CHARACTERIZATION OF THE CARBOHYDRATE RECEPTORS
OF
CLOSTRIDIUM DIFFICILE ENTEROTOXIN

by

Kenneth Davis Tucker

Dissertation submitted to the graduate faculty of the
Virginia Polytechnic Institute and State University
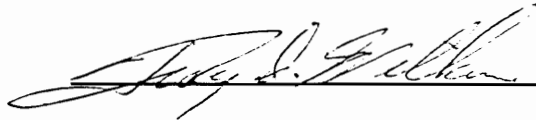in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
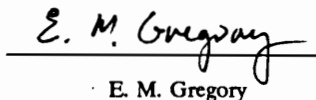
in

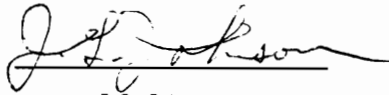Microbiology

(Department of Anaerobic Microbiology)

APPROVED:

T. D. Wilkins, Chairman

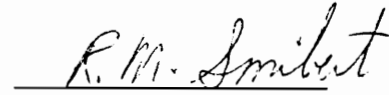E. M. Gregory   J. L. Johnson

N. R. Krieg   R. M. Smibert

May, 1990

Blacksburg, Virginia

CHARACTERIZATION OF THE CARBOHYDRATE RECEPTORS
OF
CLOSTRIDIUM DIFFICILE ENTEROTOXIN

by

Kenneth Davis Tucker

Committee Chairman: Tracy D. Wilkins
Anaerobic Microbiology

(ABSTRACT)

Clostridium difficile causes pseudomembranous colitis in humans and a similar ileocecitis in hamsters. This organism can colonize the intestines after antibiotic therapy disrupts the normal intestinal microflora. Once established in the intestines, the organism causes disease by producing two toxins, designated toxin A and toxin B. Only toxin A is active on intestinal epithelium, thus toxin A is the cause of the initial tissue damage in the intestines. In order for a toxin to affect a cell, it must first bind to the cell. Toxin A has been shown to bind to $Gal\alpha 1$-$3Gal\beta 1$-$4GlcNAc$ on the intestinal epithelium of hamsters. I provide evidence that toxin A can use this trisaccharide as a functional receptor on cell lines, and that the expression of the carbohydrate receptor increases the sensitivity of the cells to toxin A. Furthermore, the intestinal epithelium of infant hamsters bound less toxin A at 37C than did the adult tissue, and infants are less sensitive to the disease caused by *C. difficile* than are adults. This provides further evidence that the activity of toxin A is increased by the binding of the toxin to $Gal\alpha 1$-$3Gal\beta 1$-$4GlcNAc$. Even though $Gal\alpha 1$-$3Gal\beta 1$-$4GlcNAc$ was a receptor for toxin A on animal cells, it probably is not a receptor for toxin A in humans, because people

do not normally express this carbohydrate. Instead, I found that toxin A bound to the carbohydrate antigens designated I, X, and Y, which are present on the intestinal epithelium of humans. These carbohydrates could be receptors for toxin A. The possible significance of these receptors is discussed.

ACKNOWLEDGEMENTS

This dissertation is dedicated to my wife and child, Karen and Kyle, who have provided emotional support and a sense of perspective through my education.

I thank the members of my committee who encouraged me and provided many insights into the project. Each of these gentlemen have my unending respect. I especially thank Dr. T. D. Wilkins for patiently providing guidance and support as my major professor, and allowing me the freedom to pursue my own postulates, and create my own mistakes.

I thank the other members of the lab; Carol, David, Kay, Laurie, Lisa, Liz, Pauline, Roger, and Steve. Each of these individuals added to my education and made it a fun experience. They all hold a special place in my memories and heart.

Finally, I thank the members of the Anaerobe Laboratory, especially the secretaries and ladies in media and glassware: they all made my work easier with their pleasant personalities, and by shouldering some of my burdens.

iv

FORWORD

This dissertation contains seven sections. The first section is a review of the literature, which provides an introduction to *Clostridium difficile* and the disease pseudomembranous colitis. Sections II to VI are written in a format for submission for publication. Of these sections, sections II to IV, and VI are being considered as possible manuscripts; whereas section V is included to present the logic that lead to the finding presented in section VI. Section VII contains the summation of the dissertation, and additional postulates that are not presented in the previous sections.

Parts of this dissertation previously have been presented. Section III has been published (1), and is presented in this dissertation exactly as it was accepted for publication. The data in section IV were presented at the 1989 national conference for the American Society for Microbiology (K. D. Tucker and T. D. Wilkins. *Clostridium difficile* toxin A uses Galα1-3Galβ1-4GlcNAc as a functional receptor), and also is published (3). The findings presented in section VII are covered in a pending patent (4), and also will be presented at the 1990 national conference of the American Society for Microbiology (K. D. Tucker and T. D. Wilkins. A possible receptor for *Clostridium difficile* toxin A in humans). This section of the dissertation has been submitted for publication with minor changes (2).

REFERENCES

1)   Tucker, K. D., P. E. Carrig, and T. D. Wilkins. 1990. Toxin A of *Clostridium difficile* is a potent cytotoxin. J. Clin. Microbiol. **28**: 869-871.

2)   Tucker, K. D., and T. D. Wilkins. Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. Infect. Immun. (submitted for publication)

3)   Wilkins, T. D., and K. D. Tucker. 1989. *Clostridium difficile* toxin A (enterotoxin) uses Galα1-3Galβ1-4GlcNAc as a functional receptor. Microecol. Therapy **19**: 225-227.

4)   Wilkins, T. D., and K. D. Tucker. March 9, 1990. Detection, isolation, and purification of *Clostridium difficile* toxin A with toxin receptors. U. S. Patent application Serial No. 07/491,396.

TABLE OF CONTENTS

A: *CLOSTRIDIUM DIFFICILE* DISEASE IN HUMANS AND ANIMALS

The discovery of antibiotics was a major advancement for the field of medicine that radically changed the way diseases are treated, and greatly improved the prognosis for many maladies. However, like so many discoveries, the actual benefits were less than were initially hoped. Some microorganisms were resistant to antibiotics, whereas others acquired resistance as a result of antibiotic therapy. Further, there were unexpected and unexplained side effects with many antibiotics.

One side effect commonly associated with antibiotic therapy is diarrhea. This may range in severity from a mild and self-limiting disease to a life-threatening enteritis. Pseudomembranous colitis is one form of a life-threatening diarrhea associated with antibiotic treatment. This disease is characterized by the formation of pseudomembranes composed of mucin, fibrin, white blood cells, and sloughed mucosal cells on the colonic mucosa (8, 14, 61, 167). The pseudomembranes initially are sporadically located on the colonic mucosa, with each pseudomembrane covering only a 2-5 mm area (14, 61, 167). With time, these may fuse to form large patches of pseudomembranes that may be sloughed and passed as "casts" of the colon.

Treatment with a number of antibiotics can cause pseudomembranous colitis, but the use of lincomycin, and its derivative clindamycin, cause the highest incidence of the disease per patient treated (8, 16, 54, 60, 70, 159, 167, 168, 172). As early as 1969 it was known that hamsters treated with lincomycin often

1

developed an acute and rapidly fatal ileocecitis (160). In the mid to late 1970's this hamster disease became the animal model for antibiotic-associated pseudomembranous colitis (12, 25). At the microscopic level, the damage to the intestinal mucosa of the hamster is similar to that of the human; however, pseudomembranes do not form in the hamster (14, 31, 77, 140). This caused many researchers initially to reject this animal model for pseudomembranous colitis.

Fortunately, a few researchers studied the hamster disease in hopes of understanding the human disease. They found that a sterile filtrate of the cecal contents from a hamster with antibiotic-associated enteritis could cause enteritis in another hamster following intracecal injection (149). Dialysis of the cecal filtrate removed the antibiotic, but did not abolish the toxicity. Thus the antibiotic did not directly cause the enteritis, instead a toxin or virus was the apparent cause of the disease. Since the disease causing agent could not be propagated in tissue culture, this was suggestive of a toxin. After screening numerous antitoxins, gas gangrene antitoxin was found to neutralize the toxic activity in the cecal filtrate. Gas gangrene antitoxin contains antibodies to a number of clostridial toxins, so this indicated a clostridium was the etiological agent, but did not identify the actual organism. Further experiments demonstrated that the antitoxin to *Clostridium sordellii* was responsible for neutralizing the toxic activity (2, 9, 10, 96, 159). However *C. sordellii* could not be isolated from either hamsters with enteritis or from humans with

pseudomembranous colitis. Instead, it was found that the *C. sordellii* antitoxin also neutralized the toxic activity of *C. difficile*, and *C. difficile* was isolated from both hamsters and humans with antibiotic associated enteritis (10, 11, 13, 30, 95, 96, 103, 148, 149, 150). Further, sterile culture filtrates of *C. difficile* caused enteritis in the hamster (10, 95). Thus, *C. difficile* was identified as the cause of pseudomembranous colitis (9, 10, 11, 59, 62, 95, 103). This organism is estimated to cause 25% of the reported cases of antibiotic associated diarrhea (24, 165), making it second only to *Campylobacter jejuni* as the major cause of bacterial diarrhea in humans. Subsequent research demonstrated that *C. sordellii* produces two toxins (Hemorrhagic toxin and Lethal toxin) that are similar to the two toxins produced by and *C. difficile* (toxin A and toxin B) (7, 117, 136, 166). Both toxins of *C. difficile* are believed to be involved in the disease.

C. difficile is not normally present in significant numbers in the intestinal microflora of adult humans. In fact, the normal intestinal microflora creates a protective barrier that prevents *C. difficile* from colonizing the intestines (20, 21, 22, 106, 157, 180, 181); however, antibiotic therapy disrupts the normal intestinal microflora. This allows *C. difficile*, which can be acquired from the hospital environment (26, 37, 71, 84, 85, 115, 128), to colonize the intestines and grow to high numbers. The organism then produces an enterotoxin that damages the intestinal mucosa (6, 7, 75, 99, 101, 102, 108, 123, 124, 125, 166, 175), which further perturbs the intestinal microflora and allows *C. difficile* to continue

colonizing the intestines after antibiotic therapy is stopped. Ironically, this antibiotic associated disease is treated with the antibiotic vancomycin, which is bacteriocidal to *C. difficile* (12, 25, 48, 49, 80). Unfortunately, vancomycin also disrupts the normal intestinal microflora. So when the vancomycin is discontinued, *C. difficile* may recolonize the intestines, with about 24% of the patients relapsing (15). Thus the disease may become ongoing and debilitating.

Pseudomembranous colitis is most often associated with antibiotic therapy, but antibiotic therapy is not absolutely required for pseudomembranous colitis to occur. For example, one of the first reports of pseudomembranous colitis was in 1893 (50), over 40 years before the advent of antibiotic therapy. The cases of pseudomembranous colitis without prior antibiotic therapy commonly involved trauma to the intestines such as surgery or intestinal obstruction, but also involved trauma that did not directly effect the intestines, such as spinal fracture and cardiovascular disease (61). Presumably this trauma affects the normal intestinal microflora, allowing *C. difficile* to colonize the intestines. There are still reports of pseudomembranous colitis without prior antibiotic therapy (45, 127, 137), but since antibiotic therapy is common following bowel surgery, post-operative pseudomembranous colitis cannot be distinguished from an antibiotic-associated pseudomembranous colitis.

C. difficile is well established as the cause of pseudomembranous colitis; however, in rare cases it can also cause illness by infecting parts of the body other than the intestines. This organism has been reported to cause abscesses,

wound infections, septicemia, peritonitis, pleuritis, and osteomyelitis (97, 155, 163). *C. difficile* has not been implicated as the cause of necrotizing enterocolitis in neonates, inflammatory bowel disease, or Crohn's disease (18, 27, 41, 66, 83, 120, 162, 158, 161).

C. difficile often colonizes the intestines of human infants who have not been treated with antibiotics (1, 5, 38, 69, 73, 82, 94, 100, 116, 147, 164, 173, 174). This can occur in infants up to two years of age, with as many as 50% of the neonates in some hospitals being colonized (1, 82, 147, 176). However, *C. difficile* does not cause any apparent ill effects, even though the toxins can be detected in the feces (19, 39, 100, 116, 164). One possible reason for the insensitivity of infants to *C. difficile* is that human colostrum contains antibodies that can neutralize the *C. difficile* toxins (86, 178). Yet, this can not fully explain the resistance of infants to the toxins because many infants are not breast-fed. An additional explanation was suggested by Chang et al. (32), who found that cells from the intestinal mucosa of infants were more resistant to *C. difficile* toxins than were cells from the intestinal mucosa of adults. They suggested that the infant cells lack receptors for the toxins, whereas the adult cells contain these receptors; however, no supporting evidence was provided. Thus the reason for the insensitivity of infants to the *C. difficile* toxins is not known at this time.

Infant hamsters also may be colonized by toxigenic *C. difficile* from the fourth to twelfth days of life with no apparent ill effects (151). As seen in humans, mother's milk can confer passive immunity to the infant hamster (50,

87). This is of particular significance in studies with infant hamsters because the infants are maintained on mother's milk. Thus, passive immunization could explain why clindamycin-treated infant and just-weaned hamsters are less sensitive to toxigenic *C. difficile* than are adult hamsters (67). In apparent conflict with the above study, Iaconis and Rolfe reported that treatment with clindamycin increased the number of deaths with infant hamsters colonized with toxigenic *C. difficile* (78). They postulated that this was due to an increase in toxin production associated with the clindamycin treatment (78, 130). This increase in toxin apparently overwhelmed the passive immunity provided by the mother's milk. Even though infant hamsters provide an animal model to study the insensitivity of infants to *C. difficile*, the work has not progressed beyond suggesting that the insensitivity is due to passive immunity.

Germ-free mice and rats also can be colonized by *C. difficile* without prior antibiotic therapy (35, 36, 37, 132, 177). In these animals *C. difficile* does cause colitis and the formation of pseudomembranes on the colon (177). Thus the disease in these animals may be more closely related to the disease in humans. Using this model, Corthier et al. (35) found that the proteolytic activity in the intestinal tract of these animals reduced the biological activity of toxin A 100-fold without effecting the biological activity of toxin B. Further, the inactivated toxin A was still detected by immunological assays. These observations provide a potential explanation for the insensitivity of human infants to *C. difficile*. In the studies with the human infants, toxin A was assayed with an enzyme linked

immunosorbent assay, and toxin B was assayed with a cytotoxicity assay. Thus the assays did not determine if the biological activity of toxin A was reduced. As will be discussed later, toxin A must be present for enteritis to occur. So if infants inactivate toxin A by proteolysis, they could protect themselves from pseudomembranous colitis.

The use of germ-free animals for research has several disadvantages. Monoassociated animals do not have the complex microflora found in conventional animals, and this causes the physiology of the intestines to differ. The differences between the physiology of germ-free and normal animals, as well as the cost and difficulty of using germ-free animals, makes the germ-free animal model prohibitive for most researchers.

Currently, the most widely used model for pseudomembranous colitis is the antibiotic-treated hamster. This animal is relatively sensitive to the toxins of *C. difficile*, and is easy to maintain (108). Further, at the histological level, *C. difficile* causes similar tissue damage to the ceca of hamsters as it causes to the colon of humans (14, 31, 77, 140); however, since hamsters do not form pseudomembranes, their use for the study of pseudomembranous colitis may be questionable. *C. difficile* can cause antibiotic associated enteritis in a number of other laboratory animals, including rabbits, guinea pigs, and prairie dogs (88, 144, 145). Of these, only prairie dogs form pseudomembranes. The introduction of this animal model for pseudomembranous colitis has been too recent to evaluate any results.

B: *CLOSTRIDIUM DIFFICILE* TOXINS

1) Physicochemical properties

The properties of toxin A and B are summarized in Table 1. Both toxins are large proteins with a molecular mass near 300,000 that do not dissociate into smaller subunits (6, 7, 107, 119, 165, 166). This large size for toxin A has been verified from the cloning and sequencing of the toxin A gene (42, 156), and preliminary results from the toxin B gene also support its large size (personal communication from Lisa Barroso). Both toxins may exist as dimers in solution, as determined from the estimated molecular mass on native and denaturing polyacrylamide gels (6, 7, 107, 165, 166). Toxin A migrates much slower in an electric field than its pI would dictate when agarose gels are used (107). A possible explanation for this is that the toxin may interact with α-linked galactose in the agarose, thus retarding the movement of the toxin (91). Of practical importance, toxin A precipitates at pH values near its isoelectric point in low ionic strength buffers; this can be used to purify the toxin (165). Both of the toxins are inactivated by the oxidizing agents N-bromosuccinimide, H_2O_2, KO_2, and O_2 (107). Since these oxidizing agents may affect tyrosine, this suggests why it is difficult to iodinate toxin A without affecting its biological activity. Reducing agents do not affect the biological activities of either toxin, which suggests that disulfide cross-linkages are not involved in their activities (107).

TABLE 1: Physicochemical properties of toxin A and toxin B

Toxin	M_r^a	M_r^b	M_r^c	pI	Stability		Protease inactivation
					pH	Heat	
A	307,972 308,103 (42, 156)	250,000-300,000 (165)	440,000-500,000 (165)	5.2-5.7 (107)	Stable at 4 and 10 (165, 166)	Stable at 37C inactive at 56C (165, 166)	Inactivated by pronase and chymotrypsin Stable with trypsin (6, 7, 104)
B	269,987 (L. Barroso, Personal Comm)	250,000-300,000 (165)	360,000-470,000 (165)	4.1-4.5 (107)	Inactive at 4 and 10 (165, 166)	Stable at 37C inactive at 56C (165, 167)	Inactivated by trypsin, pronase and chymotrypsin (6, 7, 104)

a M_r estimated based on the nucleic acid sequence.
b M_r estimated using denaturing conditions on polyacrylamide gels.
c M_r estimated using native conditions on polyacrylamide gels.

2) Biological activities

Toxin A is a potent enterotoxin in rabbits, hamsters, mice, and suckling-mice (6, 7, 99, 101, 102, 107, 108, 111, 124, 125, 166, 174, 175). Either feeding or directly injecting the toxin into the intestinal lumen causes disruption of the intestinal epithelium, and erosion of the villus tips (99, 101, 102, 124, 125, 175). This destroys the permeability barrier that normally is formed by the intestinal epithelium (75, 123, 124), so that body fluids, including serum, may seep into the intestinal lumen (102). If an animal systemically produces antibodies to toxin A, the toxic activity can be neutralized at this point as the circulating antibodies seep into the intestines with the other serum proteins (125). However, if the toxin is not neutralized, it may continue damaging tissue into the lamina propria, which contains the blood vessels that supply the intestines. Once the blood vessels are exposed, both toxin A and toxin B increase the vascular permeability which results in blood leaking into the intestinal lumen (6, 7, 111, 124, 125, 166).

The extensive tissue damage caused by toxin A results in inflammation in the intestines (77, 99, 101, 102, 174, 175). Furthermore, toxin A is a chemoattractant for granulocytes, and stimulates granulocytes to release factors that cause secretion in the intestines (137, 138). These observations have prompted some researchers to postulate that toxin A does not directly affect the intestines; instead, they believe that toxin A causes the granulocytes to release factors that damage the intestinal epithelium and cause fluid secretion (175). Activated granulocytes can damage intestinal mucosa and release factors that

cause secretion in the intestines (68), so the activity of toxin A on the granulocytes probably does contribute to the tissue damage caused by toxin A. However, toxin A also disrupts intestinal epithelium in organ culture and tissue culture (75, 102, 123), which demonstrates that toxin A has enterotoxic activity independent of the granulocytes. So toxin A does act directly on intestinal epithelium.

Toxin A and toxin B are about equally lethal when injected subcutaneously or intraperitoneally into animals (3, 6, 7, 107, 111, 165, 166). However, the signs before and after death do not indicate the cause of death. Oral administration of toxin A also results in death of hamsters (108). Toxin A disrupts the intestinal epithelium, and apparently enters the body where it may exert its lethal activity. Toxin B alone has no observable effect on the intestinal epithelium, and is not toxic when fed to animals (5, 6, 102, 107, 111, 165, 166). However, oral doses of toxin B are lethal if the integrity of the intestinal mucosa has been damaged, such as by bruising or by the enterotoxic activity of sub-lethal amounts of toxin A (108). This suggests that toxin A and toxin B act synergistically, with toxin A damaging the intestinal mucosa and exposing cells that are sensitive to toxin B.

Both toxin A and toxin B are cytotoxic to all mammalian cell lines that have been tested and each toxin causes a similar affect to the cells (6, 7, 40, 102, 111, 165, 166). The most readily observed response of cells to the toxins is rounding. However, the cells do not die immediately, as determined by trypan blue exclusion from the cell. Instead the cells merely seem to be prevented from

growing, and begin to die after several days. Analysis at the subcellular level also demonstrates that each toxin has a similar effect on cells. Each causes a decrease in the synthesis of proteins, ribonucleic acids, and deoxyribonucleic acids (139, 146, 153). Further, after exposure to either toxin, cells show a decrease in filamentous actin and a corresponding increase in globular actin (33, 52, 75, 93, 133, 169, 170). This apparent conversion of filamentous actin to globular actin is believed to cause disruption of the cytoskeletal structure, and rounding. However, toxin B does not act directly on purified actin in vitro (122), so modification of actin may not be the mechanism of action for the toxin. The similar effects on cells caused by toxin A and toxin B suggests that the toxins may have the same mechanism of action; however, this has not been confirmed because the mechanism of action for neither toxin is known.

Even though toxin A and toxin B have similar effects on cells, their cytotoxic activities are very different: toxin B is about 1,000 to 10,000 times more cytotoxic than is toxin A on a molar basis (109, 133, 165, 166). In fact toxin B is one of the most potent cytotoxins known, with as little as 10^{-21} moles of toxin B being able to cause a cell to round. One possible explanation for the difference in the cytotoxic activities of toxin A and toxin B is that the toxins may be processed differently after entering the cells (53, 169, 170, 171). Alternately, the toxins may have different receptors, with the receptor for toxin B being more effective than the receptor for toxin A at binding and internalizing the toxin. The actual reason for the different cytotoxic activities is not known.

3) Immunochemical properties

Serology has played a major role in research concerning *C. difficile*; it was the fortuitous cross-neutralization of the toxins of *C. difficile* with *C. sordellii* antisera that led to the discovery that *C. difficile* causes pseudomembranous colitis. Ironically, the further significance of this cross-reaction was overlooked: it took three years to discover that the organism produced two toxins, even though it had been known for a decade that *C. sordellii* produced two protein toxins (4). Now it is known that toxin A and toxin B have partial immunological identity with Hemorrhagic and Lethal toxins, respectively, from *C. sordellii* (117, 136).

Antisera to toxin A or toxin B have been used to determine if these two toxins share any antigenic determinants. Affinity purified antibodies to toxin A or toxin B do not cross react as determined by western blots, Ouchterlony assays, or neutralization of biological activity (6, 7, 98, 110, 112, 165, 166). This indicates that toxin A and toxin B are not antigenically related.

The monoclonal antibody designated PCG-4 has been particularly useful for characterizing toxin A. This monoclonal antibody reacts with the portion of toxin A that binds to the carbohydrate receptor for the toxin, and neutralizes the enterotoxic activity of the toxin (102, 109, 141). Yet, this monoclonal does not reduce the cytotoxic activity of toxin A on Chinese hamster ovary cells (102, 109). This suggests that either the receptor for toxin A is different between hamster intestines and Chinese hamster ovary cells (109), or that the low cytotoxic activity

of toxin A on Chinese hamster ovary cells is due to pinocytosis of the toxin by the cells and does not involve a receptor. One property of PCG-4 that is unusual for most monoclonal antibodies is that it precipitates toxin A, which indicates that toxin A contains repeating epitopes for PCG-4 (109). This was the first evidence that toxin A did contain repeating amino acid sequences, which was later confirmed by the nucleic acid sequence of the toxin (42, 156). Like the affinity purified antibodies to toxin A, PCG-4 does not react with toxin B (109).

At the same time PCG-4 was being characterized, a number of monoclonal antibodies that cross-reacted with toxin A and toxin B were found (109, 152). This was in apparent conflict with the results obtained with polyclonal antibodies, and suggested that toxin A and B shared many antigenic determinants. This paradox was explained by Lyerly et al. (105) who found that toxin A and toxin B can bind several types of monoclonal antibodies non-specifically. Thus, the apparent serological cross-reaction between toxin A and toxin B doubtful. Even so, since both toxin A and toxin B bind antibodies non-specifically, this suggests that toxin A and toxin B do share a similar functional domain.

4) Genetics

The genes for both toxin A and toxin B have been cloned, and have now been completely sequenced (42, 156, Lisa Barroso, personal communication). Toxin A and toxin B have 6 domains that share as much as 60% identity at the amino acid level, and are similar in their relative position in each toxin (Lisa Barroso, personal communication). This indicates that the two toxins may be the result of a gene duplication, and that toxin A and toxin B could have the same mechanism of action. Further, this suggests that the toxins may have similar receptors, as will be discussed later. However, despite the identity between the toxins at the genetic level, the genes have diverged enough so that the proteins are antigenically unique; therefore, the functional properties of the toxins, such as enzyme activities and receptor recognition sites may also have diverged. So the similarity between the genes must be interpreted with caution. The sequence for the toxin B gene is preliminary, so will not be considered any further, however, the gene for toxin A is well characterized.

The toxin A gene is a single open reading frame that codes for a protein with a deduced molecular mass of 308,103 or 307,972, depending on the strain of *C. difficile* used (42, 156). The 3' end of the gene for toxin A codes for repeating amino acid sequences that comprise about 30% of the total protein (42). These repeats are involved in the binding of toxin A to a carbohydrate receptor present on the membrane of cells (Galα1-3Galβ1-4GlcNAc) (141). Antisera to this portion of toxin A neutralizes

the enterotoxic activity of toxin A in rabbit ileum and hamster cecum (42). Further, this is the portion of toxin that reacts with the monoclonal antibody PCG-4, which also neutralizes the enterotoxic activity of toxin A (102, 109).

5) Receptors

a: Toxin A

An enterotoxin must bind to the intestinal epithelium, and toxins often accomplish this by binding to specific carbohydrate structures on the membranes of cells. Likewise, toxin A binds to carbohydrates that contain Galα1-3Galβ1-4GlcNAc (34, 91, 92). The discovery that this carbohydrate is a receptor for toxin A was based on the observation that toxin A agglutinates rabbit erythrocytes (which contain Galα1-3Galβ1-4GlcNAc), and that the agglutination is inhibited by bovine thyroglobulin (which also contains Galα1-3Galβ1-4GlcNAc). Enzymatic removal of the α-linked galactose completely abolishes the binding of toxin A to the carbohydrate, which demonstrates the importance of the α-linked galactose for the binding of toxin A (34, 91). However, related carbohydrates that contain an α1-4-linked galactose do not bind the toxin (34, 91). Furthermore, blood type B erythrocytes do not bind toxin A, even though these cells contain the trisaccharide receptor with a fucose on the sub-terminal galactose (Galα1-3[Fucα1-2]Galβ1-4GlcNAc) (91). Thus toxin A appears to bind specifically to Galα1-3Galβ1-4GlcNAc.

The amount of toxin bound to Galα1-3Galβ1-4GlcNAc decreases with increasing temperatures, such that undetectable amounts of toxin A bind to the rabbit erythrocytes or bovine thyroglobulin at 37C (91, 92). This temperature effect is used to purify toxin A by binding the toxin to immobilized bovine thyroglobulin at 4C and eluting the toxin at 37C (92). Although the temperature dependant binding of toxin A is useful in research, this is an apparent

disadvantage for the toxin in vivo, because little toxin may bind at body temperature. The binding of toxin A at 37C can be increased by increasing the density of the receptor for the toxin (91, 92). A possible explanation for this observation is that as the number of receptors increase, the number of binding sites on a molecule of toxin A that actually bind a receptor also increases. This causes the avidity of toxin A for Galα1-3Galβ1-4GlcNAc to increase because the avidity of toxin for its receptor is the summation of the individual affinities of each binding site that is actually bound to a receptor. Thus with high densities of receptor, significant amounts of toxin A may bind at 37C. This appears to be the case on the hamster intestinal mucosa, which contains large amounts of Galα1-3Galβ1-4GlcNAc and binds toxin A at 37C in amounts that are similar to that observed with cholera toxin (91). Thus Galα1-3Galβ1-4GlcNAc can be a receptor for toxin A at physiological temperatures.

The importance of toxin A binding to Galα1-3Galβ1-4GlcNAc is demonstrated by the observation that antibodies to the carbohydrate binding portion of toxin A neutralize the enterotoxic activity of the toxin (42, 102, 109). The trisaccharide receptor for toxin A is expressed by almost all mammals (55); however, humans do not normally express this carbohydrate (55, 58). In humans this trisaccharide has been demonstrated only on cancerous mammary cells, and on a genetically transformed human cell line (28, 58, 79). The expression of Galα1-3Galβ1-4GlcNAc by normal cells in the human body also is limited by the fact that most people express antibodies that react with this trisaccharide (56, 57). Thus the expression of Galα1-3Galβ1-4GlcNAc in the human body may result in

an autoimmune disease (28, 58). Therefore, toxin A probably does not use

Galα1-3Galβ1-4GlcNAc as a receptor in humans.

b: Toxin B

The similar nucleic acid sequence between toxin A and toxin B suggests

that toxin B also may bind a carbohydrate receptor that is similar to the receptor

for toxin A. However, toxin B does not bind to Galα1-3Galβ1-4GlcNAc,

otherwise toxin B would co-purify with toxin A on immobilized bovine

thyroglobulin (92). Even so, the high level of cytotoxic activity of toxin B

indicates that cell lines have a receptor. This high cytotoxic activity on a large

number of cell lines suggests that the receptor for toxin B must be ubiquitous.

Conversely, the ineffectiveness of toxin B on intestinal epithelium suggests that

this tissue may lack a receptor for toxin B. Currently, the receptor for toxin B is

identified.

C: CARBOHYDRATE COMPLEXITY AS RECEPTORS

The binding of many bacterial toxins to carbohydrate receptors is well established, and the subject of several excellent reviews (44, 81). Some of the properties that make carbohydrates suitable receptors for toxins are their presence on all eucaryotic cell membranes and their structural complexity.

1) Complexity of structure

A few different monosaccharides may be used to synthesize an unlimited number of carbohydrate structures. However, unlike nucleic acids and amino acids, a single monosaccharide can covalently bond to any of the hydroxylated carbons on another carbohydrate. This exponentially increases the possible number of receptors that may be synthesized from a given number of monosaccharides. This also means that oligosaccharides and polysaccharides can be branched . Thus, if given the same number of amino acids or monosaccharides, many more receptors can be synthesized from the monosaccharides. For example, a tripeptide of glycine forms only one possible configuration, but a trisaccharide of galactose can occur in at least 88 unique configurations.

The actual number of different carbohydrate structures found on cells is limited by the requirement for enzymes to synthesize the structures (89, 90, 129, 154, 179). All oligosaccharides and polysaccharides present on the membrane of a cell are synthesized by glycosyl-transferases, and each glycosyl-transferase

synthesizes a specific carbohydrate configuration (90, 154, 179). Thus the possible

number of carbohydrate structures are limited by the ability of a cell to produce

enzymes that can synthesize the structures. Since cells can synthesize only a

limited number of enzymes, polysaccharides tend to have only a few common

core structures, which are attached to either lipids or proteins (90, 113, 154). The

structural diversity of carbohydrates is maintained by specific glycosyl-transferases

which add additional monosaccharides to these core structures. The core

structures commonly found on lipids are presented in Table 2.

TABLE 2: Carbohydrate core structures of glycolipids

Prefix	Core Structure
Lacto	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1
Lactoneo	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1
Muco	Galβ1-3Galβ1-4Galβ1-4Glcβ1-1
Gala	GalNAc?1-3Gal?1-4Galα1-4Galβ1-1
Globo	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1
Globoiso	GalNAcβ1-3Galα1-3Galβ1-4Glcβ1-1
Ganglio	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1

Sialic acid, fucose, or galactose can be α-linked to these cores to form a vast array of receptors (17, 47, 113). Furthermore, these core structures may be branched, and each branch may have different monosaccharides attached to it to create additional diversity of structure (113).

Carbohydrates may be attached to proteins via nitrogen on asparagine (N-linked), or to oxygen on threonine and serine (O-linked) (89, 90, 154). The O-linked carbohydrates can occur in a large range of sizes, from only one monosaccharide up to large and complex polysaccharides that dwarf the carrier protein (154). The configuration of the O-linked carbohydrates are diverse, but the smaller oligosaccharides often resemble the carbohydrates found on glycolipids (154). The N-linked carbohydrates all share a branched core that is high in mannose, and which is attached to the asparagine by two N-acetylglucosamines (89, 90). This carbohydrate may either be unaltered to form a high mannose structure, or each of the branches may have lactosamine (galactosyl-β1,4-N-acetylglucosamine) added to it to form a complex structure (90, 126). A hybrid structure is produced when lactosamine is added to only one or a few of the branches of a high mannose structure (90, 126). The presence of lactosamine on a N-linked carbohydrate makes the carbohydrate similar to the lactoneo-glycolipids. Likewise, glycoproteins with the lactosamine structure may be modified by the addition of α-linked sialic acid, fucose, and galactose. Hence, glycoproteins and glycolipids have a number of structures in common (142).

2) Diversity of expression

All mammalian cells are sugar-coated with a vast and complex assortment of carbohydrates being expressed on the membrane of a cell and forming the glycocalyx. These structures are the first part of a cell that are encountered by any molecule approaching the cell (81). Therefore, carbohydrates provide the first opportunity for a toxin to bind to a cell.

The particular carbohydrate structures can differ between cells. Cells from different species usually express different carbohydrates (55, 113, 142). Further, different animals within a species also may express unique carbohydrates, as is exemplified by people having different blood types which are due to carbohydrate antigens (63, 72). Even the tissues within the same animal will contain carbohydrates that are unique to the specific tissue (23, 129). Since tissues are made of several cell types, even the cells within a tissue may contain carbohydrates that are different from the carbohydrates of the cells adjacent to it (74, 76, 131, 143). Even further, the physiological state of a cell effects the expression of carbohydrates, so that the same cell types may express different carbohydrates at different times (46, 114, 129, 134, 182). Thus, by binding to a specific carbohydrate, toxins may target to specific cells within the body. This segregation of carbohydrates on certain types of cells causes different cells to have different sensitivities to the same toxin (29, 43, 65).

As if targeting to a specific cell were not enough, toxins may target to specific domains of a cell by using carbohydrate receptors. That is, carbohydrates

may be localized to specific parts of the membrane of a cell, as seen with polarized cells (118, 121). For example, the membrane of intestinal cells is separated into two functional domains: the mucosa faces the lumen of the intestines and the serosa faces the underlying tissue. Some of the carbohydrates present on the mucosal domain are not present on the serosal domain, and vise versa. Thus toxin binding can depend on the orientation of the cell, with different domains being exposed by different orientations. Therefore, carbohydrates serve as extremely selective binding sites for toxins, and target the toxins to sites that provide the greatest benefit to the organism producing the toxin.

D. INTRODUCTION TO THE THESIS

When I started these studies, Galα1-3Galβ1-4GlcNAc was the only carbohydrate receptor known for toxin A (34, 91, 92). The binding of toxin A to this carbohydrate is dependent on the presence of the α1-3-linked galactose (91), which is a relatively unique carbohydrate configuration, so other receptors for the toxin appeared to be unlikely. At that time, reports demonstrating the lack of Galα1-3Galβ1-4GlcNAc in humans had not been published; therefore, I focused my studies on the importance of toxin A binding to Galα1-3Galβ1-4GlcNAc. First, I found that the intestinal epithelium of infant hamsters bound less toxin A than did the same tissue from adult hamsters (section II). This provided an apparent explanation for why adult hamsters are more sensitive to disease caused by *C. difficile* than are infant hamsters (67, 153). Furthermore, I provided evidence that Galα1-3Galβ1-4GlcNAc acted as a biological receptor for the toxin on cells (sections III and IV). These findings supported the postulate that Galα1-3Galβ1-4GlcNAc was a biological receptor for toxin A in animals.

By this time in my research, I had started work with human colonic cell lines (section V), and had access to human intestinal tissue. Results using these tissues and cells convinced me that toxin A was indeed binding to human tissue, but I could not demonstrate α-linked galactose. Further, a survey of the literature revealed that Galα1-3Galβ1-4GlcNAc is not normally expressed in humans (28, 55, 58, 79). These observations led me to conclude that the binding of toxin A to Galα1-3Galβ1-4GlcNAc was not involved with the activity of toxin A in the human body. This resulted in a shift in the emphasis of my research, changing

the focus to identifying possible alternate receptors for toxin A in humans. Again, the cell lines provided the clue that led to the identification of other receptors for toxin A (section V): toxin A bound to the I, X, and I antigens, which are a group of similar carbohydrate receptors found on human intestinal epithelium (section VI). Fortunately, a large body of literature concerning these carbohydrates already existed, so that a number of conclusions could be made based on the identification of these new receptors.

LITERATURE CITED

1   Al-Jumaili, I. J. M. Shibley, A. H. Lishmann, and C. O. Record. 1984. Incidence and origin of *Clostridium difficile* in neonates. J. Clin. Microbiol. **19**: 77-78.

2   Alo, M., J. Silva, Jr., R. Fekety, G. D. Rifkin, and H. Waskin. 1979. Prevention of clindamycin-induced colitis in hamsters by *Clostridium sordellii* antitoxin. Gastroenterology **76**: 352-355.

3   Arnon, S. S., D. C. Mills, P. A. Day, R. V. Henrickson, N. M. Sullivan, and T. D. Wilkins. 1984. Rapid death of infant rhesus monkeys injected with *Clostridium difficile* toxins A and B: physiologic and pathologic basis. J. Pediatr. **101**: 34-40.

4   Arseculeratne, S. N., R. G. Panabokke, and S. Wijensundera. 1969. The toxins responsible for the lesions of *Clostridium sordellii* gas gangrene. J. Med. Microbiol. **2**: 37-53.

5   Bacon, A. E., R. Fekety, D. R. Schaberg, and R. G. Faix. 1988. Epidemiology of *Clostridium difficile* colonization in newborns: results using a bacteriophage and bacteriocin typing system. J. Infect. Dis. **158**: 349-354.

6   Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Uleno, and Y. Nozawa. 1984. Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from *Clostridium difficile*. Rev. Infect. Dis. **6**: S11-S20.

7   Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa. 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2**: 629-635.

8   Bartlett, J. G. 1979. Antibiotic-associated Pseudomembranous colitis. Rev. Infect. Dis. **1**: 530-539.

9   Bartlett, J. G., T.-W. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderdonk. 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N. Engl. J. Med. **298**: 531-534.

10   Bartlett, J. G., T.-W. Chang, N. Moon, and A. B. Onderdonk. 1978. Antibiotic-induced lethal enterocolitis in hamsters: studies with eleven agents and evidence to support the pathogenic role of toxin-producing clostridia. Am. J. Vet. Res. **39**: 1525-1530.

11 Bartlett, J. G., N. Moon, T.-W. Chang, N. Taylor, and A. B. Onderdonk. 1978. Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology **75**: 778-782.

12 Bartlett, J. G., A. B. Onderdonk, and R. L. Cisneros. 1977. Clindamycin-associated colitis in hamsters: protection with vancomycin. Gastroenterology **73**: 772-776.

13 Bartlett, J. G., A. B. Onderdonk, R. L. Cisneros, and D. L. Kasper. 1977. Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in hamsters. J. Infect. Dis. **136**: 701-705.

14 Bartlett, J. G., N. S. Taylor, T. Chang. J. Dzink. 1980. Clinical and laboratory observations in *Clostridium difficile* colitis. Am. J. Clin. Nutr. **33**: 2521-2526.

15 Bartlett, J. G., F. J. Tedesco, S. Shull, B. Lowe, and T. Chang. 1980. Symptomatic relapse after oral vancomycin therapy of antibiotic-associated pseudomembranous colitis. Gastroenterology **78**: 431-434.

16 Bartlett, J. G., S. H. Willey, T.-W. Chang, and B. Lowe. 1979. Cephalosporin-associated pseudomembranous colitis due to *Clostridium difficile*. J. Am. Med. Assoc. **242**: 2683-2685.

17 Blaken, W. M., D. H. Van den Eijnden. 1985. Biosynthesis of terminal Galα1-3Galβ1-4GlcNAc-R oligosaccharide sequences on glycoconjugates. J. Biol. Chem. **260**: 12927-12934.

18 Bolton, R. P., R. J. Sherriff, and A. E. Read. 1980. *Clostridium difficile* associated diarrhoea: a role in inflammatory bowel disease? Lancet i: 383-384.

19 Bolton, R. P., S. K. Tait, P. R. F. Dear, and M. S. Losowsky. 1984. Asymptomatic neonatal colonisation by *Clostridium difficile*. Arch. Dis. Childhood **59**: 466-472.

20 Borriello, S. P., and F. E. Barclay. 1984. Colonization resistance to *Clostridium difficile* infection. Microecol. Ther. **14**: 75-87.

21 Borriello, S. P., and F. E. Barclay. 1986. An in-vitro model of colonisation resistance to *Clostridium difficile* infection. J. Med. Microbiol. **21**: 299-309.

22 Bowden, T. A., A. R. Mansberger, and L. E. Lykins. 1981. Pseudomembranous enterocolitis: mechanism of restoring floral homeostasis. Am. Surg. **47**: 178-183.

23   Breimer, M. E. 1984. Tissue specificity of glycosphingolipids as expressed in pancreas and small intestine of blood group A and B human individuals. Arch. Biochem. Biophys. **228**: 71-85.

24   Brettle, R. P., and E. Wallace. 1984. *Clostridium difficile*-associated diarrhoea. J. Infect. **8**: 123-128.

25   Browne, R. A., R. Fekety, Jr., J. Silvia, Jr., D. I. Boyd, C. O. Work, and G. D. Abrams. 1977. The protective effect of vancomycin on clindamycin-induced colitis in hamsters. Johns Hopkins Med. J. **141**: 183-192.

26   Buggy, B. P., K. H. Wilson, and R. Fekety, Jr. 1983. Comparison of methods for the recovery of *Clostridium difficile* from an environmental surface. J. Clin. Microbiol. **18**: 348-352.

27   Cashore, W. J., G. Peter, M. Lavermann, B. S. Stonestreet, and W. Oh. 1981. Clostridial colonization and clostridial toxin in neonatal necrotizing enterocolitis. J. Pediatr. **98**: 308-311.

28   Castronovo, V., C. Colin, B. Parent, J.-M. Foidart, R. Lambotte, and P. Mahieu. 1989. Possible role of human natural anti-Gal antibodies in the natural antitumor defense system. J. Natl. Cancer Inst. **81**: 212-216.

29   Chan, L.-N. L., J.-S. Li, and S.-Y. Liu. 1985. Differential effects of abrin on normal and tumor cells. J. Cell. Physiol. **123**: 132-138.

30   Chang, T.-W., S. L. Gorbach, J. B. Bartlett. 1978. Neutralization of *Clostridium difficile* toxin by *Clostridium sordellii* antitoxin. Infect. Immun. **22**: 418-422.

31   Chang, T., J. G. Bartlett, S. L. Gorbach, and A. B. Onderdonk. 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect. Immun. **20**: 526-529.

32   Chang, T.-W., N. M. Sullivan, and T. D. Wilkins. 1986. Insusceptibility of fetal intestinal mucosa and fetal cells to *Clostridium difficile* toxins. Acta Phar. Sin. **5**: 448-453.

33   Ciesielski-Treska, J., G. Ulrich, B. Rihn, D. Aunis. 1989. Mechanism of action of *Clostridium difficile* toxin B: role of external medium and cytoskeletal organization in intoxicated cells. Eur. J. Cell Biol. **48**: 191-202

34   Clark, G. F., H. C. Krivan, T. D. Wilkins, and D. F. Smith. 1987. Toxin A from *Clostridium difficile* binds to rabbit erythrocyte glycolipids with terminal Galα1-3Galβ1-4GlcNAc sequences. Arch. Biochem. Biophys. **257**: 217-229.

35   Corthier, G., M. C. Muller, G. W. Elmer, F. Lucas, and F. Dubos-Ramare. 1989. Interrelationships between digestive proteolytic activities and production and quantitation of toxins in pseudomembranous colitis induced by *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **57**: 3922-3927.

36   Corthier, G., F. Dubos, and R. Raibaud. 1985. Modulation of cytotoxin production by *Clostridium difficile* in the intestinal tracts of gnotobiotic mice inoculated with various human intestinal bacteria. Appl. Environ. Microbiol. **49**: 250-252.

37   Czuprynski, C. J., W. J. Johnson, E. Balish, and T. Wilkins. 1983. Pseudomembranous colitis in *Clostridium difficile*-monoassociated rats. Infect. Immun. **39**: 1368-1376.

38   Delmee, M., G. Verellen, V. Avesani, and G. Francois. 1988. *Clostridium difficile* in neonates: serogrouping and epidemiology. Eur. J. Pediatr. **147**: 36-49.

39   Donta, S. T., and M. G. Myers. 1982. *Clostridium difficile* toxin in asymptomatic neonates. J. Pediatrics. **100**: 431-434.

40   Donta, S. T., N. Sullivan, and T. D. Wilkins. 1982. Differential effects of *Clostridium difficile* toxins on tissue-cultured cells. J. Clin. Microbiol. **15**:1157-1158.

41   Dorman, S. A., E. Liggoria, W. C. Winn, Jr., and W. L. Beeken. 1982. Isolation of *Clostridium difficile* from patients with inactive Crohn's disease. Gastroenterology **82**: 1348-1351.

42   Dove, C. H., S.-Z. Wang, S. B. Price, C. J. Phelps, D. M. Lyerly, T. D. Wilkins, and J. L. Johnson. 1990. Molecular characterization of *Clostridium difficile* toxin A gene. **58**: 480-488.

43   Draber, P., and P. Stanley. 1984. Cytotoxicity of plant lectins for mouse embryonal carcinoma cells. Somatic Cell Mol. Genet. **10**:435-444.

44   Eidels, E., R. L. Proia, and D. A. Hart. 1983. Membrane Receptors for Bacterial toxins. Microbiol. Rev. **47**: 596-620.

45   Ellis, M. E., B. M. Watson, P. J. Milewski, and G. Jones. 1983. *Clostridium difficile* colitis unassociated with antibiotic therapy. Br. J. Surg. **70**: 242-243.

46   Ernst, C. S., J-W. Shen, S. Litwin, M. Herlyn, H. Koprowski, and H. F. Sears. 1986. Multiparameter evaluation of the expression in situ of normal and tumor-associated antigens in human colorectal carcinoma. J. Nat. Cancer. Inst. **77**: 387-395.

47   Feizi, T., and R. A. Childs. 1985. Carbohydrate structures of glycoproteins and glycolipids as differentiation antigens, tumour-associated antigens and components of receptor systems. TIBS **10**: 24-29.

48   Fekety, R., J. Silva, J. Armstrong, M. Allo, R. Browne, J. Ebright, R. Lusk, G. Rifkin, and R. Toshniwal. 1981. Treatment of antibiotic-associated enterocolitis with vancomycin. Rev. Infect. Dis. **3**: S273-S281.

49   Fekety, R., J. Silva, R. Toshniwal, M. Allo, J. Armstrong, R. Browne, J. Ebright, and G. Rifkin. 1979. Antibiotic-associated colitis: effects of antibiotics on *Clostridium difficile* and the disease in hamsters. Rev. Infect. Dis. **1**: 386-397.

50   Fernie, D. S., R. O. Thomson, I. Batty, and P. D. Walker. 1983. Active and passive immunization to protect against antibiotic associated cecitis in hamsters. Dev. Biol. Stand. **53**: 325-332.

51   Finney, J. M. 1893. Gastro-enterostomy for cicatrizing ulcer of the pylorus. Johns Hopkins Hosp. Bull. **11**: 53-55.

52   Fiorentini, C., G. Arancia, S. Paradisi, G. Donelli, M. Giuliano, F. Piemonte, and P. Mastrantonio. 1989. Effects of *Clostridium difficile* toxins A and B on cytoskeleton organization in HEp-2 cells: a comparative morphological study. Toxicon **27**: 1209-1218.

53   Florin, I., and M. Thelestam. 1986. Lysosomal involvement in cellular intoxication with *Clostridium difficile* toxin B. Microb. Pathogen. **1**: 373-385.

54   Fournier, G., J. Orgiazzi, B. Lenoir, and M. Dechavanne. 1980. Pseudomembranous colitis probably due to rifampicin. Lancet. **i**: 101-102.

55   Galili, U., S. B. Shohet, E. Kobrin, C. L. M. Stults, B. A. Macher. 1988. Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J. Biol. Chem. **263**: 17755-17762.

56   Galili, U., B. A. Macher, S. B. Shohet. 1985. Human natural anti-α-galactosyl IgG. II. The specific recognition of α(1-3)-linked galactose residues. J. Exp. Med. **162**: 573-582.

57   Galili, U., J. Buehler, S. B. Shohet, B. A. Macher. 1987. The human natural anti-Gal IgG. III. The subtlety of immune tolerance in man as demonstrated by cross reactivity between natural anti-Gal and anti-B antibodies. J. Exp. Med. **165**: 693-704.

58   Galili, U. 1989. Abnormal expression of α-galactosyl epitopes in man. Lancet **ii**: 358-361.

59   George, R. H., J. M. Symonds, F. Dimock, J. D. Brown, Y. Arabi, N. Shinagawa, M. R. B. Keighley, J. Alexander-Williams, and D. W. Burdon. 1978. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br. Med. J. **1**: 695.

60   George, W. L. 1980. Antimicrobial agent-associated colitis and diarrhea. West. J. Med. **133**: 115-123.

61   George, W. L. 1984. Antimicrobial agent-associated colitis and diarrhea: historical background and clinical aspects. Rev. Infect. Dis. **6**: S208-S213.

62   George, W. L., E. J. C. Goldstein, V. L. Sutter, S. L. Ludwig, and S. M. Finegold. 1978. Etiology of antimicrobial agent-associated colitis. Lancet **i**: 802-803.

63   Gilbert, E. R. 1983. Erythrocyte antigens and antibodies. In *Hematology*. W. J. Williams, E. Beutler, A. J. Erslev, and M. A. Lichtman (Eds.). McGraw-Hill Book Co. New York. pp. 1491-1505 .

64   Gilligan, P. H., L. R. McCarthy, and V. M. Genta. 1981. Relative frequency of *Clostridium difficile* in patients with diarrheal disease. J. Clin. Microbiol. **14**: 26-31.

65   Goldstein, I. J., and R. D. Portez. 1986. Isolation, physicochemical characterization, and carbohydrate binding specificity of lectins. in *The lectins: properties, functions, and applications in biology and medicine*. I. E. Liener, N. Sharon, and I. J. Goldstein (Eds.). Academic Press Inc. pp 33-247.

66   Greenfield, C., J. R. A. Ramirez, R. E. Pounder, T. Williams, M. Danvers, S. R. Marper, and P. Noone. 1983. *Clostridium difficile* and inflammatory bowel disease. Gut **24**: 713-717.

67   Griffin, G. E., A. McDougall, S. A. Ash, and S. P. Borriello. 1984. Age related susceptibility of hamsters to clindamycin induced *Clostridium difficile* caecitis. Microecol. Therapy **14**: 269-270.

68   Grisham, M. B., and N. Granger. 1988. Neutrophil-mediated mucosal injury: role of reactive oxygen metabolites. Digest. Dis. Sci. **33**: 6S-15S.

69   Gurwith, M. J., C. Langston, and D. M. Citron. 1981. Toxin-producing bacteria in infants. Am. J. Dis. Child. **135**: 1104-1106.

70   Gurwith, M. J., H. R. Rabin, and K. Love. 1977. Diarrhea associated with clindamycin and ampicillin therapy: preliminary results of a cooperative study. J. Infect. Dis. **135**: S104-S110.

71   Hachler, H., and J. Wust. 1984. Reexamination by bacteriophage typing of *Clostridium difficile* strains isolated during a nosocomial outbreak. J. Clin. Microbiol. **20**: 604.

72   Hakomori, S. I. 1981. Blood group ABH and Ii antigens of human erythrocytes: chemistry, polymorphism, and their developmental change. Semin. Hematol. **18**: 39-62.

73   Hall, I. C., and E. O'Toole. 1935. Intestinal flora in new-born infants. Am. J. Dis. Child. **49**: 390-402.

74   Hansson, G. C., K. Simons, and G. van Meer. 1986. Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. EMBO J. **5**: 483-489.

75   Hecht, G., C. Pothoulakis, J. T. LaMont, and J. L. Madara. 1988. *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. **82**: 1516-1524.

76   Holgersson, J., N. Stromberg, and M. E. Breimer. 1988. Glycolipids of human large intestine: difference in glycolipid expression related to anatomical localization, epithelial/non-epithelial tissue and the ABO, Le and Se phenotypes of the donors. Biochem. **70**: 1565-1574.

77   Humphrey, C. D., W. B. Lushbaugh, C. W. Condon, J. C. Pittman, and F. E. Pittman. 1979. Light and electron microscopic studies of antibiotic associated colitis in the hamster. Gut **20**: 6-15.

78   Iaconis, J. P., and R. D. Rolfe. *Clostridium difficile*-associated ileocecitis in clindamycin-treated infant hamsters. Curr. Microbiol. **13**: 327-332.

79   Kagawa, Y., S. Takasaki, J. Utsumi, K. Hosoi, H. Shimizu, N. Kochibe, and A. Kobata. 1988. Comparative study of the asparagine-linked sugar chains of natural human interferon-β1 and recombinant human interferon-β1 produced by three different mammalian cells. J. Biol. Chem. **263**:17508-17515.

80   Kahn, M. Y., and W. H. Hall. 1966. Staphylococcal enterocolitis-treatment with oral vancomycin. Ann. Intern. Med. **65**: 1-8.

81   Karlson, K.-A. 1989. Animal glycosphingolipids as membrane attachment sites for bacteria. Ann. Rev. Biochem. **58**: 309-350.

82   Karsch, W., E. Strelau, W.-D. Grahlow, E. Fischer, and R. Schulz. 1989. Occurence and significance of *Clostridium difficile* in faecal specimens of hospitalized children. Zbl. Bakt. Hyg. **270**: 441-448.

83   Keighley, M. R. B., D. Youngs, M. Johnson, R. N. Allen, and D. W. Burdon. 1982. *Clostridium difficile* toxin in acute diarrhoea complicating inflammatory bowel disease. Gut **23**: 410-414.

84   Kim, K., H. L. Dupont, and L. K. Pickering. 1983. Outbreaks of diarrhea associated with *Clostridium difficile* and its toxin in day-care centers: evidence of person-to-person spread. J. Pediatr. **102**: 376-382.

85   Kim, K.-H., R. Fekety, D. H. Batts, D. Brown, M. Cudmore, J. Silva, Jr., and D. Waters. 1981. Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J. Infect. Dis. **143**: 42-50.

86   Kim, K., L. K. Pickering, H. L. DuPont, N. Sullivan, T. Wilkins. 1984. In vitro and in vivo neutralizing activity of human colostrum and milk against purified toxins A and B of *Clostridium difficile*. J. Infect. Dis. **150**: 57-62.

87   Kim, P.-H., J. P. Iaconis, and R. D. Rolfe. 1987. Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect. Immun. **55**: 2984-2992.

88   Knoop, F. C. 1979. Clindamycin-associated enterocolitis in guinea pigs: evidence for a bacterial toxin. Infect. Immun. **23**: 31-33.

89   Kobata, A. 1984. The carbohydrates of glycoproteins. In Vol. 2 *Biology of carbohydrates*. V. Gindburg and P. W. Robbins (Eds.). John Wiley and Sons. pp 87-161.

90   Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. **54**: 631-664.

91   Krivan, H. C.,G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. **53**: 573-581.

92   Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55**: 1873-1877.

93   Kushnaryov, V., and J. J. Sedmak. 1989. Effect of *Clostridium difficile* enterotoxin A on ultrastructure of Chinese hamster ovary cells. Infect. Immun. **57**: 3914-3921.

94   Larson, H. E., F. E. Barclay, P. Honour, and I. D. Hill. 1982. Epidemiology of *Clostridium difficile* in infants. J. Infect. Dis. **146**: 727-733.

95   Larson, H. E., P. Honour, A. B. Price, and S. P. Borriello. 1978. *Clostridium difficile* and the etiology of pseudomembranous colitis. Lancet i: 1063-1066.

96   Larson, H. E., and A. B. Price. 1977. Pseudomembranous colitis: presence of clostridial toxin. Lancet ii: 1312-1314.

97   Levett, P. N. 1986. *Clostridium difficile* in habitats other than the human gastro-intestinal tract. J. Infect. **12**: 253-263.

98   Libby, J. M., and T. D. Wilkins. 1982. Production of antitoxins to two toxins of *Clostridium difficile* and immunological comparison of the toxins by cross-neutralization studies. Infect. Immun. **35**: 374-376.

99   Libby, J. M., B. S. Jortner, and T. D. Wilkins. 1982. Effects of the toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. **36**: 822-829.

100   Libby, J. M., S. T. Donta, and T. D. Wilkins. 1983. *Clostridium difficile* toxin A in infants. J. Infect. Dis. **148**: 606.

101   Lima, A. M. A., D. J. Innes, Jr., K. Chadee, D. M. Lyerly, T. D. Wilkins, and R. L. Guerrant. 1989. *Clostridium difficile* toxin A: interactions with mucus and early sequential histopathologic effects in rabbit small intestines. Lab. Invest. **61**: 419-425.

102   Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultures cells in vitro. Infect. Immun. **56**: 582-588.

103   Lusk, R. H., R. Fekety, J. Silva, R. A. Browne, D. H. Ringler, and G. D. Abrams. 1978. Clindamycin-induced enterocolitis in hamsters. J. Infect. Dis. **137**: 464-475.

104   Lyerly, D. M., P. E. Carrig, and T. D. Wilkins. 1989. Susceptibility of *Clostridium difficile* toxins A and B to trypsin and chymotrypsin. Microbial Ecol. Health Dis. **2**: 219-221.

105   Lyerly, D. M., P. E. Carrig, and T. D. Wilkins. 1989. Nonspecific binding of mouse monoclonal antibodies to *Clostridium difficile* toxins A and B. Curr. Microbiol. **19**: 303-306.

106   Lyerly, D. M., H. C. Krivan, and T. D. Wilkins. 1988. *Clostridium difficile*: Its disease and toxins. Clin. Microbiol. Rev. **1**: 1-18.

107   Lyerly, D. M., M. D. Roberts, C. J. Phelps, and T. D. Wilkins. 1986. Purification and properties of toxins A and B of *Clostridium difficile*. FEMS Microbiol. Lett. **33**: 31-35.

108   Lyerly, D. M., K. E. Saum, D. K. MacDonald, and T. D. Wilkins. 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47**: 349-352.

109   Lyerly, D. M., C. J. Phelps, J. Toth, and T. D. Wilkins. 1986. Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect. Immun. **54**: 70-76.

110   Lyerly, D. M, C. J. Phelps, and T. D. Wilkins. 1985. Monoclonal and specific polyclonal antibodies for immunoassay of *Clostridium difficile* toxin A. J. Clin. Microbiol. **21**: 12-14.

111   Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins. 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1147-1150.

112   Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins. 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**: 72-78.

113   Macher, B. A., and C. C. Sweeley. 1978. Glycosphingolipids: structure, biological source, and properties. Methods Enzymol. **50**: 236-251.

114   Maddox, D. E., S. Shibata, and I. J. Goldstein. 1982. Stimulated macrophages express a new glycoprotein receptor reactive with *Griffonia simplicifolia* I-B$_4$ isolectin. Proc. Natl. Acad. Sci. USA **79**: 166-170.

115   Malamou-Ladas, H., S. O'Farrell, J. Q. Nash, and S. Tabaqchali. 1983. Isolation of *Clostridium difficile* from patients and the environment of hospital wards. J. Clin. Pathol. **36**: 88-92.

116   Mardh, P.-A., I. Helin, I. Colleen, M. Oberg, and E. Holst. 1982. *Clostridium difficile* toxin in faecal specimens of healthy children and children with diarrhoea. Acta. Pediatr. Scand. **71**: 275-278.

117   Martinez, R. D., and T. D. Wilkins. 1988. Purification and characterization of *Clostridium sordellii* hemorrhagic toxin and cross-reactivity with *Clostridium difficile* toxin A (enterotoxin). Infect. Immun. **56**: 1215-1221.

118   Matlin, K. S., and K. Simons. 1984. Sorting of apical plasma membrane glycoprotein occurs before it reaches the cell surface in cultured epithelial cells. J. Cell. Biol. **99**: 2131-2139.

119   Meador III, J., and R. K. Tweten. 1988. Purification and characterization of toxin B from *Clostridium difficile*. Infect. Immun. **56**: 1708-1714.

120   Meyers, S., L. Mayer, E. Bottone, E. Desmond. and H. D. Janowitz. 1981. Occurence of *Clostridium difficile* toxin during the course of inflammatory bowel disease. Gastroenterology **80**: 697-700.

121   Misek, D. E., E. Bard, and E. Rodriguez-Boulan. Biogenesis of epithelial cells polarity: intracellular sorting and vectorial exocytosis of an apical plasma membrane. Cell **39**: 537-546.

122   M. J. Mitchell, B. E. Laugon, and S. Lin. 1987. Biochemical studies on the effect of *Clostridium difficile* toxin B on actin in vivo and in vitro. Infect. Immun. **55**: 1610-1615.

123   Mitchell, T. J., J. M. Ketley, D. W. Burdon, D. C. A. Candy, and J. Stephen. 1987. The effects of *Clostridium difficile* crude toxins and purified toxin A on stripped rabbit ileal mucosa in Ussing chambers. J. Med. Microbiol. **23**: 199-204.

124   Mitchell, T. J., J. M. Ketley, D. W. Burdon, D. C. A. Candy, J. Stephen. 1987. Biological mode of action of *Clostridium difficile* toxin A: a novel enterotoxin. J. Med. Microbiol. **23**: 211-219.

125   Mitchell, T. J., J. M. Ketley, S. C. Haslam, J. Stephen, D. W. Burdon, D. C. A. Candy, and R. Daniel. 1986. Effect of toxin A and B of *Clostridium difficile* on rabbit ileum and colon. Gut **27**: 78-85.

126   Montreuil, J., 1980. Primary structure of glycoprotein glycans: basis for the molecular biology of glycoproteins. Advan. Carbohydrate Chem. Biochem. **37**: 157-223.

127   Moskovitz, M., and J. G. Bartlett. 1981. Recurrent pseudomembranous colitis unassociated with prior antibiotic therapy. Arch. Intern. Med. **141**: 663-664.

128   Mulligan, M. E., R. D. Rolfe, S. M. Finegold, and W. L. George. 1979. Contamination of a hospital environment by *Clostridium difficile*. Curr. Microbiol. **3**: 173-175.

129   Muramatsu, Takashi. 1988. Alterations of cell-surface carbohydrates during differentiation and development. Biochem. **70**: 1587-1596.

130   Nakamura, S., M. Mikawa, N. Tanabe, Y. Yamakawa, and S. Nishida. 1982. Effect of clindamycin on cytotoxin production of *Clostridium difficile*. Microbiol. Immunol. **26**: 985-992.

131   Nichols, G. E., J. C. Lovejoy, C. A. Borgman, J. M. Sanders, and W. W. Young, Jr. 1986. Isolation and characterization of two types of MDCK epithelial cell clones based on glycosphingolipid pattern. Biochim. Biophys. Acta. **887**: 1-12.

132   Onderdonk, A. B., R. L. Cisneros, and J. G. Bartlett. 1980. *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **28**:277-282.

133   Ottlinger, M. E., and S. Lin. 1988. *Clostridium difficile* toxin B induces reorganization of actin, vinculin, and talin in cultured cells. Experimental Cell Res. **174**: 215-229.

134   R. Parekh, I. Roitt, D. Isenberg, R. Dwek, and T. Rademacher. 1988. Age-related galactosylation of the N-linked oligosaccharides of human IgG. J. Exp. Med. **167**: 1731-1736.

135   Peikin, S. R., J. Galdibini, and J. G. Bartlett. 1980. Role of *Clostridium difficile* in a case of non-antibiotic-associated pseudomembranous colitis. Gastroenterology **79**: 948-951.

136   Popoff, M. R. 1987. Purification and characterization of *Clostridium sordellii* lethal toxin and cross-reactivity with *Clostridium difficile* cytotoxin. Infect. Immun. **55**: 35-43.

137   Pothoulakis, C., D. A. Sullivan, A. J. Melnick, T. Gadenne, T. Meshulam, and J. T. LaMont. 1986. *Clostridium difficile* toxins A and B stimulate intracellular calcium release in human neutrophils (abstr.). Clin. Res. **34**: 530A.

138   Pothoulakis, C., T. Theoharides, P. E. McAndrew, and J. T. LaMont. 1986. *Clostridium difficile* toxin A releases mediators from mucosal-like rat basophilic leukemia (RBL) cells (abstr.). Clin. Res. **34**: 871A.

139   Pothoulakis, C., G. Triadafilopoulos, M. Clark, C. Franzblau, and J. T. LaMont. 1986. *Clostridium difficile* cytotoxin inhibits protein synthesis in fibroblasts and intestinal mucosa. Gastroenterology **91**: 1147-1153.

140   Price, A. B., H. E. Larson, and J. Crow. 1979. Morphology of experimental antibiotic-associated enterocolitis in the hamster: a model for human pseudomembranous colitis and antibiotic associated diarrhoea. Gut **20**: 467-475.

141   Price, S. B., C. J. Phelps, T. D. Wilkins, and J. L. Johnson. 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. **16**: 55-60.

142   Rauvala, H., J. Finne. 1979. Structural similarity of the terminal carbohydrate sequences of glycoproteins and glycolipids. FEBS Lett. **97**: 1-8.

143   Regan, L. J., J. Dodd, S. H. Barondes, and T. M. Jessell. 1986. Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons. Proc. Natl. Acad. Sci. USA **83**: 2248-2252.

144   Rehg, J. E. 1980. Cecal toxin(s) from guinea pigs with clindamycin-associated colitis, neutralized by *Clostridium sordellii* antitoxin. Infect. Immun. **27**: 387-390.

145   Rehg, J. E., and S. P. Pakes. 1982. Implication of *Clostridium difficile* and *Clostridium perfringens* iota toxins in experimental lincomycin-associated colitis of rabbits. Lab. Anim. Sci. **32**: 253-257.

146   Rihn, B, G. Beck, H. Monteil, F. Lecuf, and R. Girardot. 1985. Effect of the cytotoxin of *Clostridium difficile* on cultured hepatoma cells. Biol. Cell **53**: 23-32.

147   Rietra, P. J. G. M., K. W. Slaterus, H. C. Zanen, and S. G. M. Meuwissen. 1978. Clostridial toxins in healthy infants. Lancet **ii**: 319.

148   Rifkin, G. D., R. Fekety, and J. Silva. 1978. Neutralization by *Clostridium sordellii* antitoxin of toxins implicated in clindamycin-induced cecitis in the hamster. Gastroenterology **75**: 422-424.

149   Rifkin, G. D., J. Silva, Jr., and F. R. Fekety. 1978. Gastrointestinal and systematic toxicity of fecal extracts from hamsters with clindamycin-induced colitis. Gastroenterology. **74**: 52-57.

150   Rifkin, G. D., J. Silva, Jr., F. R. Fekety, and R. B. Sack. 1977. Antibiotic-induced colitis. Implication of a toxin neutralized by *Clostridium sordellii* antitoxin. Lancet ii: 1103-1106.

151   Rolfe, R. D., and J. P. Iaconis. 1983. Intestinal colonization of infant hamsters with *Clostridium difficile*. Infect. Immun. **42**: 480-486.

152   Rothman, S. W., M. K. Gentry, J. E. Brown, D. A. Foret, M. J. Stone, and M. P. Strickler. 1988. Immunochemical and structural similarities in toxin A and toxin B of *Clostridium difficile* shown by binding to monoclonal antibodies. Toxicon **26**: 583-597.

153   Rothman S. W., and J. E. Brown. 1981. Inhibition of membrane function in intact HeLa cells by *Clostridium difficile* cytotoxic culture filtrates. Curr. Microbiol. **6**: 221-224.

154   Sadler, J. E. 1984. Biosynthesis of glycoproteins: formation of O-linked oligosaccharides. In Vol. 2 *Biology of carbohydrates*. V. Ginsburg, and P. W. Robbins (Eds.). John Wiley and Sons. pp. 199-288.

155   Saginur, R., R. Fogel, L. Begin, B. Cohen, and J. Mendelson. 1983. Splenic abscess due to *Clostridium difficile*. J. Infect. Dis. **147**: 1105.

156   Sauerborn, M., and C. von Eichel-Streiber. 1990. Neucleotide sequence of *Clostridium difficile* toxin A. Nucl. Acids Res. **18**: 1629-1630.

157   Schwan, A., S. Sjolin, U. Trottestam, and B. Aronsson. 1984. Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of normal faeces. Scand. J. Infect. Dis. **16**: 211-215.

158   Sherertz, R. J., and F. A. Sarubbi. 1982. The prevalence of *Clostridium difficile* in a nursery population: a comparison between patients with necrotizing enterocolitis and a asymptomatic group. J. Pediatr. **100**: 435-441.

159   Silva, J., R. Fekety, C. Werk, J. Ebright, M. Cudmore, D. Batts, C. Syrjamaki, and J. Lukens. 1984. Inciting and etiologic agents of colitis. Rev. Infect. Dis. **6**: S214-S221.

160   Small, J. D. 1968. Fatal enterocolitis in hamsters given lincomycin hydrochloride. Lab. Anim. Care. **18**: 411-420.

161   Small, M. F., S. P. Borriello, G. S. Clayden, and M. W. Casewell. 1980. Clinical and bacteriological findings in necrotizing enterocolitis: a controlled study. J. Infect. Dis. **7**: 23-31.

162   Smith, M. F., S. P. Borriello, G. S. Clayden, and M. W. Casewell. 1980. Clinical and bacteriological findings in necrotizing enterocolitis: a controlled study. J. Infect. Dis. **7**: 23-31.

163   Sofianou, D. C. 1988. Pancreatic abscess caused by *Clostridium difficile*. Eu. J. Clin. Infect. Dis. **7**: 528-530.

164   Stark, P. L., A. Lee, and B. D. Parsonage. 1982. Colonization of the large bowel by *Clostridium difficile* in healthy infants: quantitative study. Infect. Immun. **35**: 895-899.

165   Sullivan, N. M., S. Pellett, and T. D. Wilkins. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1032-1040.

166   Taylor, N. S., G. M. Thorne, and J. G. Bartlett. 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect Immun. **34**: 1036-1043.

167   Tedesco, F. J., 1976. Clindamycin-associated colitis. Review of the clinical spectrum of 47 cases. Digest. Dis. **21**: 26-32.

168   Tedesco, F. J., R. W. Barton, and D. H. Alpers. 1974. Clindamycin-associated colitis: a perspective study. Ann. Intern. Med. **81** 429-433.

169   Thelestam, M., and M. Bronnegard. 1980. Interaction of cytopathic toxin from *Clostridium difficile* with cells in tissue culture. Scand. J. Dis. **22**: 16-29.

170   Thelestam, M, and I. Florin. 1984. Cytopatogenic action of *Clostridium difficile* toxins. J. Toxicol. Toxin. Rev. **3**: 139-180.

171   Thelestam, M., and I. Florin. 1984. Interaction of *Clostridium difficile* toxin B (cytotoxin) with cultured cells. In *Antibiotic associated diarrhoea and colitis: Clostridium difficile in gastrointestinal disorders*. S. P. Borreillo (Ed.). Martinus Nijhoff Publishers, Boston. pp. 103-118.

172   Toffler, R. B., E. G. Pingould, and M. I. Burrell. 1978. Acute colitis related to penicillin derivatives. Lancet **ii**: 707-709.

173   Torres, J. F., R. Cedillo, J. Sanchez, C. Dillman, S. Giono, and O. Munoz. 1984. Prevalance of *Clostridium difficile* and its cytotoxin in infants in Mexico. J. Clin. Microbiol. **20**: 274-275.

174   Triadafilopoulos, C. Pothoulakis, R. Weiss, C. Giampaolo, and J. T. LaMont. 1989. Comparative study of *Clostridium difficile* toxin A and cholera toxin in rabbit ileum. Gastroenterology **97**: 1186-1192.

175   Triadafilopoulos, G., C. Pothoulakis, M. J. O'Brien, and J. T. LaMont. 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology **93**: 273-279.

176   Tullus, K., B. Aronsson, S. Marcus, and R. Mollby. 1989. Intestinal colonization with *Clostridium difficile* in infants up to 18 months of age. Eur. J. Clin. Microbiol. Infect. Dis. **8**: 303-393.

177   Vernet, A., G. Corthier, F. Dubos-Ramare, and L. Parodi. 1989. Relationship between levels of *Clostridium difficile* toxin A and toxin B cecal lesions in gnotobiotic mice. Infect. Immun. **57**: 2123-2127.

178   Wada, N., N. Nishida, S. Iwaki, H. Ohi, T. Miyawaki, N. Taniguchi, and S. Migita. 1980. Neutralizing activity against *Clostridium difficile* toxin in the supernatents of cultures colostral cells. Infect. Immun. **29**: 545-550.

179   Watkins, W. M., P. Greenwell, A. D. Yates, and P. H. Johnson. 1988. Regulation of expression of carbohydrate blood group antigens. Biochemie **70**: 1597-1611.

180   Wilson, K. H., and R. Freter. 1985. Suppression of *Clostridium difficile* by isolates from indigenous hamster flora. Microecol. Ther. **15**: 323-327.

181   Wilson, K. H., J. Silva, and F. R. Fekety. 1981. Suppression of *Clostridium difficile* by normal hamster cecal flora and prevention of antibiotic-associated cecitis. Infect. Immun. **34**: 626-628.

182   Youakim, A., A. Herscovics. 1987. Differentiation-associated decrease in the proportion of fucosylated polylactosaminoglycans of CaCo-2 human colonic adenocarcinoma cells. Biochem. J. **247**: 299-306.

BINDING OF *CLOSTRIDIUM DIFFICILE* ENTEROTOXIN
TO ADULT AND INFANT HAMSTER INTESTINAL EPITHELIUM

Kenneth D. Tucker

Department of Anaerobic Microbiology

Virginia Polytechnic Institute and State University

Blacksburg, Virginia 24061

ABSTRACT

Clostridium difficile can cause a severe diarrheal disease in both hamsters and humans following antibiotic therapy. However, the infants of both species may be colonized by *C. difficile* without prior antibiotic therapy, and apparently with no ill effects. Previously, toxin A has been shown to bind to carbohydrates that contain Galα1-3Galβ1-4GlcNAc on hamster intestinal epithelium. I have found that toxin A bound to the intestinal epithelium of both infant and adult hamsters by this receptor. Yet, the amount of toxin A that bound to the infant tissue at 37C was 10-fold less than the amount of toxin A that bound to the adult tissue. This suggests that the insensitivity of infant hamsters to toxin A could be due to the relatively low binding of toxin A to their intestinal epithelium.

INTRODUCTION

Clostridium difficile causes pseudomembranous colitis in humans. This organism can colonize the intestines and grow to high numbers if antibiotic therapy has disrupted the normal microflora. Once established in the intestines, the organism can cause diarrhea by producing a protein toxin, toxin A, that erodes the intestinal epithelium (23, 24, 25). Further tissue damage may be caused by a second toxin, toxin B, which is not active on intestinal mucosa (23, 24), but is toxic to the underlying tissue that is exposed by the action of toxin A. Infant humans also may be colonized by *C. difficile*, but do not require prior antibiotic therapy for this to occur (1, 3, 6, 7, 16, 21, 22, 26, 27, 29, 30). Yet *C. difficile* causes no obvious ill effects in infants, even though infants may be colonized by toxigenic *C. difficile*. Chang et al. (5) reported that human infant intestinal cells are less sensitive to both toxin A and toxin B than are adult intestinal cells. This indicates the low activity of toxin A and toxin B on the intestinal cells results in the insensitivity of infants to the toxins. However, the reason for the apparent insensitivity of the cells to toxin A and toxin B is not known

Like humans, adult hamsters also may be colonized by *C. difficile* after antibiotic treatments, and this results in a severe and fatal diarrhea in this animal (13, 28). Also like humans, infant hamsters may be colonized by *C. difficile* without prior antibiotic treatments, and with no obvious ill effects (13, 15, 28). Yet, the reason for the lack of sensitivity of the infants to toxin A is not known.

In this study, I examined the binding of toxin A to intestinal tissue from both infant and adult hamsters to determine if lack of toxin binding is a possible explanation for the infants' insensitivity to toxin A. Since toxin A is known to bind to carbohydrates that contain Galα1-3Galβ1-4GlcNAc on adult hamster intestinal tissue (19), I also determined if toxin A bound to this receptor. I found that toxin A bound to Galα1-3Galβ1-4GlcNAc on the intestinal mucosa of both adult and infant hamsters, but only the adult tissue bound toxin A at body temperature.

MATERIALS AND METHODS

Toxin A purification: Cultures of *C. difficile* VPI strain no. 10463 were grown in dialysis flask cultures of brain heart infusion as previously described (25). Toxin A was purified by affinity chromatography using immobilized bovine thyroglobulin (20). The toxin A was determined to be homogeneous using procedures previously described (25). The concentration of toxin A was estimated with the method of Bradford (4) using bovine gamma globulin as a reference.

Tissue preparation: Adult golden Syrian hamsters between 9-14 weeks of age, and infant hamsters at 8 days of age were obtained from a colony at the laboratory animal facilities at Virginia Polytechnic Institute and State University. Intestinal epithelial brush border membranes were prepared as previously described (9). In the infants the thinness of the tissue prevented the separation of the mucosa from the serosa, so the entire intestine was used to prepare the brush border membranes. This should not have affected the results because the purification procedure removes connective tissue, so the serosa was removed from the mucosa later in the procedure. Both the adult and infant tissue preparations contained predominantly brush border membranes, as determined by observation with phase contrast microscopy. The tissues were quantitated based on the amount of protein present in each preparation, as determined by the method of Bradford (4) using bovine gamma globulin as a reference.

Binding assay: The binding of toxin A to the adult and infant tissues was assayed in parallel at 4C and at 37C using an ELISA to quantitate the residual toxin as

previously described (19). The lectin *Griffonia simplicifolia* I isolectin B_4 (GSI-B_4), which specifically binds to carbohydrates that contain Galα1-3Gal on their non-reducing end (12), was used to ascertain that the receptor to which toxin A bound was the proposed Galα1-3Galβ1-4GlcNAc receptor for the toxin. The results presented in this paper are from one experiment that was representative of the results from three repeats of the binding assay.

RESULTS AND DISCUSSION

Intestinal epithelium from either adult or infant hamsters bound similar amounts of toxin A at 4C (Figure 1). In both adults and infants the binding of toxin A was blocked by the lectin GSI-B$_4$, which indicates the toxin was binding via the previously described receptor for the toxin (Galα1-3Galβ1-4GlcNAc). Both the infant and adult tissue bound much less toxin at 37C than at 4C; however, the adult tissue bound much more toxin at 37C than did the infant tissue (Figure 1). This suggests that the apparent insensitivity of infant hamsters to toxin A, relative to adult hamsters, may be due to the amount of toxin A bound to the epithelium of each at 37C.

The temperature dependent binding of toxin A to hamster intestinal epithelium was initially observed by Krivan et al. (19), who postulated that the temperature dependent binding was due to the density of the receptor. Receptor density is developmentally regulated in humans with a specific example being the iI antigen system (11, 14). The i antigen is a linear carbohydrate that ends with Galβ1-4GlcNAc (8), which is the biosynthetic precursor of the receptor for toxin A (2). This antigen is commonly found in infants up to 2 years of age (11). The I antigen (Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Gal) is a branched form of the i antigen (8). The I antigen begins to appear as the i antigen starts to disappear (11, 14). The branching of the I antigen creates a large number of i antigens on a single carbohydrate core, thus creating a higher density of receptor. If this developmental regulation of the iI antigens also occurs in hamsters, this

could explain why only the adult hamster intestinal epithelium bound toxin A at 37C.

Some researchers have suggested that infant humans and hamsters are passively immunized against toxin A by mother's milk, and that this provides protection from the activity of toxin A (17, 18, 31). This could be one reason infant hamsters are resistant to toxin A because they are always breast fed, but infant humans are not. In addition to passive immunization, my results indicate that infants may evade the activity of toxin A by not binding the toxin to their intestinal epithelium.

Humans have not been shown to express Galα1-3Galβ1-4GlcNAc (10). Even so, the density of any carbohydrate receptor with the core structure Galβ1-4GlcNAc (i.e., the iI antigen system) would be under developmental regulation in humans. Thus the apparent insensitivity of human infants may be related to the reduced binding of the toxin on low density receptors.
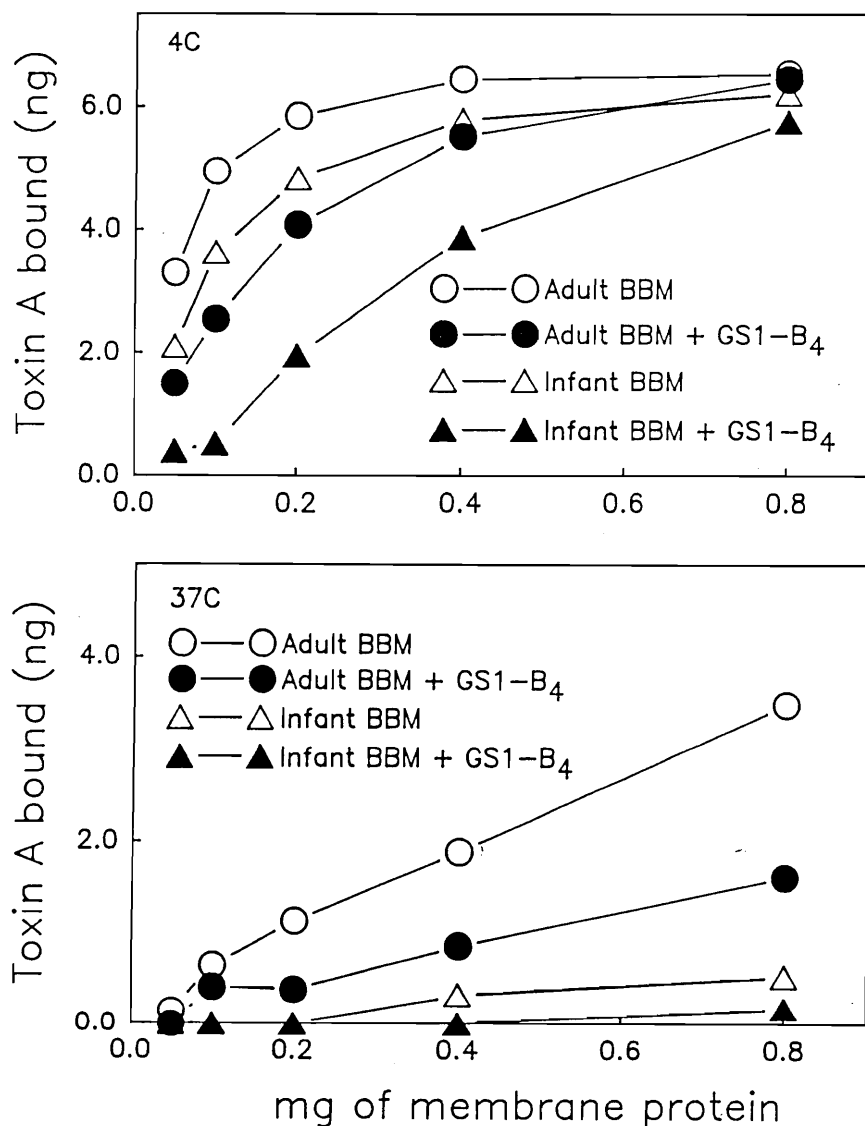
Figure 1. Binding of toxin A at 4C and at 37 to brush border membranes (BBM) isolated from adult and infant hamsters. GS1-B$_4$ is a lectin that binds to Galα1-3Gal and was used to competitively inhibit the binding of toxin A to the BBM, which indicates toxin A was binding to the carbohydrate receptor Galα1-3Galβ1-4GlcNAc.

LITERATURE CITED

1   Bacon, A. E., R. Fekety, D. R. Schaberg, ans R. G. Faix. 1988. Epidemiology of *Clostridium difficile* colonization in newborns: results using a bacteriophage and bacteriocin typing system. J. Infect. Dis. **158**: 349-354.

2   Blaken, W. M., D. H. Van den Eijnden. 1985. Biosynthesis of terminal Galα1-3Galβ1-4GlcNAc-R oligosaccharide sequences on glycoconjugates. J. Biol. Chem. **260**: 12927-12934.

3   Bolton, R. P., S. K. Tait, P. R. F. Dear, and M. S. Losowsky. 1984. Asymptomatic neonatal colonisation by *Clostridium difficile*. Arch. Dis. Childhood **59**: 466-472.

4   Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

5   Chang, T.-W., N. M. Sullivan, and T. D. Wilkins. 1986. Insusceptibility of fetal intestinal mucosa and fetal cells to *Clostridium difficile* toxins. Acta Phar. Sin. **5**: 448-453.

6   Delmee, M., G. Verellen, V. Avesani, and G. Francois. 1988. *Clostridium difficile* in neonates: serogrouping and epidemiology. Eur. J. Pediatr. **147**: 36-49.

7   Donta, S. T., and M. G. Myers. 1982. *Clostridium difficile* toxin in asymptomatic neonates. J. Pediatrics. **100**: 431-434.

8   Egge, H., M. Kordowicz, J. Peter-Katalinic, and P. Hanfland. 1985. Immunochemistry of I/i-active oligo- and polyglycosylceramides from rabbit erythrocyte membranes. J. Biol. Chem. **260**: 4927-4935.

9   Forstner, G. G., S. M. Sabesin, and K. J. Isselbacher. 1968. Rat intestinal microvillus membranes: purification and biochemical characterization. Biochem. J. **106**: 381-390.

10   Galili, U., S. B. Shohet, E. Kobrin, C. L. M. Stults, B. A. Macher. 1988. Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J. Biol. Chem. **263**: 17755-17762.

11   Gilbert, E. R. 1983. Erythrocyte antigens and antibodies. In *Hematology*. W. J. Williams, E. Beutler, A. J. Erslev, and M. A. Lichtman (Eds.). McGraw-Hill Book Co. New York. pp. 1491-1505 .

12   Goldstein, I. J., and R. D. Portez. 1986. Isolation, physicochemical characterization, and carbohydrate binding specificity of lectins. in *The lectins: properties, functions, and applications in biology and medicine.* I. E. Liener, N. Sharon, and I. J. Goldstein (Eds.). Academic Press Inc. pp 33-247.

13   Griffin, G. E., A. McDougall, S. A. Ash, and S. P. Borriello. 1984. Age related susceptibility of hamsters to clindamycin induced *Clostridium difficile* caecitis. Microecol. Thrapy **14**: 269-270.

14   Hakomori, S. I. 1981. Blood group ABH and Ii antigens of human erythrocytes: chemistry, polymorphism, and their developmental change. Semin. Hematol. **18**: 39-62.

15   Iaconis, J. P., and R. D. Rolfe. *Clostridium difficile*-associated ileocecitis in clindamycin-treated infant hamsters. Curr. Microbiol. **13**: 327-332.

16   Karsch, W., E. Strelau, W.-D. Grahlow, E. Fischer, ans R. Schulz. 1989. Occurence and significance of *Clostridium difficile* in faecal specimens of hospitalized children. Zbl. Bakt. Hyg. **270**: 441-448.

17   Kim, K., L. K. Pickering, H. L. DuPont, N. Sullivan, T. Wilkins. 1984. In vitro and in vivo neutralizing activity of human colostrum and milk against purified toxins A and B of *Clostridium difficile.* J. Infect. Dis. **150**: 57-62.

18   Kim, P.-H., J. P. Iaconis, and R. D. Rolfe. 1987. Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect. Immun. **55**: 2984-2992.

19   Krivan, H. C.,G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence $Gal\alpha$1-3$Gal\beta$1-4GlcNAc. Infect. Immun. **53**: 573-581.

20   Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55**: 1873-1877.

21   Larson, H. E., F. E. Barclay, P. Honour, and I. D. Hill. 1982. Epidemiology of *Clostridium difficile* in infants. J. Infect. Dis. **146**: 727-733.

22   Libby, J. M., S. T. Donta, and T. D. Wilkins. 1983. *Clostridium difficile* toxin A in infants. J. Infect. Dis. **148**: 606.

23   Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultures cells in vitro. Infect. Immun. **56**: 582-588.

24   Lyerly, D. M., K. E. Saum, D. K. MacDonald, and T. D. Wilkins. 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47**: 349-352.

25   Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins. 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1147-1150.

26   Mardh, P.-A., I. Helin, I. Colleen, M. Oberg, and E. Holst. 1982. *Clostridium difficile* toxin in faecal specimens of healthy children and children with diarrhoea. Acta. Pediatr. Scand. **71**: 275-278.

27   Rietra, P. J. G. M., K. W. Slaterus, H. C. Zanen, and S. G. M. Meuwissen. 1978. Clostridial toxins in healthy infants. Lancet **ii**: 319.

28   Rolfe, R. D., and J. P. Iaconis. 1983. Intestinal colonization of infant hamsters with *Clostridium difficile*. Infect. Immun. **42**: 480-486.

29   Torres, J. F., R. Cedillo, J. Sanchez, C. Dillman, S. Giono, ans O. Munoz. 1984. Prevalance of *Clostridium difficile* and its cytotoxin in infants in Mexico. J. Clin. Microbiol. **20**: 274-275.

30   Tullus, K., B. Aronsson, S. Marcus, and R. Mollby. 1989. Intestinal colonization with *Clostridium difficile* in infants up tp 18 months of age. Eur. J. Clin. Microbiol. Infect. Dis. **8**: 303-393.

31   Wada, N., N. Nishida, S. Iwaki, H. Ohi, T. Miyawaki, N. Taniguchi, and S. Migita. 1980. Neutralizing activity against *Clostridium difficile* toxin in the supernatents of cultures colostral cells. Infect. Immun. **29**: 545-550.

TOXIN A OF *CLOSTRIDIUM DIFFICILE* IS A POTENT CYTOTOXIN

Kenneth D. Tucker

Pauline E. Carrig, and Tracy D. Wilkins

Department of Anaerobic Microbiology

Virginia Polytechnic Institute and State University

Blacksburg, Virginia 24061

ABSTRACT

Clostridium difficile is the cause of antibiotic associated colitis in humans. The organism produces toxin A, which is generally known as the enterotoxin, and toxin B, which is known as the cytotoxin. Toxin A has been reported to have slight cytotoxic activity; in this study we show that cell lines (F9, OTF9-63 and P19) which express a carbohydrate to which toxin A binds are more sensitive to the toxin. These cell lines can be used as research tools for determining concentrations of biologically active toxin A and should also prove useful for studies of the mechanism of action of the toxin.

INTRODUCTION

Clostridium difficile, which causes antibiotic associated pseudomembranous colitis in humans, produces two toxins, A and B. Toxin B is one of the most potent cytotoxins known; only a picogram is required to cause a cytotoxic effect on cultured cells (1,11,13,14). Because of the high cytotoxic activity of this toxin, the neutralization of the cytotoxic activity by specific antisera is the "gold standard" for diagnosis of the disease. Interestingly, although toxin B is the toxin detected by the neutralization assay, it is not the toxin thought to be responsible for most of the symptoms of pseudomembranous colitis. Toxin A is thought to cause both the diarrhea and the destruction of the colonic mucosa which is characteristic of this disease (1,10,13,14). Toxin B appears to have no effect on undisturbed colonic mucosa (1,10,13,14).

Toxin A is not a classical enterotoxin in that the fluid response is not a clear liquid but rather a hemorrhagic exudate composed of serum components and cellular debris (10). There is some controversy over whether toxin A also is cytotoxic to cultured cells. Banno et al. (1) reported that toxin A had no cytotoxic activity; other laboratories reported the activity of the toxin to be 1,000- to 10,000-fold less than the activity of toxin B on the same cell lines (5,11,13,14). Preparations of either toxin A or toxin B cause the same change in morphology of cultured cells, thus increasing the suspicion that the cytotoxic activity of the toxin A preparations might be due to contamination with toxin B. The main fact which would refute this concept is that specific antibodies to toxin B do not

neutralize the cytotoxicity of toxin A (13). However, a direct demonstration that toxin A has cytotoxic activity independent of toxin B has not been presented.

In this paper, we show that some cell lines are much more sensitive to toxin A than those that have been tested previously. The morphological effect on these cultured cells appeared to be the same for each toxin, and these effects were neutralized by antibodies specific for the toxin tested.

MATERIALS AND METHODS

C. difficile VPI 10463 was grown in brain heart infusion dialysis flasks as previously described (13). Toxin A was purified by affinity chromatography using immobilized bovine thyroglobulin (8). Toxin B was purified by ion exchange chromatography on DEAE-Sepharose CL-6B and immunoadsorption (11). The purity of the toxins was demonstrated by crossed immunoelectrophoresis and polyacrylamide gel electrophoresis as previously described (13). Protein concentrations were estimated by the method of Bradford (2) with bovine gamma globulin as a standard.

Affinity-purified antibodies to toxin A were purified as previously described using homogeneous toxin A coupled to AffiGel 10 (Bio-Rad Laboratories, Richmond, Calif.) (12). Affinity purified antibodies to toxin B were prepared similarly using immobilized toxin B. The concentration of the affinity-purified antibodies was estimated using an $E_{280,1\%}$ of 14. For neutralization assays, the antibodies to the toxins were mixed 1:1 with serial dilutions of toxin, and the mixtures were incubated for one hour on ice before addition to the culture wells (7).

The cytotoxic activities of toxins A and B on the mouse teratocarcinoma cell lines F9, OTF9-63, P19, and P19S18O1A1 were determined by using the Chinese hamster ovary-K1 (CHO) cell line as a reference as previously described (7). The F9 cells were grown in Dulbecco modified Eagle medium containing 15% heat inactivated fetal bovine serum on tissue culture plates coated with 0.1%

gelatin. The OTF9-63, P19, and P19S18O1A1 cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. The CHO cells were grown in Ham F12 medium containing 2% fetal bovine serum. A cytotoxic unit was defined as the least amount of toxin that caused rounding of 90% of the cells after 18 hours of exposure to the toxin.

RESULTS

Our initial hypothesis was that cell lines which expressed the trisaccharide to which toxin A binds would be more sensitive to the toxin than other cell lines. We chose to test three mouse teratocarcinoma cell lines which had been reported to express this carbohydrate structure on their membranes (4,6). We compared the activity of toxin A on these cell lines to the cytotoxicity on CHO cells, which are not known to express this trisaccharide. Toxin A was 100-fold more active on these three cell lines than on CHO cells (Figure 1).

We also tested the effect of toxin A on a subclone of the P19 cell line (P19S1801A1) which has been reported to express less of the trisaccharide than the other cell lines do (6). This cell line was 16-fold less sensitive than the parent P19 cell line. The sensitivity of four other cell lines to toxin A has been reported previously by Donta et al. (5) (Table 1). The Y1 cells were the most sensitive of these cell lines, but they are ten fold less sensitive than the most sensitive cell lines reported on in this paper.

All of the cells we tested reacted to toxin A in the same manner as cells react to toxin B: they lost all of their distinctive morphology and became round, with very little attachment to the plastic. Cells treated with toxin A were thus indistinguishable on a morphological basis from the same cells treated with toxin B.
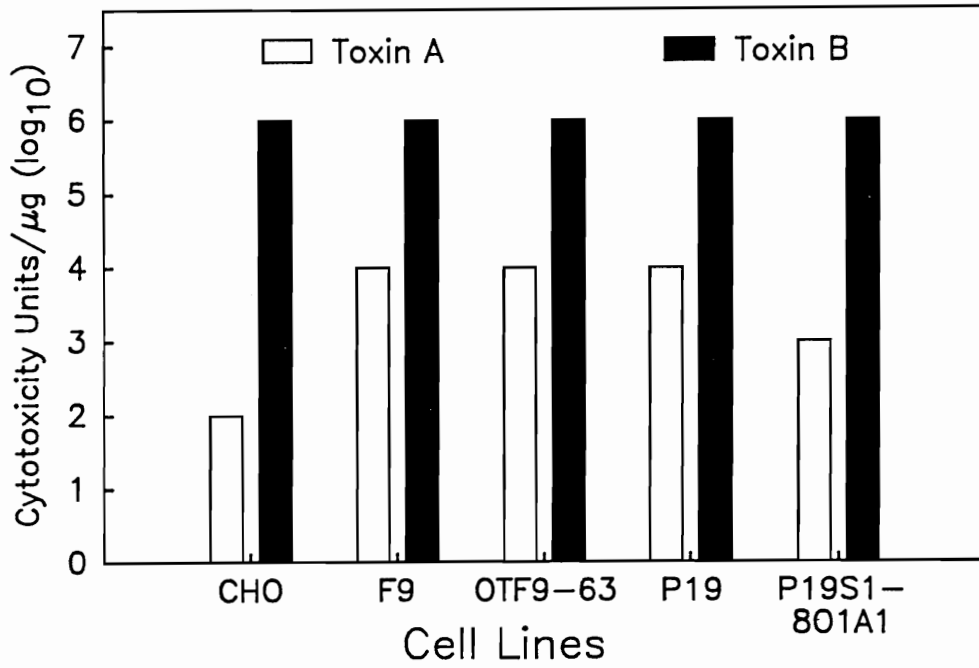
FIGURE 1. Cytotoxic units (the least amount of toxin to cause rounding of 90% of the tissue culture cells after 18 hours of exposure to the toxin) per microgram of toxin.

TABLE 1. Activities of toxins A and B previously reported[a]

Cell line	Minimal cytotoxic dose		Reference
	Toxin A (ng/ml)	Toxin B (pg/ml)	
Y1	3	1.5	5
CHO	50	5	5
HeLa	200	5	5
MHC	13	5	5
WI-38	200	5	14

[a] Because of differences in the assays used in reference 5, activities of the toxins were normalized by using the CHO cell line as a reference to determine the relative sensitivities of the other cell lines. This table is limited to cell lines that were assayed for sensitivity to toxin A.

TABLE 2. Minimal dose of toxin A required for positive reaction

Assay	Organism or cell line	Minimum dose (ng) for positive reaction	Reference
Enterotoxicity	Rabbit	1000	10
Lethality (LD_{100}[a])	Mice	50	11
Cytotoxicity	CHO	10	11
Indirect ELISA[b]		1	12
Cytotoxicity	F9,OTF9-63, or P19	0.1	This article

DISCUSSION

Our results support my initial hypothesis that cell lines which express the trisaccharide (4,6) to which toxin A binds (3,9) will be more sensitive to the toxin than other cell lines. The fact that a subclone of one of these cell lines which expresses less of this trisaccharide was eight-fold less sensitive than the parent clone also strengthens this argument. This is not proof that this trisaccharide is a functional receptor for the toxin, but it is suggestive; considerably more work will have to be done to determine if this is the case, and we are now involved in this investigation.

The increase in cytotoxic activity of toxin A in these cell lines with no increase in the activity of toxin B demonstrates that toxin A definitely has a cytotoxic activity. The cytotoxic response of cells to toxin A appear to be very similar if not identical to the responses to toxin B. The mechanism of action of these toxins has not been defined at the molecular level, so there is currently no way to determine whether these toxins have the same enzymatic mechanism of action. We believe that the enterotoxicity of toxin A could be the result of the same cytotoxic response that we report here. The enterotoxicity could result from disorganization of cells in the brush border, which would allow serum proteins and fluids to pass into the lumen.

The cell lines we tested should prove useful to investigators who desire to determine levels of biologically active toxin A. These cells are much more sensitive than the rabbit ileal loop test for enterotoxicity and are more sensitive than the enzyme linked immunosorbent assay which our laboratory previously

used to detect toxin A (Table 2). The cytotoxicity assay, when performed with any of these three cell lines, is the most sensitive test for the toxin thus far reported. The cytotoxicity test for toxin B is also the most sensitive method available for detecting this toxin. The cytotoxicity test for toxin B is about 100-fold more sensitive than the toxin A cytotoxicity test when these more sensitive cell lines are used; thus, the cytotoxicity of toxin B would still mask the effect of toxin A unless specific antibody is used to neutralize toxin B.

The cytotoxicity assay for toxin B in feces is the standard to which other methods for diagnoses of *C. difficile* colitis are compared. Toxin B does not initiate the diarrhea and colitis and, in fact, has no effect on normal intestinal mucosa. Assaying for toxin B is useful because a positive test indicates that both toxins are present. From about 150 isolates, our laboratory has never found a case of a strain producing toxin B without also producing toxin A, and in general about equal amounts of both toxins are produced in culture media. Because of the lack of sensitive tests for active toxin A, there have not been any studies of the levels of active toxin A versus levels of active toxin B in the feces of patients. The tissue culture cell lines we describe here could be used for such determinations. If these studies showed that the levels of active toxin A correlated better with the clinical course of the patient than did concentrations of toxin B, then assays for toxin A could be developed for routine use in clinical laboratories.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa. 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2:**629-635.

2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.

3. Clark, G. F., H. C. Krivan, T. D. Wilkins, and D. F. Smith. 1987. Toxin A of *Clostridium difficile* binds to rabbit erythrocyte glycolipids with terminal Galα1-3Galß1-4GlcNAc sequences. Arch. Biochem. Biophys. **257:**217-229.

4. Cummings, R. D., and S. A. Mattox. 1988. Retinoic acid-induced differentiation of the mouse teratocarcinoma cell line F9 is accompanied by an increase in the activity of UDP-galactose:ß-D-galactosyl-α1,3-galactosyl transferase. J. Biol. Chem. **263:**511-519.

5. Donta, S. T., N. Sullivan, and T. D. Wilkins. 1982. Differential effects of *Clostridium difficile* toxins on tissue-cultured cells. J. Clin. Microbiol. **15:**1157-1158.

6. Draber, P., and P. Stanley. 1984. Cytotoxicity of plant lectins for mouse embryonal carcinoma cells. Somatic Cell Mol. Genet. **10:**435-444.

7. Ehrich, M., R. L. van Tassell, J. M. Libby, and T. D. Wilkins. 1980. Production of *Clostridium difficile* antitoxin. Infect. Immun. **28:**1041-1043.

8. Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55:**1873-1877.

9. Krivan, H. C., G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galß1-4GlcNAc. Infect. Immun. **53:**573-581.

10. Lima, A. A., D. M. Lyerly, T. D. Wilkins, D. J. Innes, R. L. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestines in vivo and on cultured cells in vitro. Infect. Immun. **56:**582-588.

11. Lyerly, D. M., M. D. Roberts, C. J. Phelps, and T. D. Wilkins. 1986. Purification and properties of toxins A and B of *Clostridium difficile*. FEMS Microbiol. Lett. **33:**31-35.

12.   Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins. 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**:72-78.

13.   Sullivan, N. M., S. Pellett, and T. D. Wilkins. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032-1040.

14.   Taylor, N. S., G. M. Thorne, and J. G. Bartlett. 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036-1043.

EVIDENCE THAT *CLOSTIRDIUM DIFFICILE* TOXIN A

USES GALα1-3GALß1-4GLCNAC

AS A PHYSIOLOGICAL RECEPTOR.

Kenneth D. Tucker

Department of Anaerobic Microbiology

Virginia Polytechnic Institute and State University

Blacksburg, Virginia. 24061

ABSTRACT

Clostridium difficile causes pseudomembranous colitis in humans, and ileocecitis in hamsters. Toxin A from this organism has cytotoxic, enterotoxic, and lethal activities. Previous reports from this laboratory demonstrated that toxin A binds to the carbohydrate Galα1-3Galß1-4GlcNAc on rabbit erythrocytes and a similar, if not identical, carbohydrate on the hamster ileum. However, Galα1-3Galß1-4GlcNAc has not been shown to be a physiological receptor (i.e., a binding site on the cell surface that facilitates the activity of a toxin). I found that the mouse teratocarcinoma cell line F9, which expresses large amounts of Galα1-3Galß1-4GlcNAc, is 100-fold more sensitive to toxin A than cell lines which lack the carbohydrate. I studied the role of Galα1-3Galß1-4GlcNAc in the sensitivity of the F9 cell line to toxin A, and provide evidence that Galα1-3Galß1-4GlcNAc acts as a physiological receptor for toxin A on the F9 cells.

INTRODUCTION

Clostridium difficile is the cause of pseudomembranous colitis in humans and ileocecitis in hamsters. This microorganism can colonize the intestines after antibiotic therapy has disrupted the normal intestinal microflora. In the intestines *C. difficile* produces two potent cytotoxins, designated toxin A and toxin B. Toxin B does not appear to be toxic to intact intestinal mucosa, whereas toxin A causes extensive tissue damage to intestinal mucosa (1, 14, 16, 20, 26). This tissue damage caused by toxin A destroys the normal permeability barrier formed by the intestinal epithelium so that body fluids seep into the intestinal lumen resulting in diarrhea (11, 14). Furthermore, the disruption of the intestinal epithelium allows toxin B to exert its toxic activities (17). Therefore toxin A is believed to be responsible for the initial pathology in the diarrheal disease caused by *C. difficile.*

Before a toxin can exert its toxic effect on a cell, it must bind to its physiological receptor on the cell. A physiological receptor has been described By Eidels et al. (6) as "the binding component on the cell surface that is involved in the initial step which ultimately leads to the productive expression of the toxicity of a particular toxin." The carbohydrate sequence $Gal\alpha 1$-$3Gal\beta 1$-$4GlcNAc$ is a binding component on cell membranes for toxin A (3, 12, 13). Furthermore, toxin A binds to this carbohydrate on the intestinal epithelium of hamsters (13), but it has not been proven to be a physiological receptor for toxin A. In this report, I present evidence that $Gal\alpha 1$-$3Gal\beta 1$-$4GlcNAc$ can be a physiological receptor for toxin A.

MATERIALS AND METHODS

Toxin preparation: *C. difficile* VPI 10463 was grown in dialysis flasks (24) containing brain heart infusion. Toxin A was purified by affinity chromatography using immobilized bovine thyroglobulin (12). The purity of the toxin was demonstrated by crossed immunoelectrophoresis and polyacrylamide gel electrophoresis as previously described (19). The concentration of toxin A was estimated by the method of Bradford (2) using bovine gamma globulin as a standard.

Cell lines: The OTF9-63, P19, P19S1801A1, and PA1 cell lines were maintained in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal bovine serum. The F9 cells were grown in Dulbecco modified Eagle medium containing 15% heat-inactivated fetal bovine serum on tissue culture plates coated with 0.1% gelatin. CHO cells were grown in Ham F12 medium containing 2% heat-inactivated fetal bovine serum. Cells were removed from the tissue culture plates with either 0.25% trypsin in Hanks balanced salt solution (P19, P19S1801A1, PA1, and CHO) or with 2 mM EDTA in Hanks balanced salt solution with 2% heat-inactivated fetal bovine serum (F9 and OTF9-63). All cells were grown in an atmosphere of 5% CO_2 at 37C.

Antibodies: PCG-4, an IgG2a monoclonal antibody to toxin A (15), was purified from ascites fluid with immobilized protein A. The concentration of PCG-4 was estimated by the method of Bradford (2) using bovine gamma globulin as the

standard. An IgG2a monoclonal antibody (UPC-10, Sigma Chemical Co., St. Louis, Mo.) was used as a neutral monoclonal antibody.

Cytotoxicity assay: All assays were performed at least three times, with representaative results being reported in this paper. Cytotoxicity assays were performed in 96 well microtiter tissue culture plates as previously described (25). Briefly, cells were plated at approximately 1000 cells per well in 0.18 ml of the media. Cells were allowed to attach for at least 18 hours before additional reagents were added. For all cytotoxicity assays, toxin A was serially diluted 1:2 in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal bovine serum, and 0.02 ml of this stock was added to the wells of the microtiter plate. A cytotoxic unit was defined as the least amount of toxin A that caused rounding of at least 90% of the cells in a microtiter well after 18 hours of exposure to the toxin. The activity of toxin A on a given cell line, or with a given treatment, was reported relative to the activity of toxin A on F9 cells. This was calculated by dividing the number of cytotoxicity units per μg of toxin A on a given cell line by the number of cytotoxicity units per μg of toxin A on F9 cells, then multiplying by 100.

Cell treatments: All assays were performed at least three times, with representative results from a single experiment being presented in this paper.

(i) **tunicamycin:** PA1, CHO and F9 cells were treated with tunicamycin by replacing the original media with media containing tunicamycin

(5 μg/ml). The cells were incubated for 12 hours, then serially diluted toxin was added to the plate. To control for the effect of tunicamycin on toxin A, the media in a second set of wells containing tunicamycin was replaced with normal media before the addition of the toxin. These plates were read 8 hours after the addition of toxin.

(ii) lectin: Four μg of the lectins GS1-B$_4$ or DBA were added to the test wells of a 96 well plate containing either CHO, PA1 or F9 cells. As a control, cells not treated with a lectin were also exposed to toxin A. The cultures were incubated for five minutes, then the serially diluted toxin A was added to the wells. After a five minute incubation period, all the media was removed and the cells were washed twice with 0.2 ml of media. Then 0.2 ml of media was added back to each well and the cytotoxic effect was read after 18 hours.

(iii) Monoclonal antibody: Serially diluted toxin A was mixed 1:1 with either a monoclonal antibody to toxin A (PCG-4) or neutral a IgG2a (UPC-10) to yield a final concentration of 0.4 mg IgG per ml. The mixture was incubated for 30 minutes before being added to culture wells containing either CHO, PA1, or F9 cells. The cytotoxic effect was read after 18 hours.

Hemagglutination inhibition assay: Hemagglutination assays were performed as previously described (13). Toxin A was serially diluted 1:2 in TBS. An equal volume of PCG-4 (0.8 mg/ml, or 0.08 mg/ml) was added to each dilution of toxin. This mixture was incubated for 30 minutes on ice. Then 50 μl of toxin A or 50 μl of toxin A with PCG-4 was added to the wells of a 96 well V-bottom

microtiter plate that was chilled on ice. Fifty μl of 1.5% rabbit erythrocytes was added to each well and the plate was incubated 3 hours at 4C. Hemagglutination is reported as the least amount of toxin required for hemagglutination.

Binding assay. Cells were grown until about 40% of the tissue culture plate was covered. Cells were removed from the tissue culture plate on the same day as the binding assay using 2 mM EDTA in Hanks balanced salt solution containing 2% heat-inactivated fetal bovine serum. The cells were washed once in two volumes of media. Cell numbers were estimated using a hemacytometer. The binding of toxin A to the cells was determined as previously described (13). Briefly, various numbers of cells, not exceeding 5×10^6 cells, were exposed to 7.5 ng of toxin A contained in 0.3 ml of TBS-BSA at 4C for ten minutes. The cells were pelleted by centrifugation, and the unbound toxin A was quantitated by an ELISA (19). Binding of toxin A to cells treated with tunicamycin was assayed using F9 cells grown in the presence of tunicamycin (5μg/ml) for 12 hours. Competitive binding assays with GS1-B$_4$ were performed by exposing the cells to 25 μg of lectin in one ml of TBS-BSA for ten minutes before exposing the cells to toxin A.

RESULTS

To determine if Galα1-3Galß1-4GlcNAc could be a physiological receptor for toxin A, I examined the cytotoxic activity of toxin A on cell lines that either had large amounts of the carbohydrate, or undetectable amounts (Table 1). I found that the activity of toxin A on cell lines that do not express Galα1-3Galß1-4GlcNAc (i.e., CHO and PA1) was only about 1% of the activity of the toxin on the cell lines that express large amounts (i.e., F9, OTF9-63, and P19) (Figure 1). Furthermore, a subclone of the P19 cell line (i.e., P19S1801A1) that expresses less Galα1-3Galß1-4GlcNAc than the P19 cell line (5) was less sensitive to toxin A than was the P19 cell line (Figure 1). The correlation of an increase in the cytotoxic activity of toxin A with cell lines that express Galα1-3Galß1-4GlcNAc suggests that the trisaccharide is a physiological receptor for toxin A.

I focused my further work on the F9 cell line because this cell line produces large amounts of Galα1-3Galß1-4GlcNAc (4, 5), and because it was very sensitive to toxin A. Furthermore, many of the carbohydrates on F9 cells are well characterized. For comparative purposes, I also studied two cell lines (CHO and PA1) which apparently do not express Galα1-3Galß1-4GlcNAc. First I treated the cells with tunicamycin, which is an antibiotic that reduces glycosylation of proteins on eucaryotic cells (23). F9 cells treated with tunicamycin bound less toxin A (Figure 2) and were 75% less sensitive to toxin A than were untreated F9 cells (Figure 3). Since CHO and PA1 cells did not bind detectable amounts of toxin A, the binding of toxin A to tunicamycin treated CHO or PA1 cells was not assayed. Tunicamycin did not affect the activity of toxin A on the PA1 cells.

TABLE 1. Origin of the cell lines, and expression of Galα1-3Galß1-4GlcNAc by the cells.

Cell Line	Lineage	Expression of Galα1-3Galß1-4GlcNAc	References	Source
F9	Mouse teratocarcinoma derived from cell line OTT 6050	Yes*	4,5	a
OTF9-63	Mouse teratocarcinoma derived from cell line F9	Yes§	5	b,c
P19	Mouse teratocarcinoma derived form fetal tissue	Yes§	5	b,d
P19S1801A1	Mouse teratocarcinoma derived from cell line P19	Yes§	5	b,d
PA1	Human teratocarcinoma derived from ovarian tumor	No		e
CHO	Hamster carcinoma derived from ovary biopsy	No		f

* Presence of Galα1-3Galß1-4GlcNAc was directly demonstrated on cells.
§ Presence of Galα1-3Galß1-4GlcNAc is suggested by toxic effect of GS1 upon cells.
a = Dr. D. Smith of the Virginia Polytechnic Institute and State University.
b = Dr. P. Stanley of the Albert Einstein College of Medicine of Yeshiva University.
c = Dr. A. Levine of the Princeton University.
d = Dr. M. McBurney of the University of Ottawa.
e = American Type Culture Collection.
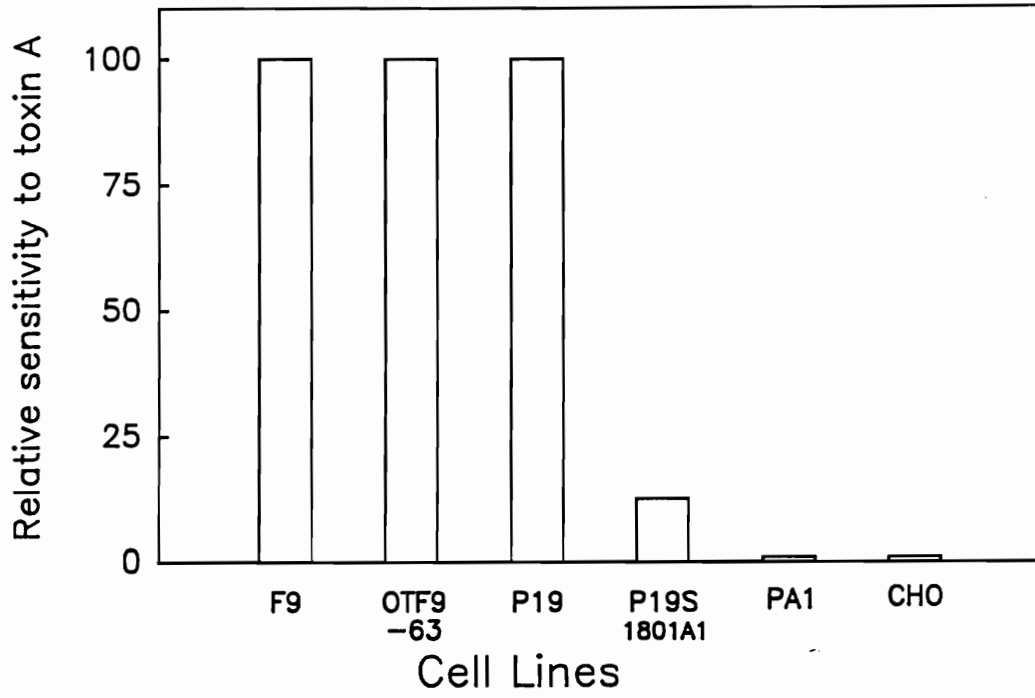f = Dr. B. Storrie of the Virginia Polytechnic Institute and State University.

FIGURE 1. Relative sensitivity of the cell lines to toxin A after exposure to the toxin For 18 hours.
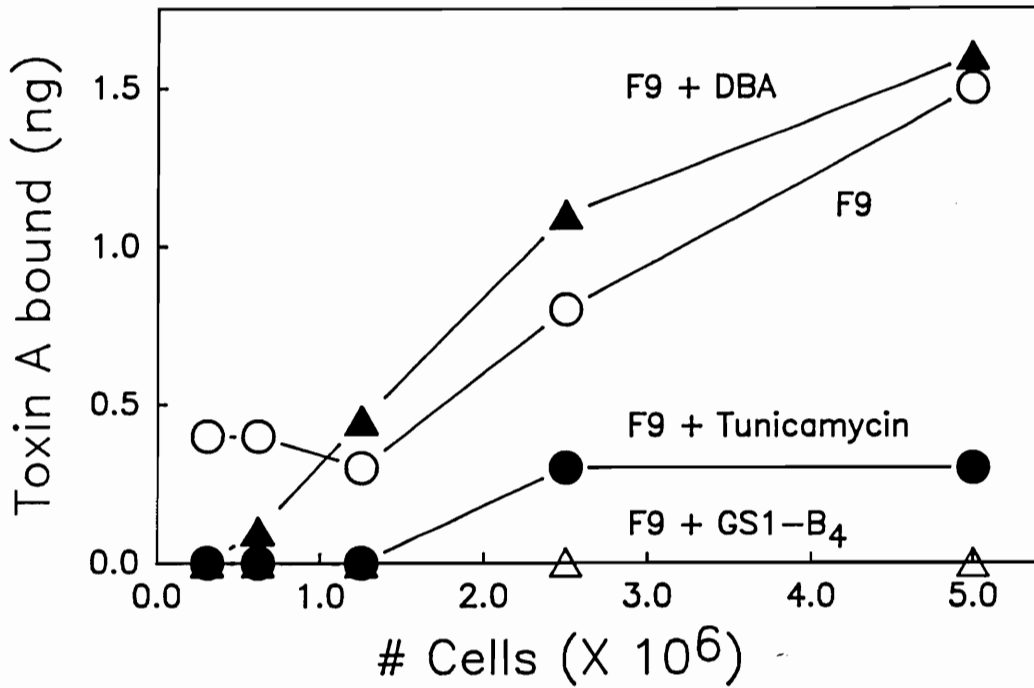
FIGURE 2. Binding of toxin A to F9 cells after the cells had undergone various treatments. Tunicamycin is an antibiotic that reduces glycosylation of protein in eucaryotic cells (23). GS1-B$_4$ and DBA are lectins that bind to carbohydrates that contain α-linked galactose or α-linked N acetylgalactosamine, respectively (9, 21). See text for the parameter of the treatments and discussion.
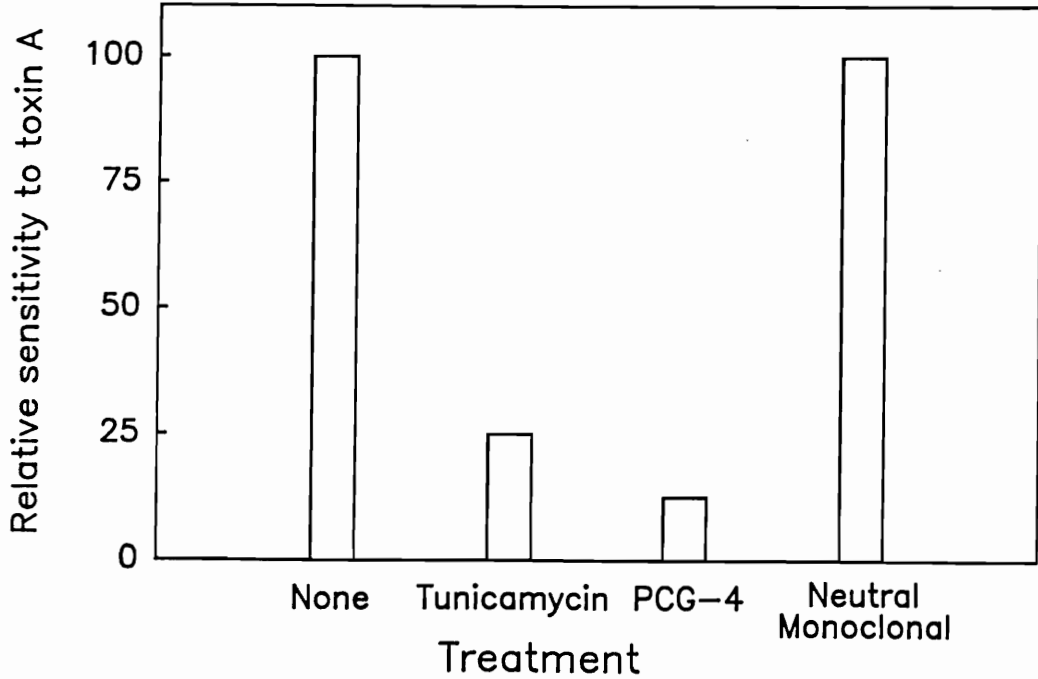
FIGURE 3. Sensitivity of F9 cells to toxin A after the cells had undergone various treatments. Tunicamycin is an antibiotic that reduces glycosylation of protein in eucaryotic cells (23). PCG-4 is a monoclonal antibody to toxin A that reacts with the carbohydrate binding portion of toxin A (22). The neutral monoclonal antibody was the same class as PCG-4, but does not react with toxin A in an antibody/antigen reaction. See text for the parameter of the treatments and discussion.

CHO cells did not remain attached to the culture plate after the tunicamycin treatment, so the activity of toxin A on tunicamycin treated CHO cells could not be determined. Leaving tunicamycin in the tissue culture, or removing it before the addition of toxin A had no effect on the cytotoxicity results, which indicates tunicamycin did not directly interact with toxin A. Therefore, the results with the F9 and PA1 cell lines suggest that a carbohydrate receptor that bound toxin A to the F9 cells was responsible for the relatively high activity of toxin A on F9 cells.

I then assayed the cytotoxic activity of toxin A after treating the toxin with PCG-4, which is a monoclonal antibody that reacts with the carbohydrate-binding portion of toxin A (22). I found that treating serially diluted toxin A with 200 μg/ml of PCG-4 increased the amount of toxin A required to hemagglutinate rabbit erythrocytes (which contain large amounts of Galα1-3Galβ1-4GlcNAc; 7, 10) from 3 μg/ml without PCG-4 to 50 μg/ml with PCG-4. This indicated that the monoclonal antibody inhibited the binding of toxin A to the carbohydrate receptor. PCG-4 also reduced the cytotoxic activity of toxin A on F9 cells by 87% (Figure 3), but did not affect the activity of toxin A on CHO or PA1 cells. The neutral monoclonal antibody (UPC-10) did not affect the cytotoxic activity of toxin A on any of the cell lines. This provides further evidence that the high activity of toxin A on F9 cells is due to toxin A binding to a receptor.

To further define the nature of the carbohydrate that was acting as a physiological receptor for toxin A on F9 cells, I attempted to competitively prevent the binding of the toxin to the cells by using the lectins GS1-B$_4$ or DBA. Since receptors may be recycled by cells, I limited the time of the competition,

and removed the toxin after a short exposure time. This short incubation period reduced the cytotoxic activity of toxin A 100-fold relative to the 18 hour incubation with toxin A. Treating the cells with DBA, which binds to α-linked N-acetylgalactosamine (9), did not reduce the activity of toxin A on any of the cell lines nor did it prevent the binding of toxin A to the F9 cells (Figure 2). Furthermore, when the CHO or PA1 cells were treated with GS1-B$_4$, which binds to α-linked galactose (9, 21), there was no reduction in the cytotoxic activity of toxin A. However, treating the F9 cells with GS1-B$_4$ reduced the cytotoxic activity of toxin A by 87% (Figure 4), and GS1-B$_4$ prevented toxin A from binding to the cells (Figure 2). This indicated that a carbohydrate which contained a terminal α-linked galactose bound toxin A to the F9 cells, and was responsible for the high activity of toxin A on the F9 cells.
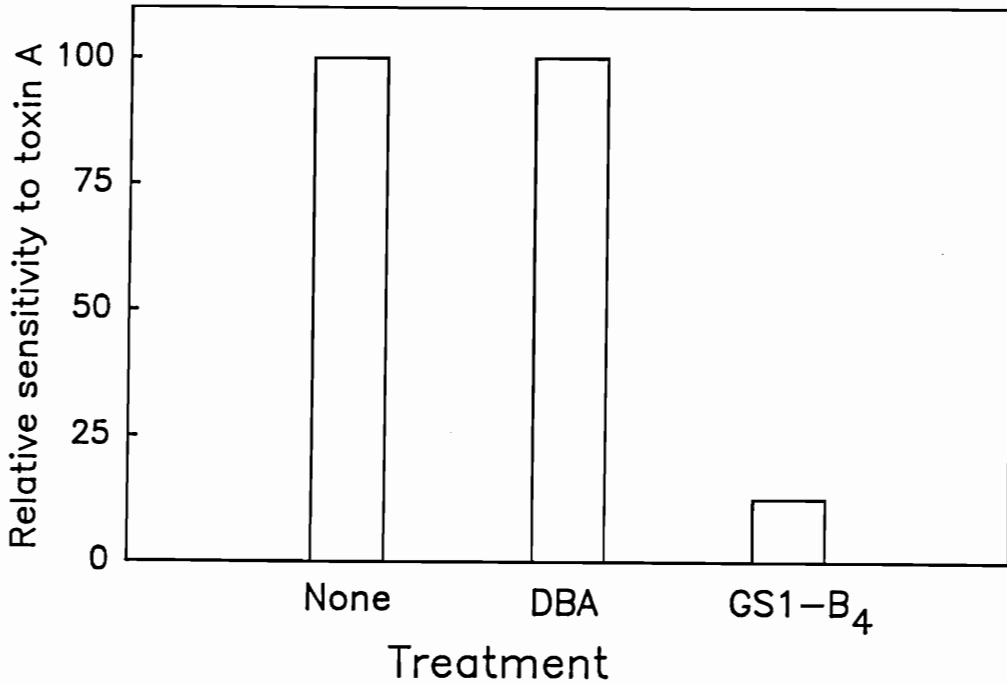
FIGURE 4. The sensitivity of F9 cells to toxin A after the cells had been treated with lectins to competitively prevent the binding of toxin A to the cells. GS1-B$_4$ and DBA are lectins that bind to carbohydrates that contain α-linked galactose or α-linked N acetylgalactosamine, respectively (9, 21). See text for the parameters of the treatments and discussion.

DISCUSSION

Previously, this laboratory demonstrated that toxin A binds to carbohydrates that terminate with Galα1-3Galß1-4GlcNAc (3, 13). This study was undertaken to determine if toxin A could use Galα1-3Galß1-4GlcNAc as a physiological receptor. I found that toxin A has a 100-fold higher cytotoxic activity on cell lines that express Galα1-3Galß1-4GlcNAc than on cell lines that do not express this carbohydrate. The high activity of toxin A on these cell lines was due to toxin A binding to a carbohydrate receptor on the cells, and this carbohydrate receptor contained a terminal α-linked galactose. Since toxin A binds to carbohydrates that terminate with Galα1-3Galß1-4GlcNAc, but does not bind to other carbohydrate structures that contain an α-linked galactose, I believe the data demonstrates that toxin A uses Galα1-3Galß1-4GlcNAc as a physiological receptor.

The monoclonal antibody PCG-4 neutralizes the enterotoxic activity of toxin A in vivo (14, 18), as well as the cytotoxic activity on F9 cells. This suggests that the cytotoxic activity of toxin A on the F9 cell line may be related to the enterotoxic activity of the toxin in vivo. Since PCG-4 prevents the binding of toxin A to Galα1-3Galß1-4GlcNAc, and this carbohydrate binds toxin A to both F9 cells and hamster intestinal mucosa, I believe this is further evidence that Galα1-3Galß1-4GlcNAc can act as a physiological receptor for toxin A in vivo.

This is the first demonstration that the monoclonal antibody to toxin A, designated PCG-4, can neutralize the cytotoxic activity of toxin A (18). Apparently, the neutralization is the result of PCG-4 preventing toxin A from

binding to the carbohydrate receptor on the F9 cells. The ineffectiveness of PCG-4 at neutralizing toxin A on some cell lines (e.g., CHO) indicates these cell lines may not contain a receptor for toxin A, and that the cytotoxic activity of toxin A on these cells may be the result of non-specific up-take of toxin A, such as by pinocytosis. However, the possibility of a second binding site on toxin A that is not affected by PCG-4 cannot be excluded at this time.

Galα1-3Galβ1-4GlcNAc is expressed on the cells of most mammals (8); therefore, toxin A could use this carbohydrate as a physiological receptor in most animals. However, old world monkeys, apes and humans do not normally express this trisaccharide (8), thus toxin A must use an alternate receptor in humans. The presence of an alternate receptor is supported by the observation that the human colonic cell line T84 is 10 times more sensitive to toxin A than are the CHO cells, and PCG-4 reduces the cytotoxic activity (see section V of this dissertation). Therefore, an unidentified receptor for toxin A apparently exists on human intestinal epithelium.

ABBREVIATIONS

Gal, galactose

GlcNAc, N-acetylglucosamine

CHO, Chinese Hamster Ovary cell line K1

DBA, *Dolichos biflorus* agglutinin

GS1, *Griffonia simplicifolia* lectin I

GS1-B$_4$, *Griffonia simplicifolia* lectin I, isolectin B$_4$

TBS 100 mM Tris, 150 mM NaCl, 5 mM CaCl$_2$, 5mM MgCl$_2$, ph 7.0

TBS-BSA 100 mM Tris, 150 mM NaCl, 0.2% bovine serum albumin, 5 mM

   CaCl$_2$, 5mM MgCl$_2$, ph 7.0.

LITERATURE CITED

1   Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa. 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Internatl. **2**: 629-635.

2   Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

3   Clark, G. F., H. C. Krivan, T. D. Wilkins, and D. F. Smith. 1987. Toxin A of *Clostridium difficile* binds to rabbit erythrocyte glycolipids with terminal Galα1-3Galß1-4GlcNAc sequences. Arch. Biochem. Biophys. **257**: 217-229.

4   Cummings, R. D., and S. A. Mattox. 1988. Retinoic acid-induced differentiation of the mouse teratocarcinoma cell line F9 is accompanied by an increase in the activity of UDP-galactose:ß-D-galactosyl-α1,3-galactosyl transferase. J. Biol. Chem. **263**: 511-519.

5   Draber, P., And P. Stanley. 1984. Cytotoxicity of plant lectins for mouse embryonal carcinoma cells. Somat. Cell Mol. Genet. **10**: 435-444.

6   Eidels, L., R. L. Proia, and D. A. Hart. 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. **47**: 596-620.

7   Eto, T., Y. Ichikawa, K. Nishimura, S. Ando, and T. Yamakawa. 1968. Chemistry of lipid of the posthemolytic residue or stroma of erythrocytes. XVI. Occurrence of ceramide pentasaccharide in the membrane of erythrocytes and reticulocytes of rabbit. J. Biochem. **64**: 205-213.

8   Galili, U., S. B. Shohet, E. Kobrin, C. L. M. Stults, and B. A. Macher. 1988. Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J. Biol. Chem. **263**: 17755-17762.

9   Goldstein, I. J., and R. D. Portez. 1986. Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins in *The lectins: properties,functions,and applications in biology and medicine.* I. E. Liener, N. Sharon, and I. J. Goldstein (Eds.). Academic Press Inc. pp 33-247.

10   Handfland, P., H. Egge, U. Dabrowski, S. Kuhn, D. Roelcke, and J. Dabrowski. 1981. Isolation and characterization of an I-active ceramide decasaccharide from rabbit erythrocyte membranes. Biochem. **20**: 5310-5319.

11   Hecht, G., C, Pothoulakis, J. T. LaMont, and J. L. Madara. 1988. *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. **82**: 1516-1524.

12   Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55**: 1873-1877.

13   Krivan, H. C., G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galβ1-3Galß1-4GlcNAc. Infect. Immun. **53**: 573-581.

14   Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant. 1988. Effects of *Clostridium difficile* Toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect. Immun. **56**: 582-588.

15   Lyerly, D. M., C. J. Phelps, and T. D. Wilkins. 1985. Monoclonal and specific polyclonal antibodies for immunoassay of *Clostridium difficile* toxin A. J. Clin. Microbiol. **21**: 12-14.

16   Lyerly, D. M., M. D. Roberts, C. J. Phelps, and T. D. Wilkins. 1986. Purification and properties of toxins A and B of *Clostridium difficile*. FEMS Microbiol. Lett. **33**: 31-35.

17   Lyerly, D. M., K. E. Saum, D. K. McDonald, and T. D. Wilkins. 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47**: 349-352.

18   Lyerly, D. M., C. J. Phelps, J. Toth, and T. D. Wilkins. 1986. Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect. Immun. **54**: 70-76.

19   Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins. 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**: 72-78.

20   Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins. 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1147-1150.

21   Peters, B. P., and I. J. Goldstein. 1979. The use of fluorescein-conjugated *Bandeiraea simplicifolia* B$_4$-isolectin as a histochemical reagent for the detection of α-D-galactopyranosyl groups. Exp. Cell. Res. **120**: 321-324.

22   Price, S. B., C. J. Phelps, T. D. Wilkins, and J. L. Johnson. 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. **16**: 55-60.

23   Romero, P. A., and A. Herscovics. 1986. Effects of tunicamycin, N-methyl-1-deoxynojirimycin, and manno-1-deoxynojirimycin on the biosynthesis of lactosaminoglycans in F9 teratocarcinoma cells. Carbohydrate Res. **151**: 21-28.

24   Sterne , M., and L. M. Wentzel. 1950. A new method for the large scale production of high-titer botulinum formol-toxoid types C and D. J. Immunol. **65**: 175-183.

25   Sullivan, N. M., S. Pellett, and T. D. Wilkins. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1032-1040.

26   Triadafilopoulos, G., C. Pothoulakis, M. J. O'Brien, and J. T. LaMont. 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology **93**: 273-279.

CYTOTOXIC ACTIVITY OF TOXIN A AND TOXIN B

OF *CLOSTRIDIUM DIFFICILE*

Kenneth D. Tucker

Department of Anaerobic Microbiology

Virginia Polytechnic Institute and State University

Blacksburg, Virginia 24061

INTRODUCTION

Many bacterial toxins are cytotoxins, but the specific activity of the toxin can differ depending on the cell line used to assay the cytotoxic activity. There are several factors that can cause a toxin to have different specific cytotoxic activities on different cells lines, including uptake and processing of the toxin by the cell. One factor that often increases the cytotoxic activity is the expression of a receptor for the toxin on the membrane of the cell (7). Such a receptor serves to target the toxin to the cell, which increases the interaction of the toxin with the cell. Conversely, a relatively high cytotoxic activity for a toxin is indicative that the cell line expresses a receptor for the cell line. Such cell lines may then be use to characterize and identify the receptor for the specific toxin.

Both toxin A and toxin B of *Clostridium difficile* are cytotoxins (15, 18, 19), and the specific activity of each toxin can differ depending on the cell line used to determine the cytotoxic activity. To gain insights into the receptors of toxin A and toxin B, I assayed the cytotoxic activities of the toxins on a variety of cells. I also used a monoclonal antibody to toxin A, PCG-4, as an aid in this study. PCG-4 reacts with the carbohydrate binding portion of toxin A (17), and prevents the binding of toxin A to carbohydrate receptors (see section IV in this dissertation), thereby neutralizing the cytotoxic activity of the toxin. Thus PCG-4 was used to indicate if toxin A bound to carbohydrate receptors on the different cell lines. This provided the preliminary evidence that toxin A may bind to the X antigen.

MATERIALS AND METHODS

Cultures of *C. difficile* were grown in dialysis flask cultures as previously described (18). Toxin A was purified by affinity chromatography on immobilized bovine thyroglobulin (10). Toxin B was purified by ion exchange chromatography, and affinity adsorption chromatography (15). Each toxin was determined to be homogeneous using procedures previously described (15). The concentration of the toxins were estimated using the method of Bradford (1) with bovine gamma globulin as a reference.

Cell lines were grown as previously described(5, 7, 12, 13), or as recommended by the American Type Culture Collection. Cell lines were kindly provided by the following: CaCo2 and T84 by Cynthia Weikel at Johns Hopkins University; 3T3 and chinese hamster ovary cell line K1 (CHO) by Brian Storrie, and F9 by David Smith at Virginia Polytechnic Institute and State University; OTF9-63, P19, and P19S1801A1 by Pamela Stanley at Yeshevia University; and Ehrlich Lettre ascites carcinoma strain E (EAT), PA1, and TIB85 from the American Type Culture Collection.

Cytotoxicity assays were performed in 96 well microtiter plates as previously described (18). The ability of PCG-4 to neutralization of the cytotoxic activity of toxin A was assayed by mixing 200 μg/ml of PCG-4 with serially diluted toxin A and incubating one hour on ice before adding the toxin to the tissue culture. All results are expresses as the number of cytotoxic units (CTU) contained in 1 μg of toxin, with a CTU being defined as the least amount of toxin that caused 90% of the cells to round after 18 hours of exposure to the toxin.

RESULTS AND DISCUSSION

Many bacterial toxins use carbohydrates as receptors; likewise toxin A binds to the carbohydrate Galα1-3Galβ1-4GlcNAc (2, 10, 11). The use of Galα1-3Galβ1-4GlcNAc as a physiological receptor for toxin A was suggested by the observation that the cell lines EAT, 3T3, F9, OTF9-63, and P19, which express Galα1-3Galβ1-4GlcNAc (4, 7, 8), were more sensitive to toxin A than were cell lines that do not express this carbohydrate (i.e., CHO and PA1) (Figure 1). Furthermore, the monoclonal antibody to toxin A (PCG-4) reduced the cytotoxic activity of toxin A on the cell lines that express the carbohydrate receptor, but did not affect the cytotoxic activity of toxin A on cell lines that do not express Galα1-3Galβ1-4GlcNAc (Figure 2). However, two other cell lines (i.e., CaCo2 and T84) that should not express Galα1-3Galβ1-4GlcNAc (8) were also more sensitive to toxin A than were the CHO and PA1 cell lines. PCG-4 also reduced the cytotoxic activity of toxin A on the T84 cell line. This suggested to me that toxin A had a receptor other than Galα1-3Galβ1-4GlcNAc.

The cytotoxic activity of toxin A on specific cell lines suggested a possible alternative receptor for the toxin. The cell lines that expressed Galα1-3Galβ1-4GlcNAc could be separated into two groups based on their relative sensitivity to toxin A with the EAT and 3T3 cell lines being less sensitive to toxin A than the F9, OTF9-63, and P19 cell lines. Interestingly, all the cell lines in the latter group were mouse teratocarcinomas (the origin of each cell line is summarized in Table 1) which express large amounts of Galβ1-4(Fucα1-3)GlcNAc (9, 16), whereas the EAT and 3T3 cell lines do not express this carbohydrate. This

suggested that Galβ1-4(Fucα1-3)GlcNAc could be acting as a receptor for toxin A. Further, Galβ1-4(Fucα1-3)GlcNAc (i.e., X antigen) is also on the membrane of T84 and CaCo2 cells as part of the glycoprotein carcinoembryonic antigen (3). Thus this was the first suggestive evidence that the X antigen may be a receptor for toxin A on human colonic epithelial cells.
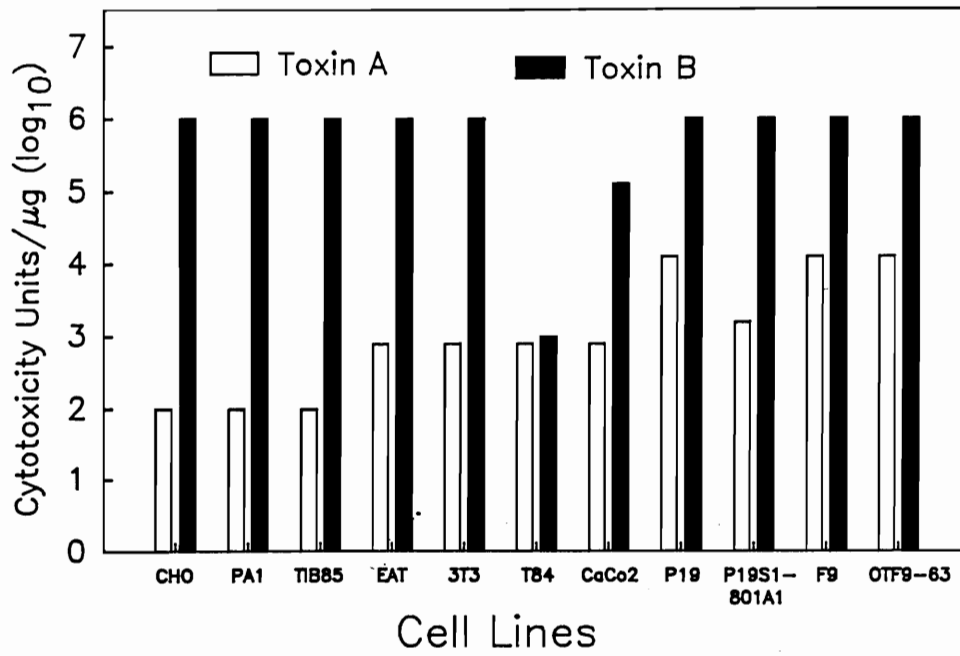
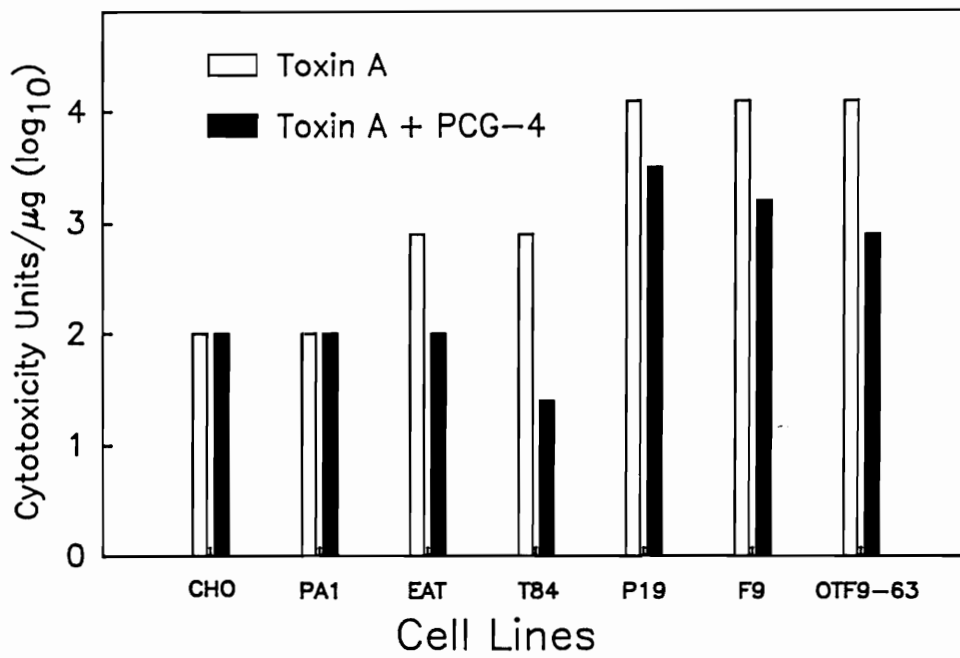Figure 1. Cytotoxic activity of toxin A or toxin B on cell lines.

Figure 2. Neutralization of the cytotoxic activity of toxin A by PCG-4 on selected cell lines.

Table 1. Origin of cell lines

Cell line	Derived from:
CHO	Chinese hamster ovary tumor
PA1	Human ovary tumor (human teratocarcinoma)
TIB85	Mouse epithelial cells
EAT	Mouse ascites tumor
3T3	Mouse epithelial cells
T84	Human intestinal epithelium tumor
CaCo2	Human intestinal epithelium tumor
P19	Mouse embryo (mouse teratocarcinoma)
F9	Cell line OTT 6050 (mouse teratocarcinoma)
OTF9-63	Cell line F9 (mouse teratocarcinoma)

The gene for toxin A has been cloned and sequenced (6). The 3' end of this gene codes for the carbohydrate binding portion of toxin A (17). Interestingly, the 3' end of the toxin A gene shows a striking identity (i.e., greater than 50%) with the 3' end of the toxin B gene, which has also been cloned and sequenced (Lisa Barroso, personal communication). The similarity between toxin A and toxin B suggests that toxin B also may have a carbohydrate receptor that could be very similar to the receptor for toxin A. However, since toxin B has a high cytotoxic activity on almost every cell line tested, the receptor for toxin B must be ubiquitous. Even so, it is interesting to note that the only cell line that was relatively insensitive to toxin B was the T84 cell line, which is derived from human intestinal epithelium, and toxin B has little or no activity on intact intestinal epithelium in vivo (13, 14, 19, 20).

LITERATURE CITED

1   Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

2   Clark, G. F., H. C. Krivan, T. D. Wilkins, and D. F. Smith. 1987. Toxin A of *Clostridium difficile* binds to rabbit erythrocyte with terminal Galα1-3Galβ1-4GlcNAc sequences. Arch. Biochem. **257** 217-229.

3   Chandrasekaran, E. V., M. Davila, D. W. Nixon, M. Goldfarb, and J. Mendicino. 1983. Isolation and structures of the oligosaccharide units of carcinoembryonic antigen. J. Biol. Chem. **258:** 7213-7222.

4   Cummings, R. D., and S. A. Mattox. 1988. Retinoic acid-induced differentiation of the mouse teratocarcinoma cell line F9 is accompanied by an increase in the activity of UDP-galactose: β-D-galactosyl-α1-3-galactosyl transferase. J. Biol. Chem. **263:** 511-519.

5   Donta, S. T., N. Sullivan, and T. D. Wilkins. 1982. Differential effects of *Clostridium difficile* toxins on tissue cultured cells. J. Clin. Microbiol. **15:** 1157-1158.

6   Dove, C. H., S.-Z. Wang, S. B. Price, C. J. Phelps, D. M. Lyerly, T. D. Wilkins, and J. L. Johnson. 1990. Molecular characterization of *Clostridium difficile* toxin A gene. **58:** 480-488.

7   Draber, P., and P. Stanley. 1984. Cytotoxicity of plant lectins for mouse embryonal carcinoma cells. Somat. Cell. Mol. Genet. **10:** 435-444.

8   Galili, U., S. B. Shohet, E. Kobrin, C. L. M. Stults, and B. A. Macher. 1988. Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J. Biol. Chem. **263:** 17755-17762.

9   Gooi, H. C., T. Feizi, A. Kapadia, B. B. Knowles, D. Solter, and M. J. Evans. 1981. Stage specific embryonic antigen involves α1-3 fucosylated type 2 blood group chains. Nature. **292:** 156-158.

10   Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55:** 1873-1877.

11   Krivan, H. C., G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. **53**: 573-581.

12   Leppanen, A., A. Korvuo, K. Puro, and O. Renkonen. 1986. Glycoproteins of human teratocarcinoma cells (PA1) carry both anomers of O-glycosyl-linked D-galacto-pyranosyl-(1-3)-2-acetamido-2deoxy-α-D-galacto-pyranosyl group. Carbohydr. Res. **153**: 87-95.

13   Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect. Immun. **56**: 582-588.

14   Lyerly, D. M., K. E. Saum, D. K. McDonald, and T. D. Wilkins. 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47**: 349-352.

15   Lyerly, D. M., M. D. Roberts, C. J. Phelps, and T. D. Wilkins. 1986. Purification and properties of toxins A and B of *Clostridium difficile*. FEMS Microbiol. Lett. **33**: 31-35.

16   Muramatsu, Takashi. 1988. Alterations of cell-surface carbohydrates during differentiation and development. Biochem. **70**: 1587-1596.

17   Price, S. B., C. J. Phelps, T. D. Wilkins, and J. L. Johnson. 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. **16**: 55-60.

18   Sullivan, N. M., S. Pellett, and T. D. Wilkins. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1032-1040.

19   Taylor, N. S., G. M. Thorne, and J. G. Bartlett. 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**: 1036-1043.

20   Triadafilopoulos, G., C. Pothoulakis, M. J. O'Brian, and J. T. LaMont. 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology **93**: 273-279.

TOXIN A OF *CLOSTRIDIUM DIFFICILE*

BINDS TO THE HUMAN CARBOHYDRATE ANTIGENS I, X, AND Y

Kenneth D. Tucker

Department of Anaerobic Microbiology

Virginia Polytechnic Institute and State University

Blacksburg, Virginia 24061

ABSTRACT

Clostridium difficile causes pseudomembranous colitis in humans. The enterotoxin (i.e., toxin A) from this organism is believed to be responsible for the initial intestinal pathology associated with this disease. Previous work shows this toxin binds to carbohydrates that contain Galα1-3Galβ1-4GlcNAc. However, this carbohydrate is not present on normal human cells. Therefore, this study was undertaken to identify potential receptors for toxin A that do exist on human intestinal epithelium. I found that toxin A bound to the carbohydrate antigens designated I, X, and Y. Each of these carbohydrates exist on the intestinal epithelium of humans.

INTRODUCTION

Clostridium difficile is one of the most common causes of bacterial diarrhea and colitis among hospitalized patients. The organism can not compete successfully with the normal microflora of the adult colon, but can grow to high numbers when the colonic flora has been disturbed by antibiotic treatments (51,63). *C. difficile* then keeps the colonic flora from reestablishing by producing an enterotoxin, toxin A, which causes destruction of the colonic epithelium resulting in fluid secretion into the intestine (38, 39, 41). A second toxin produced by *C. difficile*, toxin B, has no effect on undisturbed intestinal epithelium, but is an extremely active cytotoxin on non-epithelial cells (3, 38, 39, 55, 56, 58). This toxin may gain access to the underlying cells through the destructive action of toxin A on the epithelial cells (39). Hamsters are the most susceptible laboratory animal to this disease and in these animals the two toxins seem to act synergistically. Toxin B alone has no effect when given orally to hamsters, but kills them when it is combined with sublethal amounts of toxin A (39).

An enterotoxin must bind to the intestinal epithelial cells, and toxins often accomplish this by binding to specific carbohydrate structures on the membrane of the epithelial cells. Previous work from this laboratory has shown that toxin A binds to a trisaccharide (Galα1-3Galβ1-4GlcNAc) which occurs in large amounts on the intestinal cells of hamsters (11, 32). Hamsters can be protected from this disease by immunizing them with a recombinant peptide which contains the

carbohydrate binding portion of toxin A (14). This recombinant protein contains repeating amino acid domains which bind multiple copies of the trisaccharide receptor for the toxin (14, 49). Recently, I have shown that cell lines which express large amounts of this carbohydrate on their cell membranes are more sensitive to the cytotoxic action of toxin A than other cell lines (59, 63). This evidence taken together led me to speculate that Galα1-3Galβ1-4GlcNAc was the biological receptor for toxin A in humans as well as hamsters.

A search of the literature revealed that there was a problem with this postulate. Normal human cells do not express this trisaccharide (18). Furthermore, most people produce antibodies against this carbohydrate (19). Preliminary experiments showed that toxin A did bind to human intestinal epithelium; therefore, I started to test for the binding of toxin A to carbohydrate structures that have been reported to exist on human intestinal mucosa. The first structure I tested was a carbohydrate (Galβ1-4[Fucα1-3]GlcNAc) which appeared to be conformationally similar to the reported receptor for toxin A (Galα1-3Galβ1-4GlcNAc). I found that toxin A bound to this carbohydrate, and this finding lead to the identification of two other oligosaccharides which bind toxin A. I report here that toxin A binds to three well described carbohydrates which exist on human intestinal epithelial cells: the I, X, and Y antigens.

MATERIALS AND METHODS

Biochemicals: Carbohydrates isolated from human milk and chemically conjugated to human serum albumin (HSA) were kindly provided by Dr. Howard Krivan of BioCarb Chemicals, or were purchased from BioCarb Chemicals (Lund, Sweden). See Table 1 for a description of the oligosaccharides and their names. In order to determine if toxin A from *Clostridium difficile* bound to these oligosaccharide-HSA conjugates, I preformed two assays: crossed immunoelectrophoresis (IEP), and enzyme linked immunosorbent assay (ELISA) as described below. I also assayed the binding of toxin A to bovine thyroglobulin (Sigma Chemical Co., St. Louis, Mo.), which contains $Gal\alpha 1\text{-}3Gal\beta 1\text{-}4GlcNAc$ (53), and to the following human proteins: secretory component (Chemicon, Temecula, Calif.) and carcinoembryonic antigen (CEA) (Calbiochem, San Diego, Calif.), which contain the X antigen (9, 45).

Toxin purification: *C. difficile* VPI 10463 was grown in dialysis cultures (55). Culture filtrate was prepared by filtering the contents of the dialysis sac with a 0.45 μm pore size filter. Toxin A was purified from the culture filtrate by affinity chromatography using immobilized bovine thyroglobulin (33). The toxin was determined to be homogenous using procedures previously described (55). The concentration of toxin A was estimated using the method of Bradford (7) with bovine gamma globulin as the standard.

Antiserum: Antiserum to culture filtrate of *C. difficile* was prepared in goats (15). Affinity purified antibody to toxin A was prepared as previously described (42).

The affinity purified antibody to the toxin was biotinylated with biotinamidocaproate N-hydroxysuccinimide ester (Sigma Chemical Co.) (23). The biotin-antibody conjugate was used at a 1:1000 dilution.

IEP: The binding of toxin A to glycoproteins was assayed using crossed immunoelectrophoresis (32) by mixing 100 μg toxin A with 500 μg/ml of glycoprotein, and incubating on ice for 10 minutes before loading on the gel. The migration of toxin A with the glycoprotein was compared to the migration of toxin A alone. Interpretation of the results is based on the fact that toxin A normally migrates slowly in IEP. If toxin A binds to a glycoprotein that migrates rapidly on IEP, then the bound toxin will be pulled by the more rapid migrating glycoprotein, thus causing an increase in the migration of toxin A on IEP. If toxin A does not bind to the glycoprotein, then the mixture does not affect the migration of the toxin.

ELISA: ELISA were performed in Immulon II 96 well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). Each well was coated with 150 μl of 1:2 serially diluted glycoprotein, starting at 20 μg/ml of glycoprotein in 50 mM carbonate buffer (pH 9.6). Plates were incubated 16 hours at 4C then washed twice with PBS-T (0.8% NaCl, 0.02% KH_2PO_4, 0.22% Na_2HPO_4, 0.02% KCl, 0.05% tween 20, 0.02% NaN_3, pH 7.4). The plates were blocked with 0.5% casein in 100 mM Tris, 150 mM NaCl, pH 7.0 for 1 hour at 22C and then were washed twice with PBS-T. To each well was added 100 μl of toxin A at 10 μg/ml in PBS-T. The plates were incubated at either 4C or at 37C for 3 hours. After this step, all buffers were at 4C and all treatments were performed in a 4C cold

room, unless otherwise stated. The plates were washed twice with PBS-T, and

$100\,\mu$l of a 1:1000 dilution of biotinylated antibody to toxin A in PBS-T was

added to each well, and was incubated for 1 hour. The plates were washed twice

with PBS-T, and $100\,\mu$l of 0.1 U/ml of alkaline phosphatase conjugated to avidin

(Sigma Chemical Co.) was added to each well. The plates were incubated for 1

hour, then washed twice with PBS-T. Then the plates were moved to room

temperature, and $100\,\mu$l of 1 mg/ml of para nitrophenylphosphate in 100 mM

diethanolamine buffer (pH 9.8) was added to each well. All assays were

developed for 90 minutes at room temperature before the absorbance was read at

405 nm. Control wells included wells without toxin A and/or without

glycoprotein. The highest A_{405} obtained with wells that either did not contain

toxin A or glycoprotein was used as the value for background, which was

subtracted from the values presented in the figures.

The possibility that mono- or disaccharides could inhibit the binding of

toxin A to the Y antigen was assayed at 37C using the ELISA procedure

described, except the Immulon II plates were coated with $2\,\mu$g/ml of Y antigen-

HSA. One μg of toxin A was added to each well either alone or with 1 mg of

carbohydrate. See Table 2 for the sugars used in this experiment.

Latex agglutination: In order to confirm the reaction of toxin A with

carbohydrates that contain the X antigen, I assayed the binding of toxin A to

chemically synthesized carbohydrates linked to latex beads (Chembiomed Ltd.,

Edmonton, Canada). The oligosaccharide-latex reagents were blocked with 0.5%

casein for 4 hours. Five μl of latex beads was added to either $10\,\mu$l of toxin A

(100 μg/ml) or 10 μl of TBS (100 mM Tris, 150 mM NaCl, pH 7.0) on a glass plate. This was incubated on ice for 2 minute, then 10 μl of a monoclonal antibody to toxin A (i.e., PCG-4 at 1 mg/ml; 40) was added to the mixtures. The plate was rocked for 3 minute, and then observed for agglutination.

Toxin A isolation: Isolation of toxin A from all other *C. difficile* antigens was attempted using the basic protocol developed for the affinity purification of toxin A on immobilized Galα1-3Galβ1-4GlcNAc (33) however, I substituted the X antigen in place of Galα1-3Galβ1-4GlcNAc. Culture filtrate was concentrated five-fold on a MiniCon concentrator (Amicon; Danvers, Ma.), then 25 μl of this was mixed with a 20 μl pellet of latex beads containing the X antigen. This was incubated on ice for 10 minutes, then the latex beads were pelleted by centrifugation. The supernatant was saved, and the pellet was washed three times with ice cold TBS. The latex beads were then warmed to 37C in 25 μl of TBS, then pelleted by centrifugation. Each step of the isolation was monitored using IEP with 5 μl of sample in the first dimension, and 100 μl of antiserum to culture filtrate of *C. difficile* in the second dimension.

RESULTS

Examination of molecular models suggested to me that the X antigen

(Galβ1-4[Fucα1-3]GlcNAc) was conformationally similar to the previously

identified receptor Galα1-3Galβ1-4GlcNAc (Figure 1). This carbohydrate

structure is present on two commercially available glycoproteins:

carcinoembryonic antigen, and the secretory component of antibodies (9, 45).

When I mixed these proteins with toxin A, they caused an alteration in the

migration of toxin A in an electric field; this was most easily observed in crossed

immunoelectrophoresis. I then confirmed that toxin A was binding to these two

glycoproteins by using an ELISA. This assay showed that toxin A was binding at

4C, but not at 37C. Similar results were obtained with bovine thyroglobulin

which contains the previously described trisaccharide receptor (Galα1-3Galβ1-

4GlcNAc; Figure 2).

Although the binding of toxin A to these proteins was suggestive that the

toxin was binding to the X antigen, there was a possibility that toxin A was

binding to other carbohydrate structures shared by these two glycoproteins (e.g.,

BiAO, see Table 1 for structure; 9, 45). The X antigen trisaccharide also exists

as part of the pentasaccharide LNF III. Therefore, I tested the binding of toxin

A to the purified pentasaccharide which had been conjugated to human serum

albumin (HSA). This pentasaccharide conjugate also altered the migration of

toxin A in immunoelectrophoresis (data not shown) and bound to the conjugate

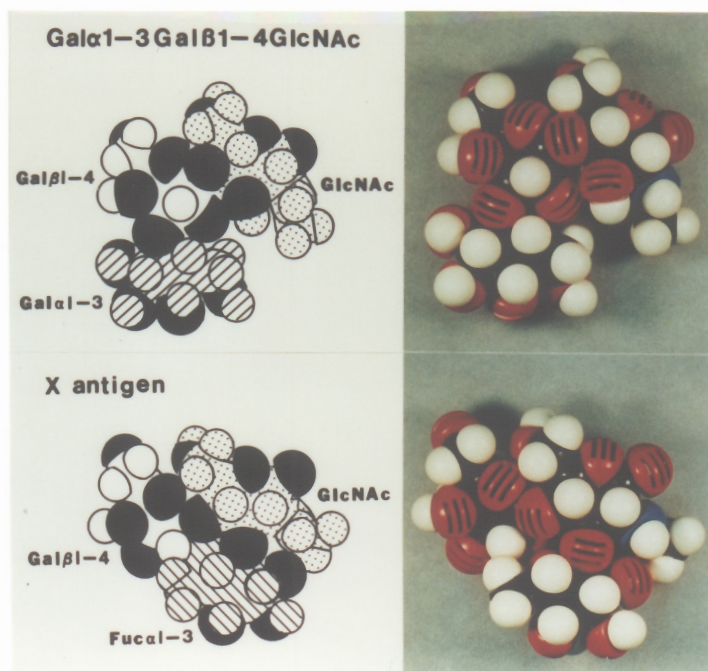in ELISA (Figure 2): the BiAO conjugate did not bind toxin A. Further, a

Figure 1: CPK space filling models showing a somewhat similar conformation between Galα1-3Galβ1-4GlcNAc and Galβ1-4(Fucα1-3)GlcNAc. Oxygen is represented by red, nitrogen by blue, hydrogen by white, and carbon by black. The left hand column contains schematic representations of the models illustrating the position of the monosaccharides in these oligosaccharides.
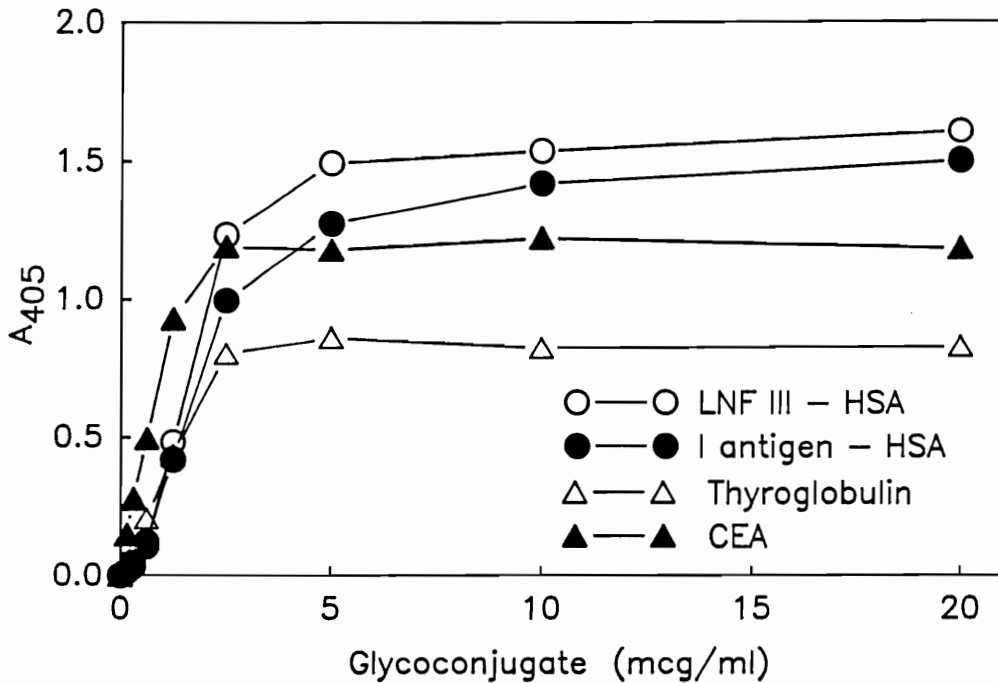
Figure 2: ELISA with toxin A binding to the glycoconjugates at 4C. CEA and LNF III-HSA contain the X antigen, and thyroglobulin contains $Gal\alpha 1\text{-}3Gal\beta 1\text{-}4GlcNAc$. The assay was developed for 90 minutes at room temperature before the A_{405} was recorded. The A_{405} for toxin incubated with the glycoproteins at 37C was insignificant. See Table 1 for carbohydrates that did not bind toxin A as determined by the IEP and ELISA.

Table 1: The oligosaccharide-HSA glycoconjugates tested for the binding of toxin A.

Name[a]	Trivial name[b]	Structure[c]	Binding of toxin A[d]
LNF I	Le[d]	Galß1-3GlcNAcß1-3Galß1-4(Glc) 2 Fucα1[/]	No
LNF II	Le[a]	GalB1-3GlcNAcB1-3GalB1-4(Glc) 4 Fucα1[/]	No
LND I	Le[b]	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc) 2 4 Fucα1[/] Fucα1[/]	No
LNF III	X Le[x]	GalB1-4GlcNAcB1-3GalB1-4(Glc) 3 Fucα1[/]	Yes
Le[y]	Y	Galβ1-4GlcNAcβ1 2 3 Fucα1[/] Fucα1[/]	Yes
LNnT	i	GalB1-4GlcNAcB1-3GalB1-4(Glc)	No
LNnH	I	GalB1-4GlcNAcB1-3GalB1-4(Glc) 6 GalB1-4GlcNAcB1[/]	Yes
BiAO	-	GalB1-4GlcNAcB1-2Manα1-3ManB1-4(GlcNAc) 6 GalB1-4GlcNAcB1-2Manα1[/]	No

Abbreviations: Gal, galactose; Glu, glucose; GlcNAc, N-acetylglucosamine; Fuc, fucose

[a] The names used in this table are the conventional abbreviations used for these carbohydrates.
[b] The X and Y antigens are also referred to as the Lewis X (Le[x]) and lewis Y (Le[y]) antigens, respectively. I use the designation X and Y because these antigens are not actually members of the Lewis blood group (62). The X antigen is also designated by several other names, including My-1 (26), VEP8- and VEP9-antigen (22), and stage specific embryonic antigen 1 (21).
[c] () around carbohydrates indicate the carbohydrate was chemically modified during the linking of the oligosaccharide to HSA.
[d] Binding of toxin A to oligosaccharides was determined by IEP and by ELISA.

number of other carbohydrates linked to HSA did not bind toxin A; so the binding of toxin A to the LNF III conjugate appears to be specific for the carbohydrate.

LNF III contains two carbohydrates more than the X antigen, which is defined by a trisaccharide. To determine if toxin A would bind to the X antigen alone, I tested for agglutination of latex beads to which this trisaccharide had been attached. Toxin A agglutinated these coated beads, and the beads specifically removed the toxin from culture filtrate at 4C. Warming the beads to 37C eluted the purified toxin (Figure 3).

I then tested the Y antigen which is similar to the X antigen except that it contains an additional α1-2-linked fucose. Y antigen (conjugated to HSA) altered the migration of toxin A in immunoelectrophoresis (Figure 4), and in an ELISA toxin A bound to this antigen in a temperature dependent manner (Figure 5). Over ten times more toxin was bound to the Y antigen than to LNF III at 4C, and significant amounts of toxin also were bound at 37C, whereas I could not detect binding of toxin to LNF III at 37C.

All of the carbohydrates which bound toxin A share only one structure in common: Galβ1-4GlcNAc. This laboratory previously reported that toxin A did not bind to the i antigen which contains this type 2 core structure (Galβ1-4GlcNAc) (11). Unexpectedly, I found that toxin A did bind to the branched form of this antigen, which is called the I antigen (Figure 2). The I antigen contains two type 2 core structures per oligosaccharide (Table 1). The toxin did
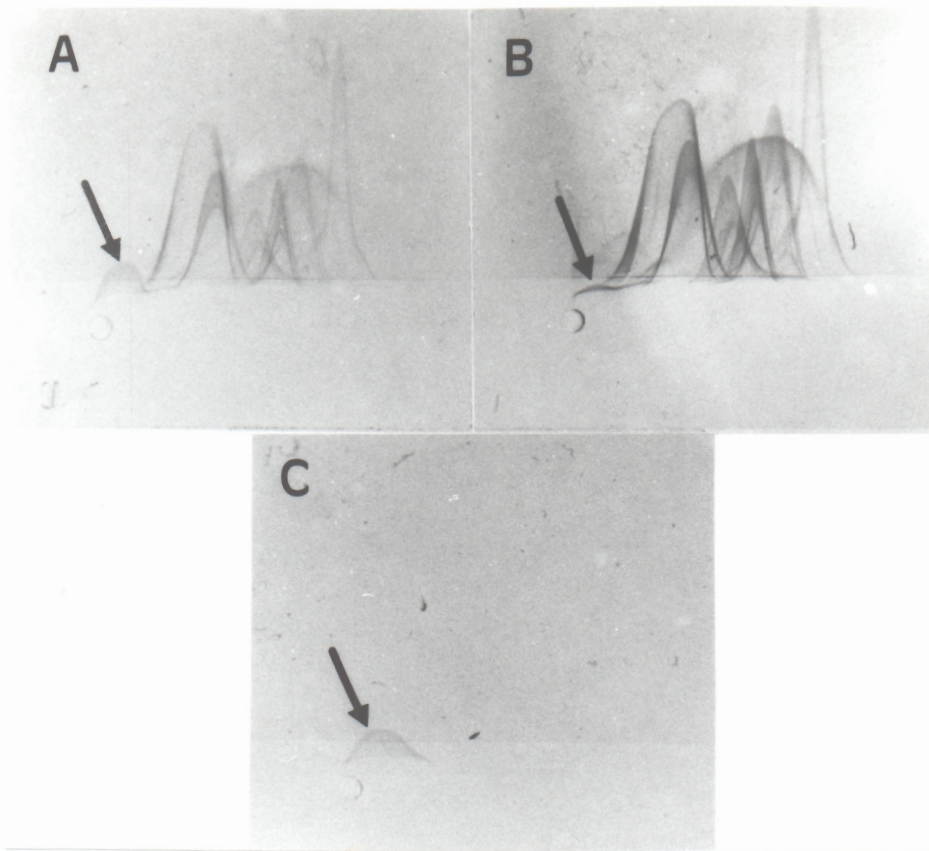
Figure 3: IEP showing the stages of the isolation of toxin A using immobilized X antigen. The arrow marks the position of toxin A. The second dimension of each plate contains antisera to culture filtrate of *C. difficile*. Culture filtrate of *C. difficile* before exposure to the immobilized X antigen (A). Culture filtrate of *C. difficile* after exposure to the immobilized X antigen at 4C (B): only toxin A was removed from the culture filtrate. The eluant obtained by warming the immobilized X antigen that had bound toxin A (C): only toxin A was detected.
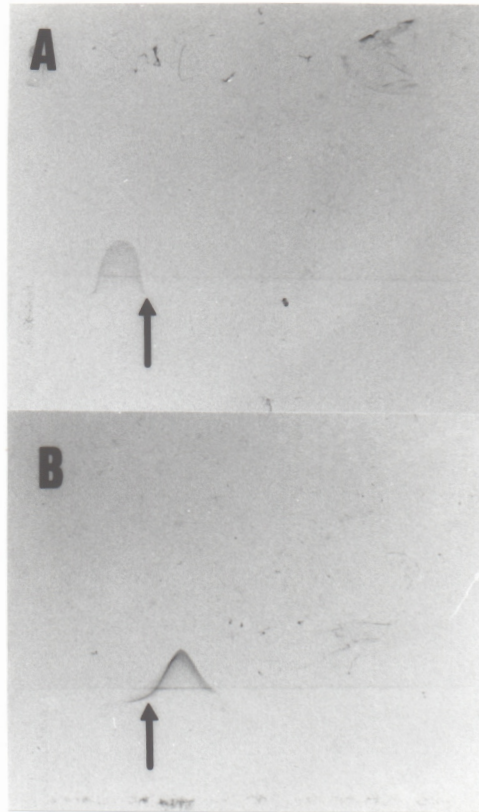
Figure 4: IEP of toxin A (A), or toxin A mixed with Y antigen-HSA (B). The arrow indicates the position of the leading edge of toxin A without the Y antigen. The Y antigen-HSA glycoconjugate caused an increased migration of toxin A. This was also observed using either I, or X antigen glycoconjugates, as well as when carcinoembryonic antigen or secretory component were mixed with toxin A. Other carbohydrates conjugated to HSA (Table 1) did not alter the migration of toxin A.
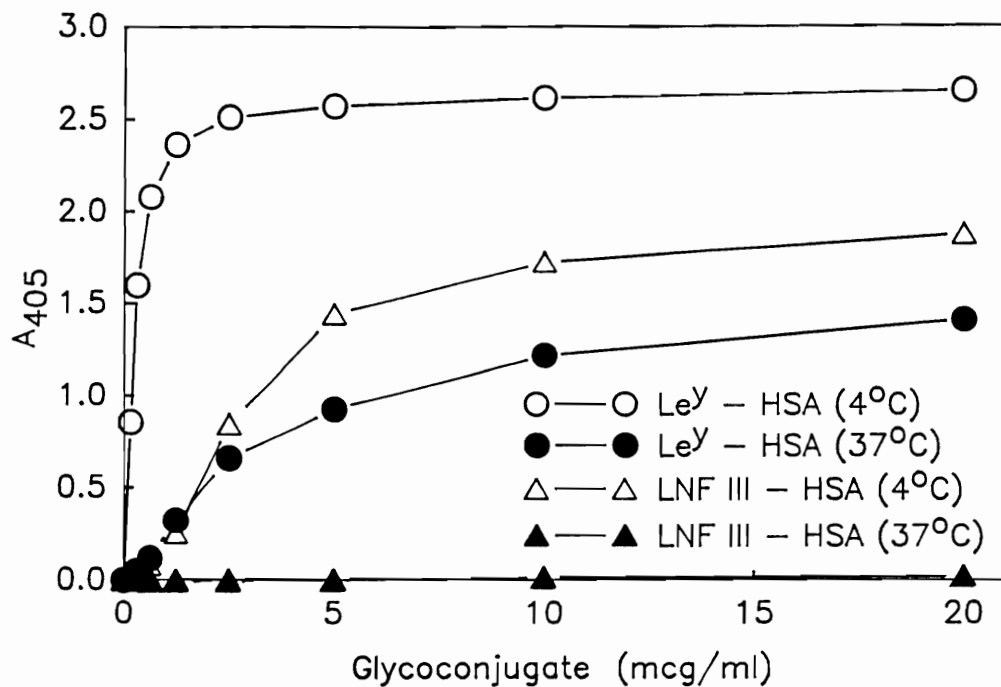
Figure 5: ELISA with toxin A binding to either LNF III, which contains the X antigen, or Y antigen conjugated to HSA. The assays were performed in parallel at either 4 or 37C. The assay was developed for 90 minutes at room temperature before recording the A_{405}.

not bind to any of the oligosaccharides I tested that contain the similar type 1 core structure (Galβ1-3GlcNAc: Table 1).

Binding of proteins to complex oligosaccharides sometimes can be inhibited by simple sugars or by disaccharides; therefore, I tested a number of these for the ability to inhibit the binding of toxin A to the Y antigen at 37C (Table 2). Of the sugars tested, only N-acetyllactosamine (Galβ1-4GlcNAc) had any effect on the binding. This compound, which is similar to the type two core, inhibited the binding by over 20%.

Table 2: Carbohydrates which did not inhibit the binding of toxin A to the Y antigen as determined by ELISA[a]

Carbohydrate	
Glucose	Galactose
N-acetylglucosamine	N-acetylgalactosamine
Mannose	Fucose
Sucrose	Fructose
Xylose	Mannitol
Lactose	Melibiose
Arabinose	Stachyose
2'-Fucosyllactose	

[a] Only N-acetyllactosamine (Galβ1-4GlcNAc) inhibited the binding of toxin A to the Y antigen. All carbohydrates were tested at a concentration of 0.5%.

DISCUSSION

All of the carbohydrates that bound toxin A contain the type 2 core (Galβ1-4GlcNAc). The β1-4-linkage of the type 2 core produces oligosaccharides with a conformation that is very different from oligosaccharides with the β1-3-linkage present in the type 1 core (Galβ1-3GlcNAc; Figure 6). This suggests that the binding of toxin A to the carbohydrates was dependent on the conformation of the type 2 core. Supporting this belief is my observation that N-acetyllactosamine (Galβ1-4GlcNAc) inhibited the binding of toxin A to the Y antigen, whereas the monosaccharides present in the type 2 core did not effect the binding. The specificity of toxin A for the type 2 core is further indicated by the observation that lactose did not inhibit the binding of toxin A. Lactose has a conformation that is identical to N-acetyllactosamine, except that lactose does not contain an N-acetyl amine (Figure 6). This indicates that toxin A requires the N-acetyl amine in order to bind to carbohydrates. Thus Galβ1-4GlcNAc appears to be the minimum carbohydrate structure that is bound by toxin A. However, the i antigen and BiAO did not bind toxin A, even though they also contain the type 2 core.

The difference between the oligosaccharides which contain the type 2 core and that bound toxin A (I, X, Y antigens), and the ones that did not bind toxin A (i antigen, BiAO) is that the former contain a branch either on or immediately adjacent to the type 2 core. These branches sterically hinder changes in the conformation of the core; this may hold the carbohydrate in a conformation that favors the binding of toxin A. Conversely, the conformations
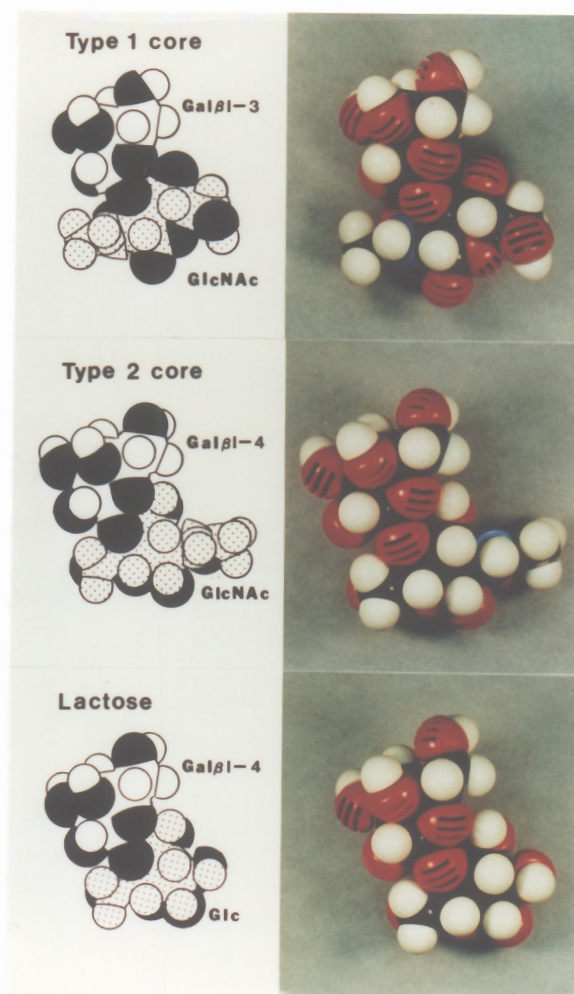
Figure 6: CPK space filling models of the type 1 core structure (Galβ1-3GlcNAc), type 2 core structure (Galβ1-4GlcNAc), and lactose (Galβ1-4Glc). Oxygen is represented by red, nitrogen by blue, hydrogen by white, and carbon by black. The left hand column contains schematic representations of the models illustrating the position of the saccharides in these oligosaccharides. The conformations in this figure are based on previously published predictions (35, 36). See text for discussion.

of the type 2 core in non-branched oligosaccharides may be too dynamic to bind toxin A. If this postulate is correct, it raises the question of how could the disaccharide N-acetyllactosamine inhibit the binding of toxin A? The answer may be that the molar concentration of N-acetyllactosamine used to inhibit the binding of toxin was at least 10,000-fold more than the concentration of either i antigen or BiAO used to assay the binding of the toxin. With such a high concentration of N-acetyllactosamine, it is possible that a small percentage of the molecules were in the correct conformation to bind to toxin A, thereby inhibiting the binding of toxin A to the Y antigen. Therefore, I believe that for toxin A to bind to a carbohydrate, the carbohydrate must contain the type 2 core.

Toxin A contains multiple binding sites for the carbohydrate receptors (14,49). Increasing the number of receptors will increase the number of binding sites on a molecule of toxin A that are actually bound to a receptor. Therefore, increasing the density of the receptor will increase the avidity of toxin A for the receptor because the avidity is determined by the summation of the affinities of the individual binding sites that are actually bound to the receptor. One way to increase the density of a carbohydrate receptor is to increase the number of branches on an oligosaccharide that contain a receptor. Initially this appeared to explain why the branched I antigen, which contains two copies of the type 2 core, bound toxin A, whereas the linear i antigen, which contains only one copy of the type 2 core, did not bind the toxin. However, BiAO did not bind toxin A, and this carbohydrate also contains two copies of the type 2 core. Therefore, a high

density of the type 2 core is not enough to bind toxin A. Instead, a receptor for toxin A apparently also must be branched immediately on or adjacent to the type 2 core.

The binding of toxin A to the I, X, and Y antigens suggests to me that these carbohydrates may be receptors for toxin A on human intestinal epithelium. Toxin A has the greatest affinity for the Y antigen, but the I and X antigens also may function as receptors even though I could not detect binding to the I and X antigens at 37C. After all, toxin A binds to the I antigen, X antigen, and Galα1-3Galβ1-4GlcNAc equally, and toxin A appears to use Galα1-3Galβ1-4GlcNAc as a receptor on hamster and rabbit intestinal mucosa (32, 46).

The I, X, and Y antigens are present on normal human intestinal epithelium (4, 6, 9, 16, 17, 25, 27, 29, 30, 44, 52, 62), but the I and Y antigens are segregated within certain subgroups of the population (62). These subgroups are defined by the expression of the Se gene, which is believed to code for an α1,2-fucosyltransferase in human epithelial cells (63). This transferase attaches a fucose onto the galactose of the type 2 core. The resulting carbohydrate then can be converted to the Y antigen by another fucosyltransferase. Thus people with an active Se gene (i.e., secretors) convert the I antigen to blood group substances and to the Y antigen (4, 17, 25, 62). This conversion effectively eliminates the I antigen from the intestinal mucosa of secretors. People with an inactive Se gene (i.e., non-secretors) do not convert the I antigen to the Y antigen, thus the I

antigen is present in non-secretors (17, 62). The X antigen is present in both secretors and non-secretors (17, 25, 62). So both secretors and non-secretors have two possible receptors for toxin A on their intestinal epithelium. Furthermore, certain neoplastic tissue can express large amounts of the I, X, or Y antigens independent of the secretor status of the individual (17, 52). Thus carbohydrates to which toxin A binds probably are present in all individuals.

Each of the receptors for toxin A are associated with hyperplasia or neoplasia (1, 6, 9, 16, 17, 27, 29). Furthermore, the expression of $Gal\alpha 1-3Gal\beta 1-4GlcNAc$ by human cells is limited to tumor cells (8, 20, 28). Thus, some hyperplastic or neoplastic tissues have a high density of receptors for toxin A. This suggests that toxin A may target to these tissues. I have no evidence to support this, but it could explain the observation that pseudomembranous colitis is observed predominantly in elderly patients, where the incidence of colonic hyperplasia and neoplasia is much higher than it is in infants or young adults. The potential targeting of toxin A to these tissues suggests that toxin A might even be a useful therapeutic agent. This would be particularly useful for maladies that are difficult to treat, such as colonic polyposis.

Human granulocytes express large amounts of the X antigen (26, 22, 54), which indicates that toxin A may be targeted to these cells. This may be of significance because large numbers of granulocytes occur in the pseudomembranes that are characteristic of pseudomembranous colitis. Furthermore, toxin A causes granulocytes to release factors that can cause

intestinal secretion, and enteritis (47, 48). This has led some researchers to speculate that toxin A is not directly active on intestinal epithelium, but that the factors released from the granulocytes cause the tissue damage and diarrhea associated with toxin A in vivo (58). I believe toxin A does act directly on intestinal epithelium because toxin A is toxic to cell lines in vitro that are physiologically similar to intestinal epithelium (24). Even so, I agree that some of the symptoms associated with pseudomembranous colitis may be due to the factors released by the granulocytes. These factors may add to the tissue damage associated with toxin A, thus increasing the severity of the disease.

Most human infants apparently are insensitive to toxin A since they can be colonized by toxigenic *C. difficile* with no ill effects (2, 5, 12, 13, 31, 34, 37, 43, 50, 57, 60, 61). Previously, this laboratory proposed that the insensitivity of infants to toxin A may be due to either the lack of a receptor for toxin A, or a low density of the receptor (10, 32). However, human infants express the Y antigen on their intestinal epithelium (29, 30), and the Y antigen binds toxin A at 37C. Therefore, the absence of a receptor for toxin A apparently is not the reason for the insensitivity of infants to the toxin.

In summary, I have found that toxin A binds to three carbohydrate antigens (I, X, and Y) that are present on human intestinal cells. These antigens may be receptors for toxin A on the human intestinal mucosa.

LITERATURE CITED

1   Abe, K., S.-i. Hakomori, and S. Ohshiba. 1986. Differential expression of difucosyl type 2 chain (Le^y) defined by monoclonal antibody AH6 in different locations of colonic epithelia, various histological types of colonic polyps, and adenocarcinomas. Cancer Res. **46**: 2639-2644.

2   Bacon, A. E., R. Fekety, D. R. Schaberg, and R. G. Faix. 1988. Epidemiology of *Clostridium difficile* colonization in newborns: results using a bacteriophage and bacteriocin typing system. J. Infect. Dis. **158**: 349-354.

3   Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa. 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2**: 629-635.

4   Bjork, S., M. E. Breimer, G. C. Hansson, K.-A. Karlsson and H. Leffler. 1987. Structures of blood group glycosphingolipids of human small intestine. J. Biol. Chem. **262**: 6758-6765.

5   Bolton, R. P., S. K. Tait, P. R. F. Dear, and M. S. Losowsky. 1984. Asymptomatic neonatal colonization by *Clostridium difficile*. Arch. Dis. Childhood **59**: 466-472.

6   Brown, A., I. O. Ellis, M. J. Embleton, R. W. Baldwin, D. R. Turner, and J. D. Hardcastle. 1984. Immunohistochemical localization of Y hapten and the structurally related H type-2 blood-group antigen on large-bowel tumours and normal adult tissues. Int. J. Cancer **33**: 727-736.

7   Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

8   Castronovo, V., C. Colin, B. Parent, J.-M. Foidart, R. Lambotte, and P. Mahieu. 1989. Possible role of human natural anti-Gal antibodies in the natural antitumor defense system. J. Natl. Cancer Inst. **81**: 212-216.

9   Chandrasekaran, E. V., M. Davila, D. W. Nixon, M. Goldfarb, and J. Mendicino. 1983. Isolation and structures of the oligosaccharide units of carcinoembryonic antigen. J. Biol. Chem. **258**: 7213-7222.

10   Chang, T.-W., N. M. Sullivan, and T. D. Wilkins. 1986. Insusceptibility of fetal intestinal mucosa and fetal cells to *Clostridium difficile* toxins. Acta Phar. Sin. **5**: 448-453.

11   Clark, G. F., H. C. Krivan, T. D. Wilkins, and D. F. Smith. 1987. Toxin A from *Clostridium difficile* binds to rabbit erythrocyte glycolipids with terminal Galα1-3Galβ1-4GlcNAc sequences. Arch. Biochem. Biophys. **257**: 217-229.

12   Delmee, M., G. Verellen, V. Avesani, and G. Francois. 1988. *Clostridium difficile* in neonates: serogrouping and epidemiology. Eur. J. Pediatr. **147**: 36-49.

13   Donta, S. T., and M. G. Myers. 1982. *Clostridium difficile* toxin in asymptomatic neonates. J. Pediatrics. **100**: 431-434.

14   Dove, C. H., S.-Z. Wang, S. B. Price, C. J. Phelps, D. M. Lyerly, T. D. Wilkins, and J. L. Johnson. 1990. Molecular characterization of *Clostridium difficile* toxin A gene. **58**: 480-488.

15   Ehrich, M., R. L. van Tassell, J. M. Libby, and T. D. Wilkins. 1980. Production of *Clostridium difficile* antitoxin. **28**: 1041-1043.

16   Ernst, C. S., J-W. Shen, S. Litwin, M. Herlyn, H. Koprowski, and H. F. Sears. 1986. Multiparameter evaluation of the expression in situ of normal and tumor-associated antigens in human colorectal carcinoma. J. Nat. Cancer. Inst. **77**: 387-395.

17   Feizi, T., and R. A. Childs. 1985. Carbohydrate structures of glycoproteins and glycolipids as differentiation antigens, tumour-associated antigens and components of receptor systems. **10**: 24-29.

18   Galili, U., S. B. Shohet, E. Kobrin, C. L. M. Stults, B. A. Macher. 1988. Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J. Biol. Chem. **263**: 17755-17762.

19   Galili, U., B. A. Macher, S. B. Shohet. 1985. Human natural anti-α-galactosyl IgG. II. The specific recognition of α(1-3)-linked galactose residues. J. Exp. Med. **162**: 573-582.

20   Galili, U. 1989. Abnormal expression of α-galactosyl epitopes in man. Lancet **8659**: 358-361.

21   Gooi, H. C., T. Feizi, A. Kapadia, B. B. Knowles, D. Solter, and M. J. Evans. 1981. Stage specific embryonic antigen involves α1-3 fucosylated type 2 blood group chains. Nature **292**: 156-158.

22   Gooi, H. C., S. J. Thorpe, E. F. Hounsell, H. Rumpold, D. Kraft, O. Forster, and T. Feizi. 1983. Marker of peripheral blood granulocytes and monocytes of man recognized by two monoclonal antibodies VEP8 and VEP9 involves the trisaccharide 3-fucosyl-N-acetyllactosamine. Eur. J. Immunol. **13**: 306-312.

23   Harlow, E., and D. Lane. 1988. Labeling antibodies with biotin. in *Antibodies:a laboratory manual.* Cold Spring Harbor Laboratory pp 340-341

24   Hecht, G., C. Pothoulakis, J. T. LaMont, and J. L. Madara. 1988. *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. **82**: 1516-1524.

25   Holgersson, J., N. Stromberg, and M. E. Breimer. 1988. Glycolipids of human large intestine: difference in glycolipid expression related to anatomical localization, epithelial/non-epithelial tissue and the ABO, Le and Se phenotypes of the donors. Biochem. **70**: 1565-1574.

26   Huang, L. C., C. I. Civin, J. L. Magnani, J. H. Shaper, and V. Ginsburg. 1983. My-1, the human myeloid-specific antigen detected by mouse monoclonal antibodies, is a sugar sequence found in lacto-N-fucopentaose III. Blood. **61**: 1020-1023.

27   Itzkowitz, S. H., M. Yuan, Y. Fukushi, A. Palekar, P. C. Phelps, A. M. Shamsuddin, B. J. Trump, S.-I. Hakomori, and Y. S. Kim. 1986. Lewis^x- and sialylated Lewis^x-related antigen expression in human malignant and nonmalignant colonic cells. Cancer Res. **46**: 2627-2632.

28   Kagawa, Y., S. Takasaki, J. Utsumi, K. Hosoi, H. Shimizu, N. Kochibe, and A. Kobata. 1988. Comparative study of the asparagine-linked sugar chains of natural human interferon-β1 and recombinant human interferon-β1 produced by three different mammalian cells. J. Biol. Chem. **263**:17508-17515.

29   Kapadia, A., T. Feizi, D. Jewell, J, Keeling, and G. Slavin. 1981. Immunocytochemical studies of blood group A, H, I, and i antigens in gastric mucosae of infants with normal gastric histology and of patients with gastric carcinoma and chronic peptic ulceration. J. Clin. Pathol. **34**: 320-337.

30   Karlsson, K.-A., and G. Larson. 1981. Molecular characterization of cell surface antigens of fetal tissue. J. Biol. Chem. **256**: 3512-3524.

31   Karsch, W., E. Strelau, W.-D. Grahlow, E. Fischer, and R. Schulz. 1989. Occurrence and significance of *Clostridium difficile* in faecal specimens of hospitalized children. Zbl. Bakt. Hyg. **270**: 441-448.

32   Krivan, H. C.,G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. **53**: 573-581.

33   Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55**: 1873-1877.

34   Larson, H. E., F. E. Barclay, P. Honour, and I. D. Hill. 1982. Epidemiology of *Clostridium difficile* in infants. J. Infect. Dis. **146**: 727-733.

35   Lemieux, R. U., K. Bock, L. T. J. Delbaere, S. Koto, and V. S. Rao. 1980. The conformations of oligosaccharides related to the ABH and lewis human blood group determinants. Can. J. Chem. **58**: 631-653.

36   Lemieux, R. U. 1982. The binding of carbohydrate structures with antibodies and lectins. in *Frontiers of Chemistry*. K. J. Laidler (ed.). pp 3-24.

37   Libby, J. M., S. T. Donta, and T. D. Wilkins. 1983. *Clostridium difficile* toxin A in infants. J. Infect. Dis. **148**: 606.

38   Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultures cells in vitro. Infect. Immun. **56**: 582-588.

39   Lyerly, D. M., K. E. Saum, D. K. MacDonald, and T. D. Wilkins. 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47**: 349-352.

40   D. M. Lyerly, C. J. Phelps, J. Toth, and T. D. Wilkins. 1986. Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect. Immun. **54**: 70-76.

41   Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins. 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**: 1147-1150.

42   Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins. 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**: 72-78.

43   Mardh, P.-A., I. Helin, I. Colleen, M. Oberg, and E. Holst. 1982. *Clostridium difficile* toxin in faecal specimens of healthy children and children with diarrhoea. Acta. Pediatr. Scand. **71**: 275-278.

44   McKibbin, J. M., W. A. Spencer, E. L. Smith, J.-E. Mansson, K.-A. Karlsson, B. E. Sammuelsson, Y.-T. Li, and S.-C. Li. 1982. Lewis blood group fucolipids and their isomers from human and canine intestines. J. Biol. Chem. **257**: 755-760.

45   Mizoguchi, A., T. Mizuochi, and A. Kobata. 1982. Structures of the carbohydrate moieties of secretory component purified from human milk. J. Biol. Chem. **257**: 9612-9621.

46   Pothoulakis, C., P. McAndrew, and J. T. LaMont. 1988. Specific binding of radiolabelled *Clostridium difficile* toxin A on rabbit intestinal brush borders (BB) and cultured rat basophilic leukemia (RBL) cells (abstr.). Gastroenterology **94**: A358.

47   Pothoulakis, C., D. A. Sullivan, A. J. Melnick, T. Gadenne, T. Meshulam, and J. T. LaMont. 1986. *Clostridium difficile* toxins A and B stimulate intracellular calcium release in human neutrophils (abstr.). Clin. Res. **34**: 530A.

48   Pothoulakis, C., T. Theoharides, P. E. McAndrew, and J. T. LaMont. 1986. *Clostridium difficile* toxin A releases mediators from mucosal-like rat basophilic leukemia (RBL) cells (abstr.). Clin. Res. **34**: 871A.

49   Price, S. B., C. J. Phelps, T. D. Wilkins, and J. L. Johnson. 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. **16**: 55-60.

50   Rietra, P. J. G. M., K. W. Slaterus, H. C. Zanen, and S. G. M. Meuwissen. 1978. Clostridial toxins in healthy infants. Lancet **ii**: 319.

51   Rolfe, R. D., S. Helebian, and S. M. Finegold. 1981. Bacterial interference between *Clostridium difficile* and normal fecal flora. J. Infect. Dis. **143**: 470-475.

52   Sakamoto, J., K. Furukawa, C. Cordon-Cardo, B. W. T. Yin, W. J. Rettig, H. F. Oettgen, L. J. Old, and K. O. Lloyd. 1986. Expression of Lewis^a, Lewis^b, X, and Y blood group antigens in human colonic tumors and normal tissue and in human tumor-derived cell lines. **46**: 1553-1561.

53   Spiro, R. G., and V. D. Bhoyroo. 1984. Occurrence of α-D-galactosyl residues in the thyroglobulins from several species. J. Biol. Chem. **259**:9858-9866.

54   Spooncer, E., M. Fukuda, J. Klock, J. Oates, and A. Dell. 1984. Isolation and characterization of polyfucosylated lactosaminoglycan from human granulocytes. J. Biol. Chem. **259**: 4792-4801.

55   Sullivan, N. M., S. Pellett, and T. D. Wilkins. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35:** 1032-1040.

53   Taylor, N. S., G. M. Thorne, and J. G. Bartlett. 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect Immun. **34:** 1036-1043.

57   Torres, J. F., R. Cedillo, J. Sanchez, C. Dillman, S. Giono, and O. Munoz. 1984. Prevalance of *Clostridium difficile* and its cytotoxin in infants in Mexico. J. Clin. Microbiol. **20:** 274-275.

58   Triadafilopoulos, G., C. Pothoulakis, M. J. O'Brien, and J. T. LaMont. 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology **93:** 273-279.

59   Tucker, K. D., P. E. Carrig, and T. D. Wilkins. 1990. Toxin A of *Clostridium difficile* is a potent cytotoxin. J. Clin. Microbiol. **28:** 869-871.

60   Tullus, K., B. Aronsson, S. Marcus, and R. Mollby. 1989. Intestinal colonization with *Clostridium difficile* in infants up to 18 months of age. Eur. J. Clin. Microbiol. Infect. Dis. **8:** 303-393.

61   Viscidi, R. P., and J. G. Bartlett. 1981. Antibiotic-associated pseudomembranous colitis in children. Pediatrics **67:** 381-386.

62   Watkins, W. M., P. Greenwell, A. D. Yates, and P. H. Johnson. 1988. Regulation of expression of carbohydrate blood group antigens. Biochemie **70:** 1597-1611.

63   Wilkins, T. D., and K. D. Tucker. 1989. *Clostridium difficile* toxin A (enterotoxin) uses Galα1-3Galβ1-4GlcNAc as a functional receptor. Microecol. Therapy **19:** 225-227.

64   Wilson, K. H., J. Silva, and F. R. Fekety. 1981. Suppression of *Clostridium difficile* by normal hamster cecal flora and prevention of antibiotic-associated cecitis. Infect. Immun. **34:** 626-628.

CONCLUSION

Krivan et al. (18) demonstrated that *Clostridium difficile* toxin A binds to carbohydrates that contain Galα1-3Galβ1-4GlcNAc. I have found that this carbohydrate acts as a physiological receptor for toxin A on cell lines, and this indicates that Galα1-3Galβ1-4GlcNAc may act as a physiological receptor in vivo. However, Galα1-3Galβ1-4GlcNAc is not found on normal human cells (10, 11), so it is unlikely that this is the receptor for toxin A in humans.

In addition to binding to Galα1-3Galβ1-4GlcNAc, I have found that toxin A binds to similar carbohydrate antigens, designated I, X, and Y. These antigens are found on human intestinal epithelium (3, 4, 8, 9, 14, 15, 24, 27); therefore, these carbohydrates may be receptors for toxin A on the colonic epithelium of humans.

Infants appear to be less sensitive to the effects of colonization by *C. difficile* than are adults (1, 2, 6, 7, 13, 19, 22, 23, 25, 28, 29). Further, Chang et al. (5) reported that intestinal cells from human infants are more resistant to toxin A compared to intestinal cells from adults. Like human infants, infant hamsters also appear to be less sensitive to the effects of colonization by *C. difficile* than are adults (12, 26). I have found that the intestinal epithelium from infant hamsters binds less toxin A at 37C than does the same tissue from adult hamsters. Thus, the lack of sensitivity of cells from human infants to toxin A may be due to less toxin A binding to these cells. However, even though toxin A binds to Galα1-3Galβ1-4GlcNAc in hamsters, this carbohydrate is not normally

133

found in humans (10, 11). Instead in humans toxin A may bind to the I, X, and Y antigens. The Y antigen will bind large amounts of toxin A at 37C, and this receptor is present on the intestinal epithelium of human infants (16, 17).

Each of the carbohydrates to which toxin A binds share only the type 2 core structure (Galβ1-4GlcNAc), thus this structure could be the actual receptor for toxin A. However, the binding of toxin A to the type 2 core appears to depend on the conformation of the carbohydrate, because not all carbohydrates with a type 2 core will bind toxin A. Toxin A has the highest affinity for the Y antigen, and large amounts of toxin A bind to this structure at body temperature. This suggests that the Y antigen may be the most important receptor for toxin A in humans. Even so, toxin A uses Galα1-3Galβ1-4GlcNAc as a physiological receptor, and toxin A binds to the I and X antigens as well as it does to Galα1-3Galβ1-4GlcNAc. Thus the I and X antigens also may serve as receptors for toxin A in humans.

The stabilization of the type two core by branches on the carbohydrates could explain why toxin A binds to the I, X, and Y antigens, but this would not explain why toxin A has such a high affinity for the Y antigen. The only difference between the X and Y antigens is the presence of an α1-2-linked fucose on the Y antigen (Figure 1). Since Galβ1-4(Fucα1-3)GlcNAc has a similar conformation in both the X and Y antigens, the α1-2-linked fucose makes the Y antigen a high affinity receptor for toxin A. I believe that the α1-2-linked fucose actually fits into the binding site of toxin A. If this is true then toxin A binds to

both the type 2 core and the α1-2-linked fucose on the Y antigen, but only to the type 2 core on the X antigen. The α1-2-linked fucose on the Y antigen is conformationally similar to the α1-3-linked galactose on Galα1-3Galβ1-4GlcNAc (Figure 1). This suggests to me that the α1-3-linked galactose also may directly interact with the binding site of toxin A, so toxin A would bind to both the α1-3-linked galactose and the type 2 core on Galα1-3Galβ1-4GlcNAc. Thus, this could explain why toxin A binds to Galα1-3Galβ1-4GlcNAc even though this carbohydrate does not contain a branch to stabilize the type 2 core: the α1-3-linked galactose may directly interact with the binding site on toxin A, thus promoting the binding of toxin A even with the non-stabilized type 2 core.

Toxin A does not bind to blood group B antigen (Galα1-3[Fucα1-2]Galβ1-4GlcNAc) (18). The B antigen contains both an α1-3-linked galactose and an α1-2-linked fucose, both linked to the penultimate galactose. The conformation of the type 2 core in this oligosaccharide is similar to the conformation in both the Y antigen and Galα1-3Galβ1-4GlcNAc (Figure 1); however, the α1-2-linked fucose and the α1-3-linked galactose are shifted as compared to the Y antigen and Galα1-3Galβ1-4GlcNAc, respectively (Figure 1). Apparently, the shift in the orientation of the α1-3-linked galactose and the α1-2-linked fucose, as well as the possible stearic hinderance produced by the presence of both monosaccharides, prevents toxin A from binding to the blood type B antigen.
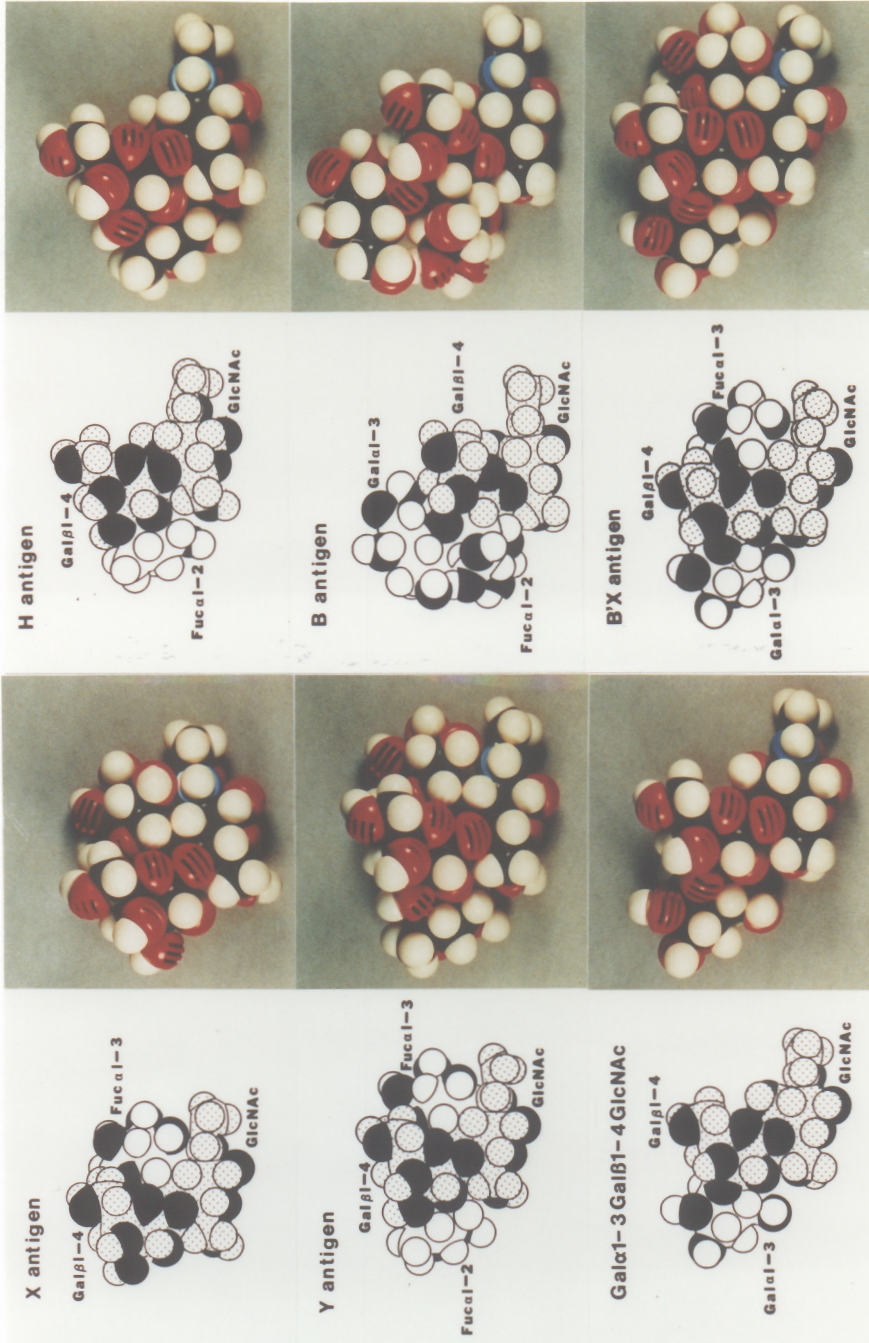
Figure 1: CPK space filling models of the X antigen, Y antigen, Galα1-3Galβ1-4GlcNAc, blood group B antigen (Galα1-3[Fucα1-2]Galβ1-4GlcNAc), and the blood group H antigen (Galα1-3[Fucα1-2]Galβ1-4GlcNAc). Also presented is a CPK space filling model of a theoretical oligosaccharide designated B'X antigen (Galα1-3Galβ1-4[Fucα1-3]GlcNAc). The conformation of the I antigen is identical to the type 2 core structure shown in figure 6 in section VI. The left hand column contains schematic representations of the models illustrating the position of the saccharides in these oligosaccharides, with the shaded area marking the type 2 core. A given carbohydrate can assume many different conformations, but the preferred conformation can be determined by analyzing nuclear magnetic resonance data and using HSEA calculations. The conformations in this figure, with the exception of the B'X antigen, are based on previously published predictions (20, 21). See text for discussion.

The conformation of blood type H antigen is almost identical to the Y antigen (Figure 1) except that the H antigen (Fucα1-2Galβ1-4GlcNAc) does not have the branch formed by the α1-3-linked fucose on the Y antigen. Thus the conformation of the H antigen is not stabilized . Even so, the conformational similarity between the H antigen, Y antigen, and Galα1-3Galβ1-4GlcNAc suggests that the H antigen should also bind toxin A; yet this carbohydrate does not bind the toxin. Thus, the α1-2-linked fucose on this non-branched carbohydrate does not promote the binding of toxin A. Since the α1-3-linked galactose on Galα1-3Galβ1-4GlcNAc does appear to promote the binding of toxin A, this suggests the α1-3-linked galactose on the type 2 core favors the binding of toxin A more than the α1-2-linked fucose. If this is true, then toxin A should bind to Galα1-3Galβ1-4(Fucα1-3)GlcNAc with a higher affinity than it binds to the Y antigen. This postulate is based on the premise that the type 2 core is stabilized by an α1-3-linked fucose in both, but the α1-2-linked fucose of the Y antigen is replaced by the preferred α1-3-linked galactose on Galα1-3Galβ1-4(Fucα1-3)GlcNAc. However, this suggestion can not be tested at this time because this carbohydrate is not available. Since Galα1-3Galβ1-4(Fucα1-3)GlcNAc has not been identified on any mammalian cells, I conclude that the Y antigen is the only high affinity receptor for toxin A on human cells.

LITERATURE CITED

1   Al-Jumaili, I. J. M. Shibley, A. H. Lishmann, and C. O. Record. 1984. Incidence and origin of *Clostridium difficile* in neonates. J. Clin. Microbiol. **19**: 77-78.

2   Bacon, A. E., R. Fekety, D. R. Schaberg, ans R. G. Faix. 1988. Epidemiology of *Clostridium difficile* colonization in newborns: results using a bacteriophage and bacteriocin typing system. J. Infect. Dis. **158**: 349-354.

3   Bjork, S., M. E. Breimer, G. C. Hansson, K.-A. Karlsson and H. Leffler. 1987. Structures of blood group glycosphingolipids of human small intestine. J. Biol. Chem. **262**: 6758-6765.

4   Brown, A., I. O. Ellis, M. J. Embleton, R. W. Baldwin, D. R. Turner, and J. D. Hardcastle. 1984. Immunohistochemical localization of Y hapten and the structurally related H type-2 blood-group antigen on large-bowel tumours and normal adult tissues. Int. J. Cancer **33**: 727-736.

5   Chang, T.-W., N. M. Sullivan, and T. D. Wilkins. 1986. Insusceptibility of fetal intestinal mucosa and fetal cells to *Clostridium difficile* toxins. Acta Phar. Sin. **5**: 448-453.

6   Delmee, M., G. Verellen, V. Avesani, and G. Francois. 1988. *Clostridium difficile* in neonates: serogrouping and epidemiology. Eur. J. Pediatr. **147**: 36-49.

7   Donta, S. T., and M. G. Myers. 1982. *Clostridium difficile* toxin in asymptomatic neonates. J. Pediatrics. **100**: 431-434.

8   Ernst, C. S., J-W. Shen, S. Litwin, M. Herlyn, H. Koprowski, and H. F. Sears. 1986. Multiparameter evaluation of the expression in situ of normal and tumor-associated antigens in human colorectal carcinoma. J. Nat. Cancer. Inst. **77**: 387-395.

9   Feizi, T., and R. A. Childs. 1985. Carbohydrate structures of glycoproteins and glycolipids as differentiation antigens, tumour-associated antigens and components of receptor systems. TIBS **10**: 24-29.

10   Galili, U., S. B. Shohet, E. Kobrin, C. L. M. Stults, B. A. Macher. 1988. Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J. Biol. Chem. **263**: 17755-17762.

11   Galili, U. 1989. Abnormal expression of α-galactosyl epitopes in man. Lancet **ii**: 358-361.

12   Griffin, G. E., A. McDougall, S. A. Ash, and S. P. Borriello. 1984. Age related susceptibility of hamsters to clindamycin induced *Clostridium difficile* caecitis. Microecol. Thrapy **14**: 269-270.

13   Gurwith, M. J., C. Langston, and D. M. Citron. 1981. Toxin-producing bacteria in infants. Am. J. Dis. Child. **135**: 1104-1106.

14   Holgersson, J., N. Stromberg, and M. E. Breimer. 1988. Glycolipids of human large intestine: difference in glycolipid expression related to anatomical localization, epithelial/non-epithelial tissue and the ABO, Le and Se phenotypes of the donors. Biochem. **70**: 1565-1574.

15   Itzkowitz, S. H., M. Yuan, Y. Fukushi, A. Palekar, P. C. Phelps, A. M. Shamsuddin, B. J. Trump, S.-I. Hakomori, and Y. S. Kim. 1986. Lewis^x- and sialylated Lewis^x-related antigen expression in human malignant and nonmalignant colonic cells. Cancer Res. **46**: 2627-2632.

16   Kapadia, A., T. Feizi, D. Jewell, J, Keeling, and G. Slavin. 1981. Immunocytochemical studies of blood group A, H, I, and i antigens in gastric mucosae of infants with normal gastric histology and of patients with gastric carcinoma and chronic peptic ulceration. J. Clin. Pathol. **34**: 320-337.

17   Karlsson, K.-A., and G. Larson. 1981. Molecular characterization of cell surface antigens of fetal tissue. J. Biol. Chem. **256**: 3512-3524.

18   Krivan, H. C.,G. F. Clark, D. F. Smith, and T. D. Wilkins. 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. **53**: 573-581.

19   Larson, H. E., F. E. Barclay, P. Honour, and I. D. Hill. 1982. Epidemiology of *Clostridium difficile* in infants. J. Infect. Dis. **146**: 727-733.

20   Lemieux, R. U., K. Bock, L. T. J. Delbaere, S. Koto, and V. S. Rao. 1980. The conformations of oligosaccharides related to the ABH and lewis human blood group determinants. Can. J. Chem. **58**: 631-653.

21   Lemieux, R. U. 1982. The binding of carbohydrate structures with antibodies and lectins. in *Frontiers of Chemistry*. K. J. Laidler (ed.). pp 3-24.

22   Libby, J. M., S. T. Donta, and T. D. Wilkins. 1983. *Clostridium difficile* toxin A in infants. J. Infect. Dis. **148**: 606.

23   Mardh, P.-A., I. Helin, I. Colleen, M. Oberg, and E. Holst. 1982. *Clostridium difficile* toxin in faecal specimens of healthy children and children with diarrhoea. Acta. Pediatr. Scand. **71**: 275-278.

24   McKibbin, J. M., W. A. Spencer, E. L. Smith, J.-E. Mansson, K.-A. Karlsson, B. E. Sammuelsson, Y.-T. Li, and S.-C. Li. 1982. Lewis blood group fucolipids and their isomers from human and canine intestines. J. Biol. Chem. **257**: 755-760.

25   Rietra, P. J. G. M., K. W. Slaterus, H. C. Zanen, and S. G. M. Meuwissen. 1978. Clostridial toxins in healthy infants. Lancet **ii**: 319.

26   Rolfe, R. D., and J. P. Iaconis. 1983. Intestinal colonization of infant hamsters with *Clostridium difficile*. Infect. Immun. **42**: 480-486.

27   Sakamoto, J., K. Furukawa, C. Cordon-Cardo, B. W. T. Yin, W. J. Rettig, H. F. Oettgen, L. J. Old, and K. O. Lloyd. 1986. Expression of Lewis[a], Lewis[b], X, and Y blood group antigens in human colonic tumors and normal tissue and in human tumor-derivedcell lines. **46**: 1553-1561.

28   Stark, P. L., A. Lee, and B. D. Parsonage. 1982. Colonization of the large bowel by *Clostridium difficile* in healthy infants: quantitative study. Infect. Immun. **35**: 895-899.

29   Tullus, K., B. Aronsson, S. Marcus, and R. Mollby. 1989. Intestinal colonization with *Clostridium difficile* in infants up tp 18 months of age. Eur. J. Clin. Microbiol. Infect. Dis. **8**: 303-393.

CURRICULUM VITAE
May 1990

NAME: Kenneth Davis Tucker

BIRTH DATE AND PLACE: February 15, 1961
                               Richmond, Virginia

ADDRESSES: Anaerobe Laboratory
                               Virginia Polytechnic Institute and State University
                               Blacksburg, Virginia 24061
                               Telephone: (703) 231-5094

EDUCATION:

1987-present   Virginia Polytechnic Institute and State University
                               (Anaerobic Microbiology)

1984-1987   Virginia Polytechnic Institute and State University
                               Master of Science (Microbiology)

1979-1983   Virginia Polytechnic Institute and State University
                               Bachelor of Science (Biology)

PROFESSIONAL EMPLOYMENT:

1987-present   Graduate Research Assistant. Department of Anaerobic
                               Microbiology, Virginia Polytechnic Institute and State
                               University, Blacksburg, Virginia 24161.

1989 Jan.-May   Laboratory Teaching Assistant.

1984-1986   Laboratory Teaching Assistant and Graduate Research
                               Assistant. Department of Biology, Virginia Polytechnic
                               Institute and State University, Blacksburg, Virginia 24061.

1983-1984   Research Assistant. Department of Dentistry, Virginia
                               Commonwealth University: Medical College of Virginia,
                               Richmond, Virginia.

PROFESSIONAL SOCIETY:

1985-present   American Society for Microbiology

HONORARY SOCIETY:

    Phi Kappa Phi

GRANTS:

1987
Title   Identification of the Physiological Receptor for Toxin A
of *Clostridium difficile.*
Graduate Student Assembly
Graduate Research Development Project

PRESENTATIONS:

1990   Tucker, K. D., and T. D. Wilkins. A possible receptor for *Clostridium difficile* toxin A in humans. American Society for Microbiology, Anaheim, California.

1989   Tucker, K. D., P. E. Carrig, and T. D. Wilkins. *Clostridium difficile* toxin A uses Galα1-3Galβ1-4GlcNAc as a functional receptor. American Society for Microbiology, New Orleans, Louisiana.

1986   Tucker, K. D., and J. L. Neal. Growth of *Bradyrhizobium japonicum* Utilizing Nitrous Oxide as the Terminal Electron Acceptor. Virginia Branch of American Society for Microbiology, Pembroke, Virginia.

PUBLICATIONS:

Tucker, K. D., P. E. Carrig, and T. D. Wilkins. 1990. Toxin A of *Clostridium difficile* is a potent cytotoxin. J. Clin. Microbiol.28: 869-871.

Wilkins, T. D., and K. D. Tucker. 1989. *Clostridium difficile* toxin A (enterotoxin) uses Galα1-3Galβ1-4GlcNAc as a functional receptor. Microecol. Therapy **19**: 225-227.

Tucker, Kenneth D., and John L. Neal. 1988. Growth and Respiration of *Bradyrhizobium japonicum* USDA 143 with Nitrous Oxide as the Terminal Electron Acceptor. Current Microbiology **17**: 89-94.

Tucker, Kenneth D., and John L. Neal. 1988. Amperometric Method for Monitoring Anaerobic Respiration with Nitrous Oxide as the Terminal Electron Acceptor. Current Microbiology **17**: 95-98.

PENDING PATENT:

Wilkins, T. D., and K. D. Tucker. March 9, 1990. Detection, isolation, and purification of *Clostridium difficile* toxin A with receptors. U. S. Patent application Serial No. 07/491,396.