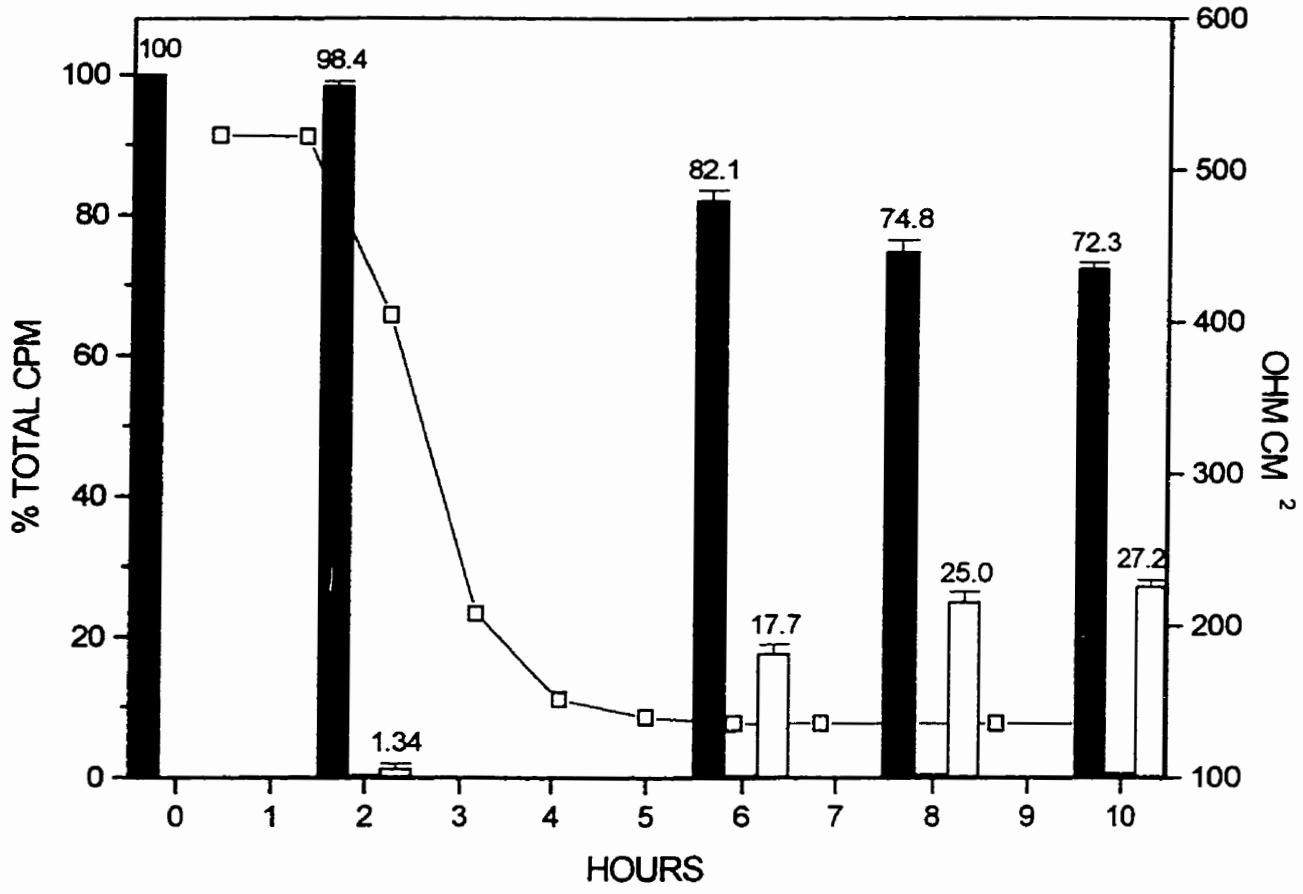


Figure 12. Radioactive Count Distribution for Inserts Treated With ^{125}I Labeled toxin A and Unlabeled toxin B.

Inserts were inoculated with 250 µl of ^{125}I labeled toxin A (0.250 µg /ml) and 250 µl of unlabeled toxin B (0.250 µg /ml). Samples from apical (■), basal (□), and insert (▨) were tested for percent of the total input cpm. The figure represents the average of 3 separate experiments. An electrical resistance curve is included to indicate the level of tight junction integrity. The total amount of radioactivity inoculated onto the insert in the form of radiolabeled toxin A was 4.6×10^3 CPM.

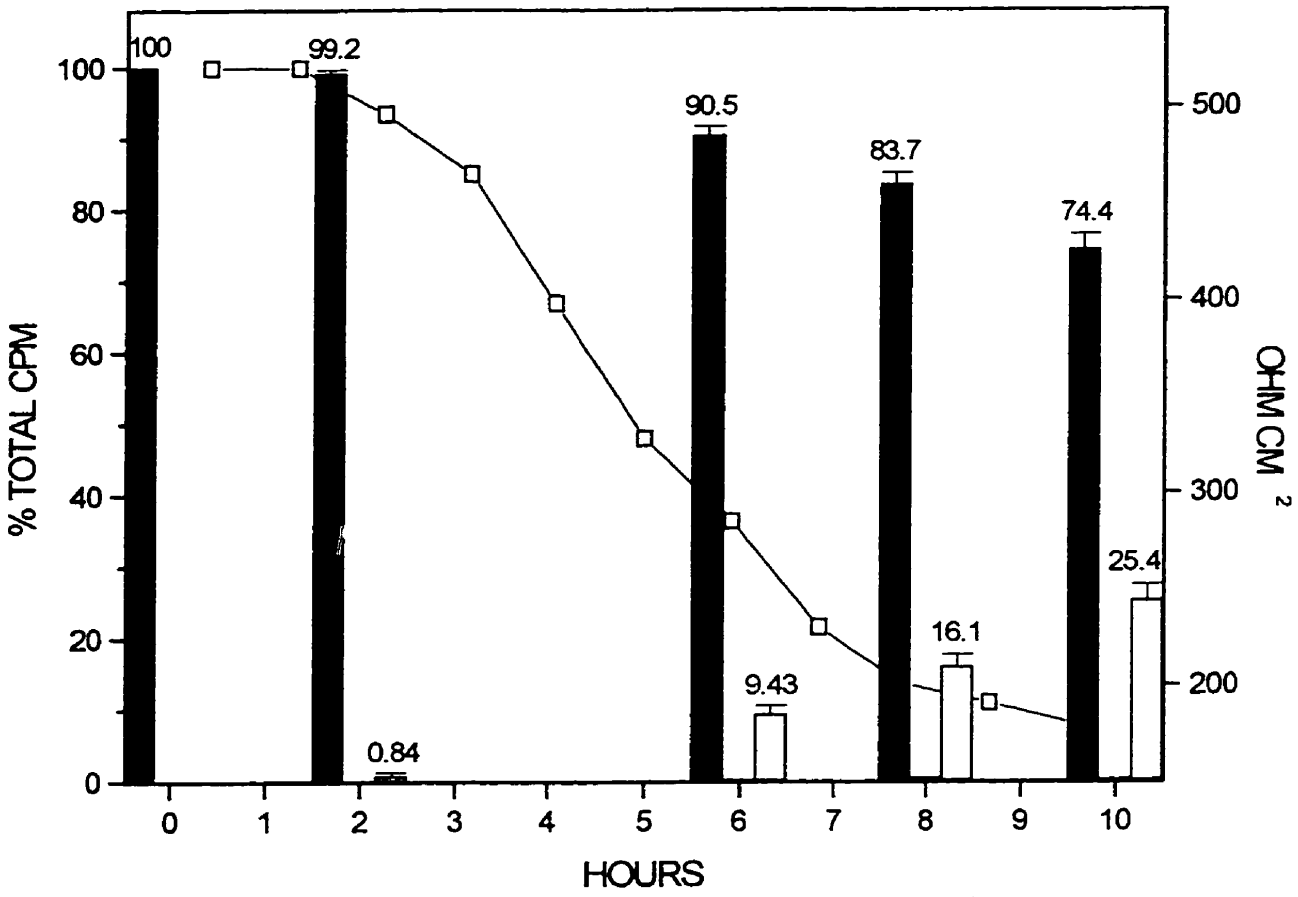


Figure 13. Radioactive Count Distribution for Inserts Treated With ^{125}I Labeled toxin B.

Inserts were inoculated with 500 µl of ^{125}I labeled toxin B (0.250 µg /ml). Samples from apical (■), basal (□), and insert (▨) were tested for percent of the total input cpm. The figure represents the average of 3 separate experiments. An electrical resistance curve is included to indicate the level of tight junction integrity. The total amount of radioactivity inoculated onto the insert in the form of radiolabeled toxin B was 7.0×10^3 CPM.

To determine if the addition of toxin A would enhance the translocation of toxin B, inserts were treated with unlabeled toxin A and ^{125}I-toxin B (Fig. 14). The addition of toxin A had a dramatic impact on the ability of ^{125}I-toxin B to translocate across intact Caco-2 cell monolayers. The amount of toxin B capable of translocating the monolayer was increased by approximately two-fold in each of the time intervals tested. The addition of toxin A also reduced the RD_{50} of the monolayer to 3 hours.

Based on our data which indicates that toxin B is preferentially translocated, we have proposed a model of pathogenesis (Fig. 15) which illustrates the synergistic roles of both *Clostridium difficile* toxins. In contrast to Sears and Kaper's model for toxin A translocation, we suggest that toxin B is the predominant toxin breaching the submucosal barrier to elicit the inflammatory responses.

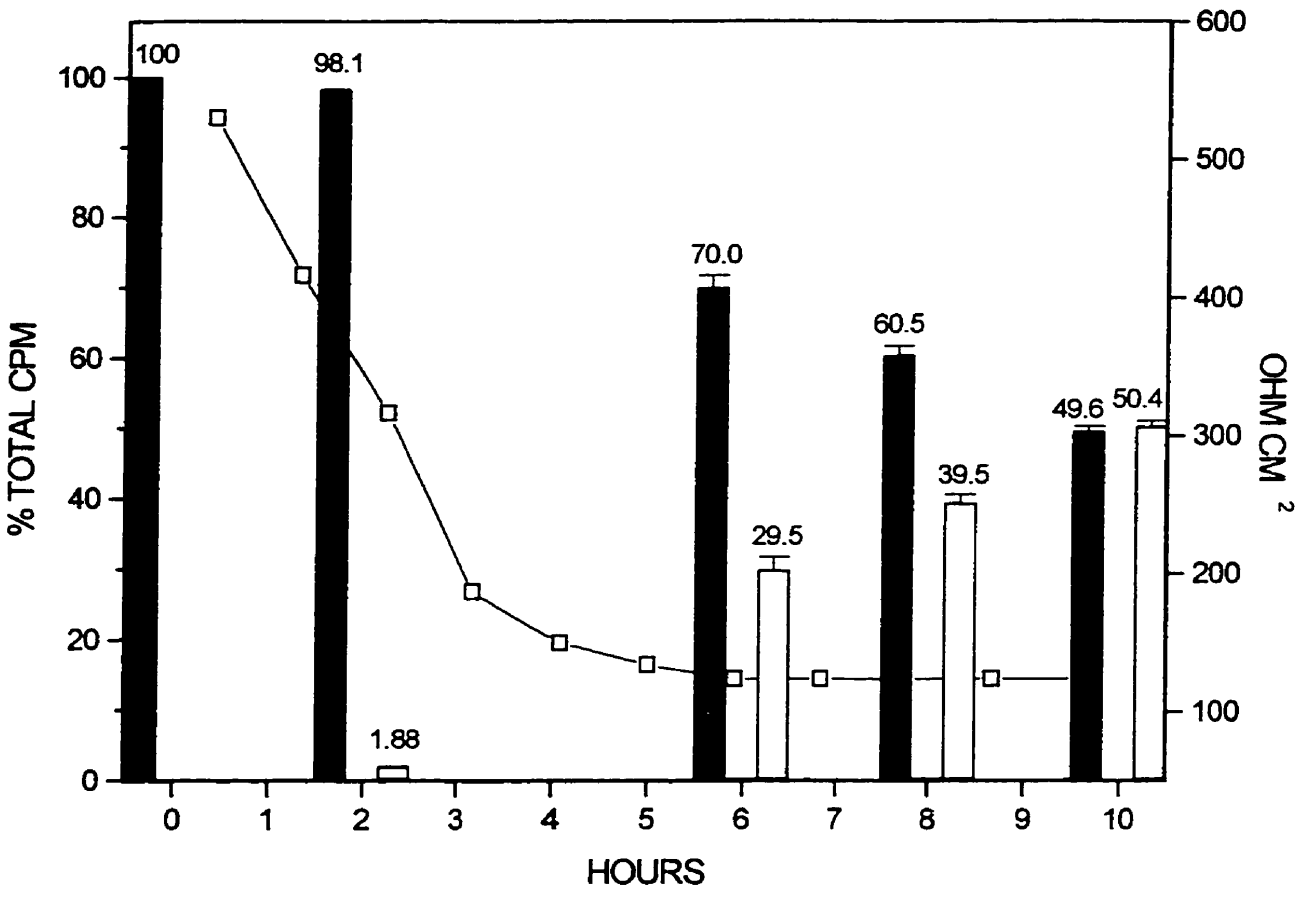


Figure 14. Radioactive Count Distribution for Inserts Treated With ^{125}I Labeled toxin B and Unlabeled toxin A.

Inserts were inoculated with 250 µl of ^{125}I labeled toxin B (0.250 µg /ml) and 250 µl of unlabeled toxin A. Samples from apical (■), basal (□), and insert (□) were tested for percent of the total input cpm. The figure represents the average of 3 separate experiments. An electrical resistance curve is included to indicate the level of tight junction integrity. The total amount of radioactivity inoculated onto the insert in the form of radiolabeled toxin b was 7.0×10^3 CPM.

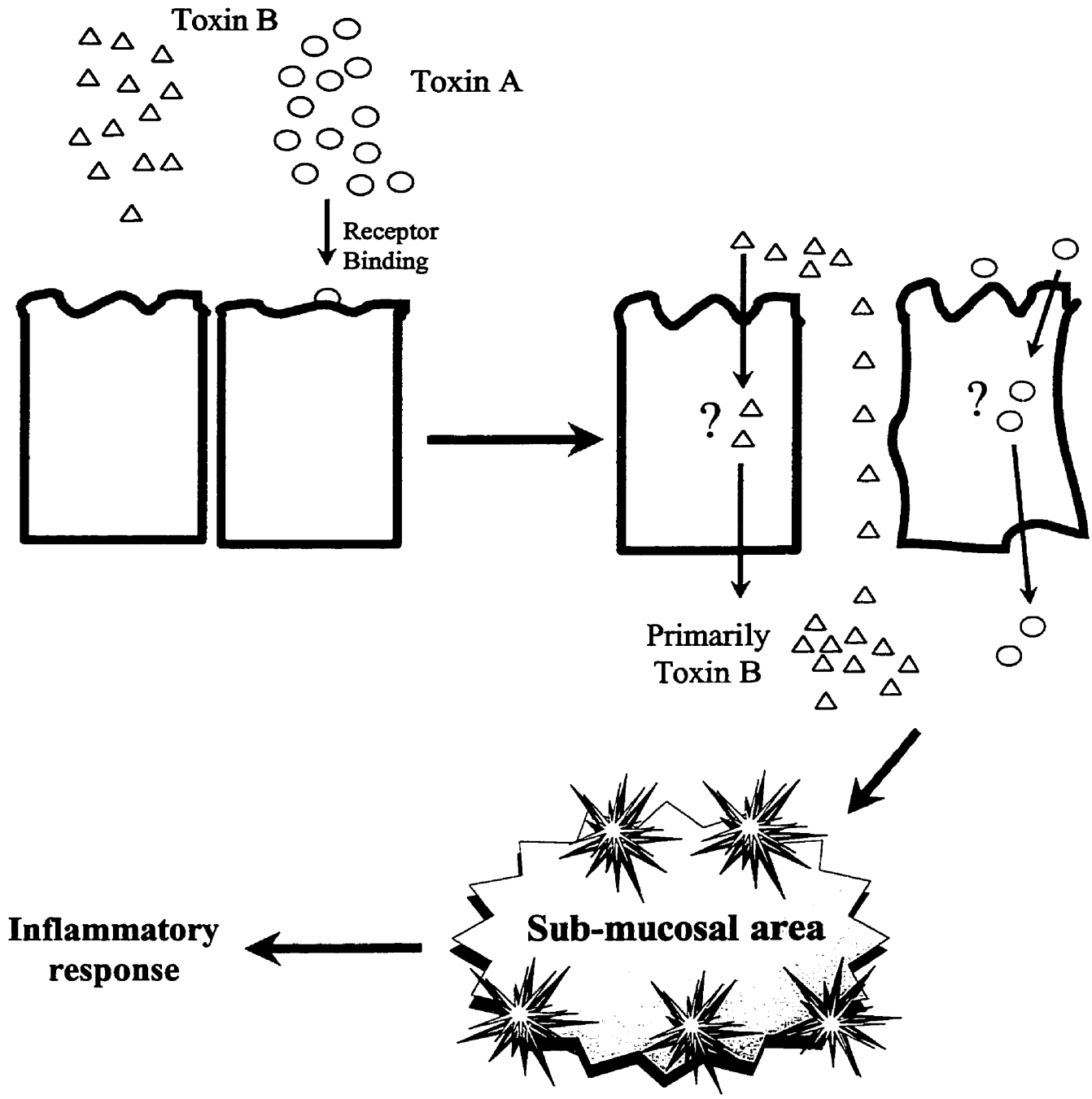


Figure 15. Proposed Model For Enhanced Translocation of *Clostridium difficile* toxin B Mediated by toxin A.

In this model, Toxin A binds to epithelial cells and disrupts epithelial tight junctions. The disruption in cell monolayer integrity allows both toxins to gain access to submucosal tissue to elicit an inflammatory response. However, toxin B has enhanced ability to translocate compared to toxin A.

Section Ten:

Inhibition of toxin A Using Synsorb CD and Cholestyramine.

Infections caused by *C. difficile* are difficult to manage because the organism can persists for extended periods of time in the environment to pose significant risks for re-infection. Current treatment regimens consist primarily of antibiotic usage. Although these treatments are efficacious in eradicating most infections, they are ineffective for a minority of complicated cases. To address the limitations of antibiotic usage, several approaches have been taken. One approach has been to devise inhibitors which can block the biological activities of *C. difficile* toxins. Synsorb CD and Cholestyramine are two such inhibitors which have been proposed for treatment of *C. difficile* infections.

To assess the efficacy of these two inhibitors, dilutions of toxin A (0.250 μg /mL) were made in either PBS or RPMI supplemented with 20% FBS. These toxin mixtures were then incubated with either Synsorb CD or Cholestyramine for 1 hours at 35°C and then inoculated onto Caco-2 cell monolayers. Untreated toxin A in PBS or RPMI supplemented with 20% FBS served as positive controls, while PBS and RPMI supplemented with 20% FBS served as negative controls. Electrical resistance changes were used to assess the ability of the inhibitors to bind to toxin A and negate its effects on Caco-2 cell monlayers. HFF cell titrations were also used to assess whether the inhibitors were effective in reducing the titre of CPE on HFF cells.

Treatment of Caco-2 cell monolayers with either PBS or RPMI resulted in no significant changes in electrical resistance measurements over a nine hour period (Fig. 16). Treatment of Caco-2 cell monolayers with toxin A in PBS also resulted in no changes in electrical resistance measurements (Fig.16). The results of this experiment were unexpected as it implied inactivation of toxin A by PBS. The reasons for this apparent inactivation of toxin A is unclear, but results have been reproduced. Similar inactivation of toxin A is recorded when toxin A is diluted in RPMI without serum. Toxin B incubated with PBS also shows reductions in titre of CPE on HFF cells. The reasons for toxin inactivation are currently under investigation. Treatment of Caco-2 cell monolayers with toxin A in RPMI supplemented with 20% FBS produced expected transepithelial resistance drops (Fig. 16). The RD_{50} of this test case was 3 hours.

Results of inhibition studies showed that neither Synsorb CD or Cholestyramine were effective in preventing electrical resistance drops in Caco-2 cell monolayers (Fig. 17). HFF cell titrations also failed to show any reductions in CPE titres in toxin A mixtures incubated with inhibitors. Despite using a wide range of concentrations of inhibitors (10 mg/mL to 60 mg/mL), no inhibition of toxin A was seen with either inhibitors tested (Optimal concentration for Synsorb CD was reported as 20 mg/mL) (Data not shown). These results were discouraging as previously published data had indicated that these products were efficacious in preventing toxin mediated damage.

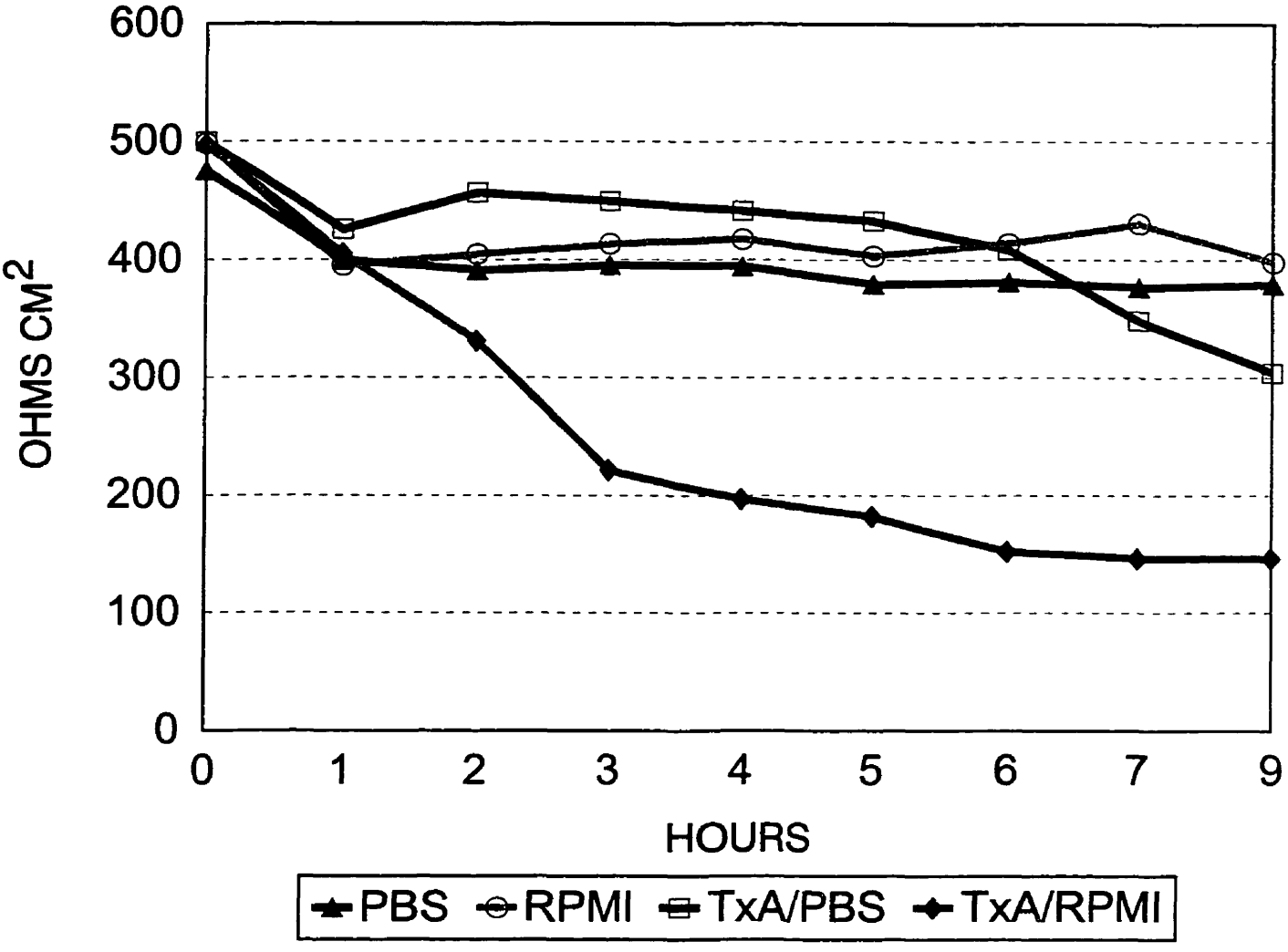


Figure 16. Positive and Negative Controls for toxin A Inhibition Studies

Negative control inserts treated with PBS and supplemented RPMI media show negligible decreases in electrical transepithelial resistance. Positive control inserts with Caco-2 cell monolayers treated with toxin A suspended in RPMI show characteristic transepithelial resistance drops. However, toxin A suspended in PBS media is inactivated and shows no appreciable electrical resistance decreases. Standard errors for electrical resistance measurements were less than 5%, therefore error bars were not included.

Inoculation of Caco-2 cell monolayers with toxin A in PBS incubated with Synsorb CD produced no changes in electrical resistance measurements (Fig. 17). As previously reported, it appears that toxin A may have been inactivated by the PBS. Surprisingly, toxin A in PBS incubated with Cholestyramine seemed to have some effect on electrical resistance measurements (Fig. 17). Paradoxically, it appears that toxin A may have been protected by the Cholestyramine.

Results of our experiments show that Synsorb CD and Cholestyramine are ineffective in preventing toxin mediated monolayer resistance changes, or HFF cell CPE. Further studies are needed to assess the ability of these inhibitors to prevent toxin mediated intestinal damage.

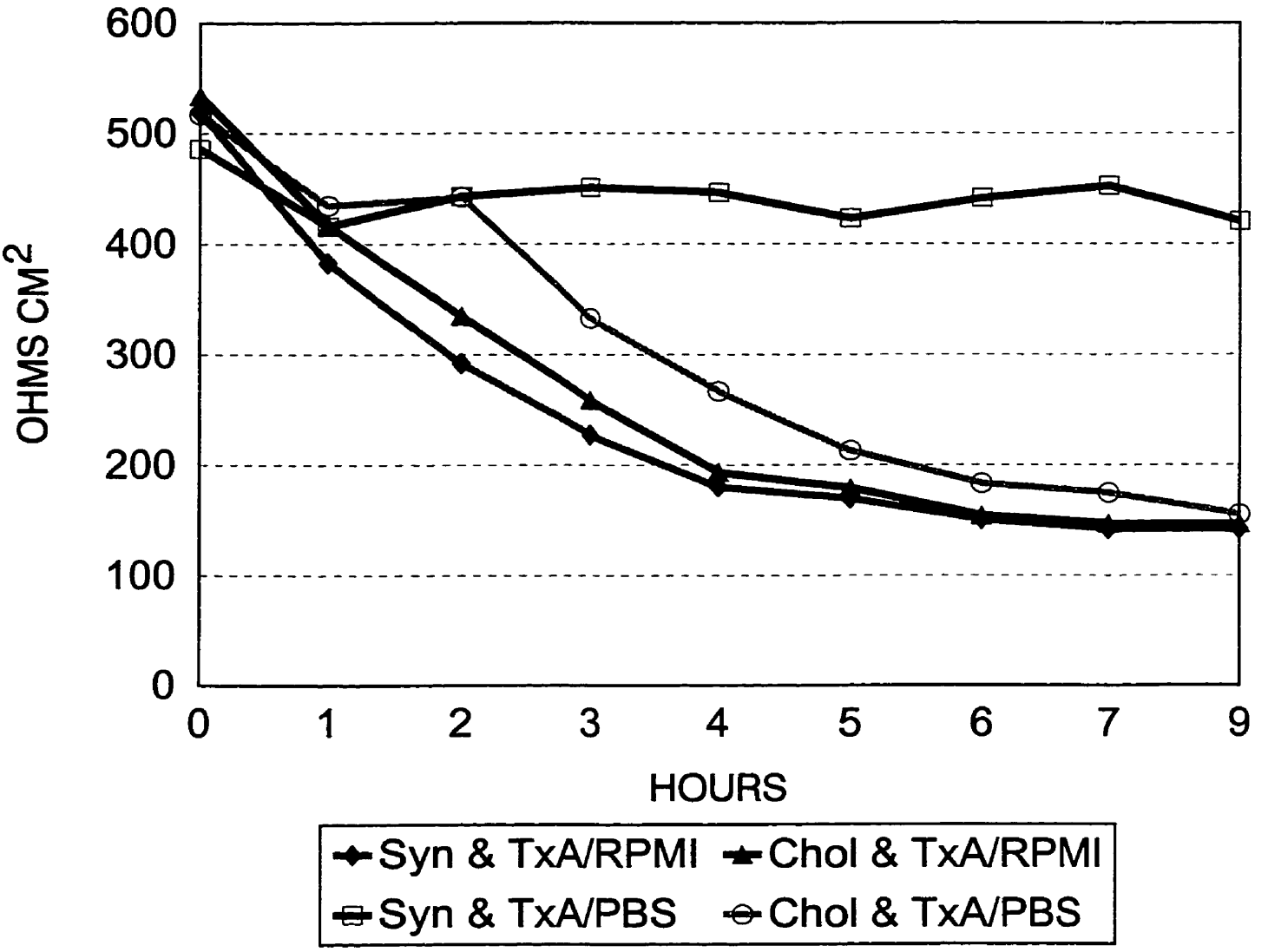


Figure 17. Efficacy of Synsorb CD and Cholestyramine in Preventing toxin A Induced Damage on Caco-2 cell Monolayers.

The efficacy of Synsorb CD and Cholestyramine in preventing toxin A induced monolayer damage was assessed by pre-incubating toxin A with either inhibitor prior to inoculation on HFF cell monolayers. In this figure, 40 mg of Synsorb CD or Cholestyramine was incubated with 1.0 mL of toxin A (0.250μg/mL) for 1 hour at 35°C. Synsorb CD and Cholestyramine were ineffective in inhibiting toxin A mediated Caco-2 monolayer damage. As previously reported, toxin A is inactivated when suspended in PBS. Standard errors for electrical resistance measurements were less than 5%, therefore error bars were not included.

DISCUSSION

Use of Caco-2 Cells For The Development of a Polarized Cell Culture Model.

Transport of compounds across the intestinal mucosal barrier is a difficult parameter to study because the organ exhibits considerable cellular heterogeneity and geometric structure. Augmenting this problem, is the lack of easy access to the basal side of the epithelium due to the presence of subadjacent tissues (65). However, with the recent use of cultured intestinal cells, the problem has been somewhat alleviated.

In our studies, we have employed a polarized tissue culture insert model utilizing Caco-2 cells to study the translocation of *Clostridium difficile* toxins A and B similar to that described by Hect et al. (69, 70). In our initial trials, both T84 (69, 70) and Caco-2 cells (124) were evaluated as possible candidate cell lines for our studies. Since both cell lines were derived from the colon, they were ideal models for our studies as we had sought to examine the translocation dynamics of *C. difficile* toxins across the intestinal epithelium. However, due to the inability of T84 cells to reach electrical resistance plateaus (Fig. 5), it was decided that Caco-2 cells would serve as the model cell line. The inability of T84 cells to reach electrical resistance plateaus posed several problems for our studies. As experimental reproducibility is crucial to every study, it was necessary for us to obtain confluent cell monolayers in similar physiological states. Since this task would have been difficult to accomplish using the T84 cell lines, we chose to utilize the Caco-2 cell lines.

The reasons for why the T84 cell line was unable to reach electrical resistance plateaus are unclear, but may have been influenced by the inherent properties of the cell line. It did, however, appear under phase contrast microscopy, that the cells may have been growing continuously on top of one another. This was suggested as the monolayer had appeared confluent after one week of incubation. If true, this may explain why the electrical resistance measurements continued to increase daily without reaching a plateau. It is also possible that the T84 cell line may have had an unusually high electrical resistance reading when fully confluent. As we had only monitored the progress of the cell line for about two weeks, it was possible that the cells may have eventually reached electrical resistance plateau after a more prolonged incubation. However, if this was true, the T84 cell line would still have been a less desirable model to use as the duration of time required for confluent monolayers to develop would have been lengthy.

Due to the difficulties associated with the use of the T84 cell line, the human colonic carcinoma cell line, Caco-2, was chosen. The Caco-2 cell line is advantageous in many respects; it is well studied (124, 65), and is also known to show signs of spontaneous in-vitro enterocytic differentiation. Confluent Caco-2 cell monolayers have fully formed tight junction and brush border microvilli (124). The formation of tight junctions was crucial to our polarized cell culture model as it ensured the integrity of the cell monolayers. The presence of brush border membranes was also important to our study as it provided receptors (Fucα1-3Galβ1-4GlcNac) for toxin A binding.

Caco-2 cells inoculated onto Transwell inserts became confluent after one week of incubation. This property of the Caco-2 cell line was very beneficial to our study as it reduced the amount of preparation time required. The ability of Caco-2 cells to form confluent monolayers within a weeks duration was verified both by phase contrast microscopy, and electrical resistance measurements. The increases in electrical resistance measurements for Caco-2 cells were similar for all inserts seeded, as was the time required for cells to reach electrical resistance plateaus. The ability of Caco-2 cells to respond similarly to *C. difficile* toxin treatments was also remarkable. The reproducibility of electrical resistance curves in all test cases were exceptional. Although triplicate inserts were used for all test cases, each insert exhibited almost identical electrical resistance responses to the toxins with minimal standard errors (<5%). This was true of inserts treated with labeled or unlabeled toxin A, toxin B, or both toxins A and B.

In summary, the polarized Caco-2 cell culture model was an ideal model for our study purposes. This cell culture model allowed us to access both apical and basolateral sides of epithelium; a feat not otherwise possible using animal models, or conventional cultured cells grown on plastic surfaces. The ability to assess the cell monolayers from both apical and basolateral domains was beneficial to our studies because it allowed us to monitor toxin translocation dynamics, as well as, transepithelial resistance. As the Transwell inserts were clear, this also allowed for good visability under phase contrast microscopy for monitoring cell progress. The Caco-2 polarized cell culture model should prove valuable for many studies assessing transport across intestinal epithelial cells.

Assessing Translocation Dynamics of *Clostridium difficile* toxins A and B.

The initial titration experiments performed on HFF cell monolayers confirmed the cytopathic capabilities of toxins A and B on cultured cells. In accordance with literature values, the cytopathic titre of toxin B was approximately 1000 fold more potent than toxin A (125).

As *C. difficile* toxins act primarily on the colon, we sought to assess the ability of these toxins to alter the integrity of the intestinal epithelium. If toxins A and B were found to disrupt the intestinal epithelium, and facilitate passage of toxin and/or other toxic factors into deeper tissues and/or systemic circulation, this knowledge would be novel in understanding the pathogenesis of *C. difficile*-associated diarrhea.

Results from preliminary toxin studies on Caco-2 cell monolayers were in agreement with previously published data which indicated that toxin A was much more effective in eliciting tight junction changes in comparison to toxin B (69, 70). In our studies we found that treatment of polarized Caco-2 cell monolayers with toxin B allowed passage of factor which caused CPE on HFF cells. When both toxins A and B were utilized, this translocation effect was enhanced. Efforts to use monoclonal and polyclonal antibodies directed against these toxins were ineffective in elucidating which toxins were able to translocate. To arrive at a more definitive conclusion ^{125}I radiolabeling of *C. difficile* toxins was employed. BSA was also radiolabeled, and served as a negative control.

For radiolabeling experiments we employed a gentle method of iodination using IODO-BEADS. As toxin A and toxin B are sensitive to oxidation, it was our primary goal to come up with a gentle radiolabeling technique. Protein radiolabeling experiments were successful as all radiolabeled samples had TCA precipitabilities greater than 75%. Although TCA precipitations were lower than those normally expected for radiolabeled proteins, they were higher than those of previously published studies which also utilized radiolabeled *C. difficile* toxins (39). To confirm that the toxins were not inactivated by the iodination reaction, the biological activity of the toxins were assessed by using HFF cell titrations, and by measuring electrical resistance changes in Caco-2 cells. The titre of biological activity of these radiolabeled toxins were essentially unchanged according to the assays we used.

In view of their large molecular sizes, our experiments still showed that toxins A and B were more efficiently translocated than BSA. These findings suggest that the translocation process for toxins A and B may be an active process. Several lines of evidence support this hypothesis: Researchers have already identified the putative receptor for toxin A in humans, the carbohydrate Lewis X blood group antigen (Fucα1-3Galβ1-4GlcNac) found on the surface of intestinal epithelial cells (165). This receptor is believed to be involved in the attachment process which leads to the internalization of toxin A. Using biochemical and electron microscopy studies, investigators have also revealed endocytic pathways for both toxins A and B (72, 175). Taken together, these lines of evidence support an active transport model for *Clostridium difficile* toxin internalization and translocation.

When Caco-2 cell monolayers were exposed to both toxins A and B, an enhanced effect was seen with respect to the amount of toxin B able to translocate at each of the time intervals tested. A molecular basis for the differential trafficking of toxin A and toxin B cannot be provided from our data, however, several explanations may be proposed: *Clostridium difficile* toxins A and B have clostridial repetitive oligopeptide (CROP) sequences at their carboxyl termini which are believed to be involved in cellular attachment. Each CROP segment consists of either 20 or 50 amino acids repeated 14-30 times (46, 74, 171). In competition assays, recombinant CROP sequences blocked binding of toxin A to cell surfaces, indicating that CROP sequences may function as ligands (144). There are 30 CROP sequences in toxin A and only 19 CROP sequences in toxin B (170). Moreover, neutralization experiments utilizing monoclonal antibodies have revealed a putative hexapeptide sequence (TIDGKK) on the toxins believed to be involved in interaction between the toxin and its cellular receptor (58, 144). This hexapeptide sequence was found in six copies in the CROP region of toxin A, however, no copies were found in toxin B. Taken together, these findings suggest that cellular attachment specificities for the two toxins may be different, and this may explain why toxin B does not bind to intestinal cells as efficiently as toxin A. Since toxin B cannot bind to cellular receptors, it would have an increased ability to pass through gaps created in the tight junctions, possibly resulting in greater translocation efficiencies. The results of our experiments are in agreement with these findings since our data indicated an increased translocation for ^{125}I-toxin B when tested in conjunction with unlabeled toxin A. Studies have also shown that toxins A and B have different sites of localization within endosomal compartments (72). Since precise cellular

trafficking mechanisms for the toxins are unclear, it is possible that the two toxins may utilize different mechanisms of translocation.

In tests which utilized radiolabeled toxin A or toxin B alone, the amount of radiolabeled toxin translocating at each time point remained at similar levels. Since toxin A is more efficient at modulating tight junctions, we would have expected an increased amount of toxin A translocating as a result of passage through holes in tight junctions. This was, however, not observed and may be attributed in part to the binding of toxin A to cellular receptors via its CROP region.

In summary, the results of our experiments suggest a synergistic role for *C. difficile* toxins in promoting disease symptoms. If correct, our model would predict that *C. difficile* isolates capable of producing both toxins would be more virulent as translocation of toxins would be more efficient. Indeed, most clinically significant infections have been caused by strains capable of producing both toxins. Strains deficient in toxin A production have been reported (103). One such strain, Serogroup F, lacks toxin A and is found only in neonates (42). Although cytotoxic activity has been detected in stool samples from patients infected with this strain, there has been no association with clinically significant disease as most carriers remain asymptomatic. Furthermore, germfree mice inoculated with this strain do not develop diarrhea. Other toxin A-, toxin B+ strains have been described, but are only rarely associated with disease. In these strains, it is not clear whether toxin A is totally deficient, or if toxin A is just truncated.

To our knowledge, our data provides the first experimental evidence of the synergistic effects of toxins A and B on the translocation process. Furthermore, it is the first report to indicate that toxin B is more efficiently translocated across the intestinal epithelium. Our results would suggest that toxin B is also an important component in sub-mucosal bowel damage seen in vivo. Although toxin A has been reputed to be the more important virulence factor in disease manifestation, it is not possible to exclude the importance of toxin B. Many researchers have speculated on the synergistic roles of the toxins in the promotion of the disease state. Several studies have shown that toxin A deficient strains were incapable to causing death in germ free animals (31, 168). Indeed Lyerly et al. speculated that toxin A facilitated the exit of toxin B from the gut (104).

Translocation of Gram Negative Lipopolysaccharide.

Gram negative bacterial lipopolysaccharide has a variety of actions on humans. It is thought to trigger a series of reactions including both septic shock and the release of cytokines, as well as, development of multiple organ failure. In major surgery, burns, and major trauma, the endotoxin which appears in the blood stream is postulated to have translocated from the patients digestive tract. This triggers a series of inflammatory reactions, which sometimes result in the grave sequelae of multiple organ failure (76).

Since our previous studies have indicated that toxins A and B were effective in compromising the integrity of the intestinal tight junctions, we sought to assess whether translocation of Gram negative lipopolysaccharides could be facilitated by the addition of *C. difficile* toxins. In our studies, we were unable to determine if *E. coli* LPS could translocate across intact Caco-2 cell monolayers. Despite complete disruption of Caco-2 cell

monolayer, as indicated by low electrical resistance readings, we were unable to detect appreciable amounts of *E. coli* LPS on the basal side of the insert. Surprisingly, positive control inserts which had no Caco-2 cell monolayers growing, also failed to reveal translocation of significant amounts of LPS.

As LPS molecules are known to form micelles in aqueous solution, it is possible that micelle formation may have excluded the translocation of LPS. Exclusion of LPS from the basal side of the insert as a result of size exclusion was not possible as the porosity of the insert membrane (0.4 μm) was much greater than the molecular size of LPS. It is, however, possible that charge exclusion may have played a role in preventing LPS translocation. As the insert membrane was made up of polyester, it is possible that the LPS molecules may have had an unfavorable charge interaction with the surface of the insert.

The results of our experiments were inconclusive in determining whether *C. difficile* toxins A and B could facilitate the translocation of *E. coli* LPS. Further studies are needed to elucidate this important aspect of *C. difficile* pathogenesis.

Assessment of Circulating Toxin Molecules in Cytotoxin Positive Patients.

One of the major functions of intestinal epithelium is to provide a barrier between the intestinal lumen and the underlying compartments. In healthy individuals, this barrier is maintained and serves as a protective mechanism. However, in patients with PMC this barrier may be breached, allowing for the passage of foreign molecules such as *C. difficile* toxins into systemic circulation. For these reasons, it was necessary to examine for the possibility of finding circulating toxin molecules in severely ill patients.

Analysis of serum samples from cytotoxin positive patients using ELISA, and a HFF cell neutralization assay, failed to reveal appreciable amounts of toxin B in systemic circulation. Although our findings did not substantiate our original hypothesis that toxic factors may contribute to shock-like systemic symptoms in patients with PMC, we cannot exclude this possibility with our experimental data. As serum samples used in the study had been previously frozen and thawed, it is possible that toxin B may have lost its potency, or had been inactivated by the storage process. Our studies have confirmed the inactivation of toxin B by freezing and thawing, as have other studies (57). Analysis of fresh serum samples obtained from three patients with PMC also failed to reveal presence of circulating cytotoxin molecules. However, due to the limited sample size, we must reserve conclusions until such a time when more samples can be analyzed.

Since it is possible for patients to have neutralizing antibodies to toxins A and B in their systemic circulation (88, 169), it is not inconceivable that these antibodies may have neutralized the cytotoxic effects of toxin B. Similarly, proteases in the serum may have had a detrimental effect on biological activity of toxin B. As *C. difficile* infection is primarily localized to the large colon, the distribution of toxin B molecules in the systemic circulation may have been at low levels. This low level of cytotoxic activity may have been undetectable by the methods we employed. Perhaps it would have been more beneficial to sample for cytotoxic activity in sub-mucosal tissues of the damaged bowel. The possible roles of circulating cytotoxin in *C. difficile*-associated diseases requires further study.

Role of Humoral Immunity in *C. difficile* Infections

Infections caused by *C. difficile* are enigmatic in that patients may present with a wide spectrum of symptoms. The basis for these differences in clinical presentation are not clearly defined but are postulated to be influenced by the presence or absence of humoral or mucosal antibody responses to *C. difficile* and its toxins (85, 99). As *C. difficile* toxins act to disrupt the epithelial tight junctions, it may be possible for toxins to gain access to deeper tissues to induce a humoral immune response. Indeed, a study by Stieglbauer et al (152) found an elevated serum antibody response to toxin a following splenic abscess due to *C. difficile.*

To assess for the prevalence of toxin B antibodies, sera from 74 cytotoxin positive (CP), and 72 cytotoxin negative (CN) patients with diarrhea were tested using an ELISA and toxin B neutralization assay. In contrast to previously reported data (85, 169), the prevalence of toxin B antibodies in our test population were quite small. Analysis of 74 CP patients revealed that only 14.9% of the population had neutralizing antibodies to toxin B. This figure was further reduced to 9.5% when specific detection of toxin B antibodies using ELISA was assessed. In addition, there did not appear to be a significant difference between prevalence of antibodies to toxin B in both CP and CN patients. The percentage of CP and CN patients found to have immune responses to toxin B according to toxin B neutralization and ELISA assays were (14.9% and 13.9%) and (9.5% and 4.2%) respectively. In examining CP patients for development of humoral immune responses to toxin B, we failed to find any significant increases in toxin B antibodies.

The results of our studies were inconclusive in elucidating the importance of humoral antibodies in resolving *C. difficile* infections. It should be noted that many patients who suffer from *C. difficile* infections, also suffer from many episodes of relapse. These findings call into question the importance of humoral antibodies in protection from *C. difficile* infections. As *C. difficile* infections are predominately localized to the intestinal mucosa of the large colon, it is possible that secretory antibodies play a more significant role in *C. difficile* pathogenesis than humoral antibodies. Our study did not address the role of either secretory antibodies, or toxin A neutralizing antibodies. Investigations into these important aspects of immunity should prove valuable for understanding the overall model of *C. difficile* pathogenesis. In conclusion, the roles of humoral immunity in *C. difficile* infections warrant further study.

Inhibitors of *C. difficile* toxins.

As toxin binding to mucosal surfaces is a prelude to disease progression, we wanted to determine if commercially available inhibitors were efficacious in preventing toxin A induced intestinal damage. For our study purposes, we examined two inhibitors; Synsorb CD and Cholestyramine. Synsorb CD is an inert resin with carbohydrate receptors specific for toxin A. Previous studies have indicated that this inhibitor was effective in preventing rabbit red blood cell agglutination (71). Cholestyramine is a non-specific inhibitor often used by patients as an adjunct therapy for high cholesterol. This resin is believed to bind to toxin A non-specifically, and has been used to treat *C. difficile* infections (158).

Results from our experiments showed that neither Synsorb CD or Cholestyramine were effective in preventing toxin A mediated Caco-2 cell monolayer damage. Transepithelial resistance responses in Caco-2 cell monolayers treated with toxin A, or toxin A incubated with Synsorb CD or Cholestyramine were very similar. The addition of Synsorb CD or Cholestyramine did not have an effect on transepithelial responses in Caco-2 cell monolayers; regardless of concentration of inhibitor used. The inability of Synsorb CD and Cholestyramine to inhibit toxin A activity was also confirmed with HFF cell titrations. Addition of Synsorb CD or Cholestyramine had no effect on reducing the titre of cytopathic effect on HFF cells. These results suggest that neither Synsorb CD or Cholestyramine were effective as inhibitors of toxin A activity as neither inhibitor was effective in reducing the enterotoxic or cytotoxic activity of the toxin A.

Although a previous study has indicated that Synsorb CD was efficacious in reducing toxin A activity (71), this study was limited in its scope and based primarily on the ability of Synsorb CD to reduce the titre of rabbit red blood cell agglutination. This was an insensitive assay as it did not look into biological effects of toxin A such as its ability to cause cytoskeletal rearrangements and induce cell rounding. Perhaps this study would have been more convincing if a hamster model of inhibition was used. Currently, there are no documented studies reporting on the efficacy of Synsorb CD using either in vitro tissue culture models, or in vivo animal models. It is clear that more studies are needed to assess the feasibility of these alternative approaches to treatment of *C. difficile* infections.

Cholestyramine has also been used as an adjunct therapy for treatment of complicated cases of *C. difficile* colitis (158). The effectiveness of this therapeutic agent is still under investigation. Similar to studies with Synsorb CD, there is also a lack of data to document the efficacy of Cholestyramine in treatment of *C. difficile* infections. Most studies involving Cholestyramine have assessed this agent in conjunction with other therapeutic agents, such as antibiotics, for treatment of *C. difficile* infections (158). Because of these confounding factors, it is hard to assess the role of Cholestyramine in promoting resolution of *C. difficile* infections.

In examining the ability of Synsorb CD and Cholestryramine to effect inhibition in toxin A, we made a serendipitous discovery. From our experimental data, it appears that toxin A is inactivated by PBS. This effect was not artifactual in nature as identical results have been reproduced several times. The inactivation effect of PBS was observed for both toxin A and toxin B. Moreover, RPMI in the absence of serum also promoted this effect. Presently, the precise reasons for these findings are unclear. As PBS and RPMI are starvation medias, they may promote cessation of eukaryotic cell metabolism. If endocytic pathways for toxin internalization by eukaryotic cells can be halted while cells are in their quiescent state, this may explain why cells do not demonstrate CPE when exposed to toxins A and B. This explanation is, however, not completely acceptable as toxic effects are not rescued when toxin A and/or B suspensions in PBS or RPMI are diluted back into media containing serum, prior to cytotoxicity testing. The exact basis for this apparent inactivation warrants further evaluation, as it may be of value in developing disease interventions.

Future directions

Clostridium difficile is an important nosocomial pathogen that has significant implications for costs associated with hospitalization, as well as, patient morbidity and mortality (136, 178). Despite extensive research into this enigmatic pathogen, the model of pathogenesis remains incomplete. Although the biological activities of toxins A and B have been extensively studied, their relative contributions to human intestinal mucosal damage is unclear. Although toxin internalization has been revealed, the exact cellular sites of toxin translocation are still not known. It is, however, clear that disease progression is much more complex than just the direct action of toxin on the gut mucosa. Initially, it was speculated that toxin A was the most important component in disease production because it elicited extensive tissue damage and fluid accumulation (17, 104). However, it is now clear that both toxins A and B are important for eliciting disease symptoms. Supporting the shared role of toxin A and toxin B are several studies that have reported that it was necessary to vaccinate hamsters against both toxins A and B in order to confer immunity (54, 101).

In addition to elucidating the roles of toxins A and B in *C. difficile* pathogenesis, it is also important to learn how *C. difficile* becomes established in the intestine. The ability of *C. difficile* and other bacteria to adhere to gastrointestinal cell surfaces is increasingly becoming recognised as a prerequisite for colonisation of the gut, expression of virulence, and development of infection (21, 108). Already, it has been clearly established that *C. difficile* organisms can associate with the intestinal mucosa of humans and hamsters (21). There appears to be an association between virulence and mucosal adherence of *C. difficile*: the highly virulent strains attach to the mucosa better than poorly virulent or avirulent strains

(21). These findings suggest that inhibition of mucosal adhesion by *C. difficile* could be one new promising strategy for the prevention of infections. For inhibition of *C. difficile* adhesion, several blocking substances, such as soluble receptor analogues or anti-receptor antibodies could be used.

Researchers have also known for years that many infants colonized with toxigenic strains of *C. difficile* are refractory to the disease, yet, the precise reasons for this are still unknown. Speculations ranging from lack of toxin receptors, to immature receptors, to lack of inflammatory responses have all been made, but not confirmed with concrete data. Research into this area may provide better insight on how to better manage and treat patients afflicted with *C. difficile* infections.

A frightening consequence of treating *C. difficile* infections with nonselective broad spectrum antibiotics is the potential for the development of widespread antibiotic resistance in other intestinal microorganisms. Vancomycin resistance is of particular concern as it is the only effective drug against many life-threatening pathogens. The consequences of rampant antibiotic resistance has already been felt; methicillin-resistant *Staphylococcus aureas* (MRSA) strains discovered in Japan and Michigan were found to have intermediate susceptibilities to vancomycin, the only antibiotic effective against multi-resistant MRSA (25, 177). With the problems of antibiotic resistance, and the limited utility of antibiotics, there is a clear need to develop more selective and effective alternatives for treating *C. difficile* infections.

Current alternative approaches to treating *C. difficile* infections include immunizations against *C. difficile* (or its toxins) (143,162), use of toxin inhibition agents, and use of biotherapeutic agents (100). Vaccination studies utilizing hamster models have shown promise, however, reports are conflicting with regards to what vaccine should be used as both inactivated toxoids (162) and recombinant toxin epitopes (143) have been assessed. The exact routes of vaccine administration also need to be elucidated as some studies have revealed that the vaccines were only protective if administered by both parenteral and mucosal routes (162). Toxin inhibition agents can also be used for treatment of *C. difficile* infections. However, this approach requires some developing as currently available agents lack convincing clinical data. The use of biotherapeutic agents has also been assessed in many studies (23, 64, 101). This approach may prove to be the most beneficial in preventing infections by toxigenic *C. difficile* as it directly interferes with the ability of organism to colonize the intestinal tract.

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