IDENTIFICATION AND CHARACTERIZATION OF A RECEPTOR FOR *CLOSTRIDIUM DIFFICILE* ENTEROTOXIN

by

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FOREWORD

This dissertaion contains four sections. Section I is a literature review which contains an introduction and ends with a statement of the research problem. Sections II and III were written in the form of two papers for publication with the concurrence of my committee. The first paper (section II) has been accepted in Infection and Immunity and will be published in September, 1986. Section III contains the second paper which has been submitted to Infection and Immunity. Section IV is an overall summary and discussion. The titles of the papers are as follows:

Krivan, H.C., G.F. Clark, D.F. Smith, and T.D, Wilkins. 1986. The Cell Surface Binding Site for Clostridium difficile Enterotoxin: Evidence for a Glycoconjugate Containing the Sequence Galα1-3Galβ1-4GlcNAc. Infection and Immunity (in press).

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In addition I contributed to the following publications:

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SECTION I. LITERATURE REVIEW

Introduction

The normal flora of the gastrointestinal tract forms an important protective barrier against infection by pathogens. Under normal conditions the concentration of bacteria in the human small intestine ranges from 10^5 to 10^8 organisms per ml and includes both aerobes and anaerobes (47,75,106). In the colon, however, a striking increase in concentration of bacteria occurs (10^{10} to 10^{11} per ml) where the anaerobic bacteria predominate (47,69,75,157,212). Moore and Holdeman (147) have reported that the total number of different kinds of bacteria in the intestinal tract at any one time probably exceeds 400 species, but most are represented by less than 10^8 cells per gram feces (less than 1/1000 of the bacterial population). Thus the normal flora represents one of the most complex ecosystems found in nature forming not only a barrier between the host and potential disease-causing organisms, but also exerting an influence on the normal physiological functioning of the gastrointestinal tract.

Disturbance of this microecology, by the administration of antibiotics, may result in an individual becoming susceptible to infection, either from an opportunistic member of the flora or from an exogenous pathogen. The ecological imbalance which follows antibiotic therapy, where nutrients are plentiful and bacteria are scarce, provides a bacterial

overgrowth syndrome where a pathogen that could normally not compete in this ecosystem can grow rapidly and cause disease. If these bacteria happen to produce a toxin the patient could be in serious trouble.

Shortly after the discovery of antibiotics, between 1950 and 1965, a disease developed that was a direct result of antibiotic treatment with chloramphenicol and tetracycline (166). This disease was characterized by inflamation and pseudomembrane formation in the small intestine and colon. The pseudomembrane, consisting of necrotic cells of the epithelium, fibrin, mucin, polymorphonuclear leukocytes and other cellular debris was thought to be caused by toxin-producing Staphylococcus aureus, a Gram-positive facultative anaerobe which was frequently isolated from the feces of these patients (2,3,111,190). Although a great deal of clinical and laboratory research was directed to proving that S. aureus caused some cases of antibiotic-associated colitis, in retrospect the proof of this association seems incomplete. Studies by Batts et al. (13) and McDonald et al. (136) have shown that S. aureus does not cause pseudomembranous colitis.

In the 1970s another form of pseudomembranous colitis was discovered that also was a direct result of treatment with antibiotics, particularly lincomycin and its derivative clindamycin. It was discovered that these antibiotics were extremely active against Bacteroides fragilis and other clinically important anaerobes. Because chloramphenicol

therapy often was associated with numerous and sometimes lethal side effects, clindamycin became an attractive alternative for serious anaerobic infections; however, this created a serious dilemma for the clinician. A number of subsequent reports linked the occurence of pseudomembranous colitis to the administration of clindamycin and linocmycin (16,31,41,45,53,63,64,77,118,141,159,161). "Clindamycin-associated colitis" could fairly be termed an epidemic coincident with a rise in prescriptions of this new antibiotic. The recognition that clindamycin caused pseudomembranous colitis became a major problem for many physicians prescribing the antibiotic as well as for the drug company that manufactured it. A major research effort to identify the cause of this disease began. Several novel microbiological observations which are described below clearly established that pseudomembranous colitis was an infection, and a disease we now know rarely occurs in uncompromised individuals.

Etiology of Pseudomembranous Colitis

In 1977 fecal extracts from patients with psuedomembranous colitis were found to be toxic to cultured mammalian cells (118). These investigators demonstrated that cytotoxicity was not caused by a cytopathic virus but instead appeared to be produced by some species of clostridia. Gas gangrene antitoxin was available in these laboratories and investigators soon

discovered that this antitoxin neutralized the cytotoxicity. Gas gangrene antitoxin is composed of a mixture of clostridial antitoxins made to culture filtrates of each of the clostridia that can cause gas gangrene: C. perfringens, C. sordellii, C. novyi, C. bifermentans, and C. histolyticum. It was discovered that the neutralizing antitoxin in this mixture was C. sordellii antitoxin (1,25,119, 170,218). Investigators were unable, however, to isolate C. sordellii from the cytotoxic fecal specimens. Studies later carried out by Bartlett et al. (9,11,12,) and Rifkin et al. (171) showed that another clostridium, C. difficile, was recovered in high numbers (greater than 10^6 organisms per gm wet wt.) and that C. sordellii antitoxin neutralized the cytotoxic activity produced by C. difficile. They also showed that C. difficile isolates from humans with pseudomembranous colitis caused a similar type of disease in hamsters and that the cytotoxic activity in the feces of these animals was neutralized by C. sordellii antitoxin. Thus, the fortuitous cross-reaction of C. difficile toxin with C. sordellii antitoxin led to the discovery of C. difficile as the causitive agent of psuedomembranous colitis (1,8,9,11,12,21,24,25,65,97,118,119, 165,218). We now know that these two organisms produce toxins that are very similar if not identical, although C. sordellii produces much smaller amounts and has not been shown to produce pseudomembranous colitis in man or other animals (220).

It is now well documented that at least 90% of cases of pseudomembranous colits related to antimicrobial therapy are due to C. difficile (6,19,55). In patients with antibiotic-associated colitis without pseudomembranes, C. difficile is isolated in at least 25% and toxin detected in 38% of the patients (11).

The contribution of the Syrian Golden hamster model to the understanding of pseudomembranous colitis in humans cannot be overemphasized. This animal model has permitted investigation of a number of aspects of the disease by methods that are neither feasible nor ethical in studies of humans. The association of antibiotic treatment and C. difficile infection can be fully reproduced in the hamster model. Clindamycin-treated hamsters develop a fatal enterocecitis if they are given viable C. difficile by mouth (202). Uncontaminated animals remain well. Every type of antibiotic associated with the human disease has been shown to have an action similar to clindamycin in hamsters.

Although C. difficile is recognized as the major cause of pseudomembranous colitis, there are reports indicating that the disease can be caused by other agents. Dickinson et al. (43) described two cases of pseudomembranous colitis which occurred without antibiotic therapy. C. difficile and its toxins were not detected in the feces of either patient. Jewkes et al, (93) have suggested that some of these cases may be due to an as yet undiscovered pathogen. There is some evidence to

incriminate C. perfringens type A in many cases of antibiotic-associated diarrhea (20); in fact, some strains of C. perfringens produce toxins which are immunologically related to toxins A and B of C. difficile (220).

Pathophysiology

Several interrelated variables are involved in the pathophysiology of C. difficile disease including a source of the organism, antibiotic exposure, toxin production, and patient age.

A. Source of the organism. It has been reported that about 3-5% of healthy adults harbor C. difficile (10,105). These people are presumably at risk in the event that they undergo antibiotic therapy. Even higher rates have been found in hospitalized patients without diarrhea who have received antibiotics, or are debilitated, or reside in parts of the hospital where the organism is abundant (52,105,116,152). C. difficile forms resistant spores that are difficult to exclude from the hospital environment. It has been reported that C. difficile can be detected on room surfaces up to 40 days after a patient with C. difficile diarrhea has vacated (153); in addition, the organism has been isolated from the hands of nursing personnel (105,132,182) and from shared commodes (178)

Some patients become healthy carriers after treatment for the disease (183). Despite investigations by a number of

workers, it is still not known whether the usual ecologic niche of C. difficile is endogenous, exogenous or both. One intriguing fact is that C. difficile is commonly found as a member of the intestinal flora of apparently healthy neonates (215). Larson and coworkers (1982) screened fecal samples from 451 newborn infants daily for C. difficile. Their results provide some evidence for nosocomial spread of the organism in this population, both from a common source and by person-to-person transmission. However, the initial source from which C. difficle might gain access to the neonatal group (or any other hospitalized population) is still not known. C. difficile was not detected in cultures of vaginal swabs and stool specimens from 17 mothers of colonized infants (116); thus there was no evidence for maternal-child transmission.

B. Antibiotic exposure. C. difficile is unprecedented as an enteric pathogen in causing disease almost exclusively in the presence of antibiotic exposure. Many different antimicrobial agents have been implicated (Table 1), although the most common are ampicillin, clindamycin, and the cephalosporins (10,205). There are a number of explanations as to how an antibiotic which is active on C. difficile might allow the pathogen to still colonize the gut. The best explanation, however, is probably that as antibiotic levels decline following the cessation of antibiotic therapy, C. difficile can become

established before the complete normal flora is restored.

C. Toxin Production. In contrast to other forms of infectious colitis, there is no bacterial invasion of the intestinal mucosa by C. difficile. Rather, there are several lines of evidence which implicate the two toxins (toxin A and toxin B) produced by the organism in the development of the disease: (i) C. difficile and both toxins are consistently detected in the stools of patients with pseudomembranous colitis (20), and the amount of toxin in the stool can exceed 10^6 tissue culture doses per gram of feces; (ii) intestinal pathology and death caused by the purified toxins in experimental animals mimics the pathology observed during the disease (125); and (iii) experimental animals must be vaccinated against both of the toxins in order to be protected against the disease (54,125). Several investigators have reported that all of the isolates of C. difficile so far examined either produce both of the toxins or neither toxin (121,131,132). This may suggest that factors controlling the expression or regulation of toxin A may be similar or identical to those regulating the expression of toxin B.

D. Patient Age. Human infants frequently harbor C. difficile and its toxins without any apparent deleterious consequences (5,18,72,74,116,120,124,169,182). This group represents the only patient population in which the toxins are frequently encountered without any clinical expression of the disease.

Table 1. Antimicrobial agents reported to be associated with pseudomembranous colitis in humans[1].

Amoxicillin	Clindamycin	Neomycin
Ampicillin	Cloxacillin	Penicillin G
Amphotericin	Erythromycin	Penicillin V
Carbenicillin	5-Fluorocytosine	Rifampin
Cefazolin	Flucloxacillin	Sulfmethoxazole
Ceforoxine	Gentamycin	Tetracycline
Cefoxitin	Kanamycin	Tobramycin
Cephaloridine	Lincomycin	Trimethoprim
Cephalothin	Metronidazole	
Chloroamphenicol	Nafcillin	

[1]Data taken from the following references: Boriello, S.P. and Larson, 1981; Bartlett, 1981; Larson et al., 1984; Tedesco, 1984; Boriello and Larson, 1985.

A possible explanation as to why infants are refractory to the toxins will be discussed later; however, the implication is that age and/or development is associated with enhanced susceptibility and older individuals appear to be most susceptible (10).

Clostridium difficile toxins

The toxigenic nature of *C. difficile* was originally described by Hall and O'Tool in 1935 who studied several strains isolated as normal flora from the feces of healthy human infants (74). Although these infants showed no signs of clinical disease, these investigators showed that cell-free filtrates prepared from cultures of the organism caused edema, respiratory arrest and death when injected subcutaneously into guinea pigs. Their conclusion was that *C. difficile* produced an exotoxin. Subsequent work by Snyder in 1937 (189) showed the exotoxin was heat labile, that antiserum against *C. difficile* cultures neutralized the toxic activity of the cell-free filtrates, and that all experimental animals studied were susceptible including guinea pigs, rabbits, dogs, cats, rats, and pigeons.

In spite of these most interesting findings over five decades ago, the organism was not known to cause disease in patients until 1977 when it was identified as the major cause of antibiotic-associated pseudomembranous colitis. This discovery provided the impetus to study the toxins produced

by C. difficile. Initially, only a single toxin was thought to be produced by the organism; however, the presence of a second toxin was demonstrated by Taylor and Bartlett in 1980 (200) and later by Banno et al. in 1981 (5). The two toxins can be separated by anion exchange chromatography using NaCl gradient elution. One toxin possesses both enterotoxic and cytotoxic activity, elutes first during anion exchange chromatography and has been designated by different investigators as toxin A (194), enterotoxin (201), and toxin D-1 (5). The second toxin is a much more potent cytotoxin with no detectable enterotoxic activity; this toxin elutes after toxin A during anion exchange chromatography, and has been designated toxin B (194), cytotoxin (201), and toxin D-2 (4,5). The nomenclature most commonly used at the present time is toxin A and toxin B. A comparison of the two toxins produced by C. difficile is presented in Table 2 and their physicochemical and biological properties are discussed below.

A. Toxin A. Sullivan et al. (194) described a procedure for preparing homogeneous toxin A from culture filtrate. The method consisted of ultrafiltration through an XM-100 membrane filter, DEAE anion exchange chromatography, and acetic acid precipitation at pH 5.6. Other procedures for purifying toxin A have been reported using vaying methods : (i) ammonium sulfate precipitation, gel filtration, batch anion exchange chromatography, hydrophobic interaction chromatography,

and precipitation of the toxin in acetate buffer, pH 5.6 (4,5,133,194,201), all of which are based on a modification of Sullivan's method; (ii) preparative electrophoresis in a discontinuous buffer system (192); (iii) chromatofocusing using FPLC (Fast Protein Liquid Chromatography) (173); and, (iv) immunoaffinity chromatography (133).

Toxin A is a large, heat labile, hydrophobic protein with an estimated molecular weight of 440,000-600,000 and an isoelectric point of 5.4-5.6 (4,5,89,175,194,199,203,219,). The toxin is stable to acid (pH 4) and alkaline (pH 10) conditions (175,194,199), but is sensitive to oxidation (220), loses activity at temperatures greater than 56C (194) and is inactivated by proteolyic enzymes (5,175,194). The high molecular weight estimates of the toxin suggest that the toxin has a subunit structure. Banno et al. (4) have reported that the toxin consists of two subunits with an estimated molecular weight for each of 200,000. Rihn et al. (173) reported two dissimilar subunits with estimated molecular weights of 41,500 and 16,000. Wilkins et al. (220) and other investigators have so far been unsuccessful in dissociating toxin A. Toxin A and toxin B may exist as aggregates which do not readily dissociate following exposure to denaturing conditions. Two lines of evidence also suggest that the toxin has repeating subunits: (i) toxin A has been found to be precipitated by a monoclonal antibody (128) which should not occur unless the same epitope occurs more than

once on the molecule, i.e. in a repeating manner; and (ii) the toxin agglutinates rabbit erythrocytes (see section III) which also requires at least two binding sites per molecule of toxin.

Amino acid compositions of highly purified preparations of toxin A have been reported by several investigators (5,129,192). Toxin A contains high amounts of the hydrophobic amino acids valine, isoleucine, and leucine; this may account for the strong interaction of the toxin with hydrophobic gels (194). Toxin A also contains relatively high amounts of aspartic acid (or asparagine), glutamic acid (or glutamine) and glycine, and very little histidine and sulfur-containing amino acids. The toxin does not appear to contain pentose, hexose, nor any detectable amino sugars or phosphorus (5,194).

Studies on the biological activity indicate that toxin A possesses two distinct toxic activities: a potent enterotoxic activity and weak cytotoxic activity. Toxin A, like toxin B, is cytotoxic in all of the mammalian cells which have been tested (20,132). In monolayer cultures of fibroblasts both toxins cause a morphological change which has been termed "actinomorphic" due to the star-like appearance of affected cells under the light microscope (25). The wide range of cytotoxic activity shared by both toxins suggests that any receptors involved in the cytotoxic process are ubiquitous.

The enterotoxic activity of C. difficle toxin A was initially detected using ligated intestinal loops in rabbits

(5,200). The fluid which accumulates in the rabbit ileal loop assay is hemorrhagic; an intense inflammatory response occurs as does necrosis in the intestinal mucosa. This enterotoxic response is markedly different from the classic "ricewater" fluid produced by the action of the enterotoxins from Vibrio cholera and Escherichia coli. Unlike toxin A these toxins elicit fluid flux with no inflammatory response. Toxin A also causes fluid accumulation in colonic loops, although the tissue damage is not as extensive (146). The fluid which occurs following treatment with toxin A may be due to an increase in the permeability of the gut mucosa (146). Toxin A also produces a positive fluid response in infant mice after intragastric challenge, a model which can also be used to study enterotoxic activity (76,127). Injection of nanogram amounts into the skin of rabbits produces erythematous and hemorrhagic lesions and increases vascular permeability (5,127,201). Intraperitoneal injection into mice causes death (5,194,201). Intracecal injection of toxin A in the hamster causes a moderate hemorrhage, acute inflammation, necrosis, fluid accumulation and death (125). Toxin A given intragastrically causes hemorrhage, fluid accumulation in the cecum and small intestine, diarrhea and death (130). Hamsters are at least ten-fold more sensitive to toxin A given intragastrically than mice or rats (130). This increase in sensitivity may be due to differences in the nature or number of receptors in the intestine, a subject which will

be discussed later.

B. **Toxin B.** Sullivan et al. (193,194) also developed a method for purifying toxin B to homogeneity. The method consisted of ultrafiltration, hydrophobic interaction chromatography, anion-exchange chromatography, and immunoaffinity chromatography. Toxin B was also found to be a large, heat labile, hydrophobic protein (MW 360,000-470,000) with an isoelectric point of 4.1-4.5 (129,194). It was shown that a homogeneous toxin B preparation contained five immunologically related cytotoxic proteins with estimated molecular weights of 70,000, 160,000, 250,000, 280,000, and 340,000 (132,193). These smaller cytotoxic components, which have been termed "multiple forms" of the toxin (193) may be varying aggregates of the toxin. Toxin B is inactivated after exposure to acid (pH 4) and alkaline (pH 9) conditions, by oxidation, temperatures of 56C for one hour, and after reaction with proteolytic enzymes (5,132,175,194,199).

The amino acid compostion of highly purified toxin B has also been reported and has been shown to contain high amounts of aspartic acid (or asparagine), glutamic acid, (or glutamine) glycine, leucine, isolecine and valine; and low amounts of histidine and sulfur-containing amino acids similar to toxin A (4,129,192). Like toxin A, toxin B does not contain phophorus but low amounts of hexose and pentose were detected (194).

The most impressive difference between toxin A and toxin B is that toxin B is completely devoid of enterotoxic activity, but is 1000 times more cytotoxic than toxin A. Toxin B is responsible for the cytotoxic activity observed in fecal specimens from persons with pseudomembranous colitis because it masks the lesser cytotoxic activity of toxin A. Like toxin A, toxin B is active against all mammalian cells tested (23,25,46,198,204). These include normal and transformed fibroblasts, epithelium-like cells and neuron-like cells from different species (human, monkey, rabbit, hamster, mouse and rat), and tissues (brain, liver, kidney, adrenal gland, lung, skin, conjunctiva, smooth muscle, tonsils, intestine, rectum, ovary, amnion fluid, chorion, and cervix). The tissue culture death dose (TCD_{100}) of toxin B is about 1 pg (4,129, 194) and its effect on tissue cultured cells has been reported to be irreversible (204). Toxin B, however, does not affect the growth of yeasts, Gram-positive and Gram-negative bacteria or mycoplasma (206).

Although toxin B does not elicit a fluid response in the rabbit ileal loop assay, it does produce erythematous, hemorrhagic lesions and increases vascular permeability when injected intradermally into rabbit skin in nanogram amounts (127,201). Intracecal injection of toxin B in hamsters causes extensive hemorrhage and inflammation in the mucosal wall, but no response is observed when the toxin is given intragastrically to hamsters (130). Toxin B, like toxin A,

when injected intraperitoneally into mice is lethal in nanogram amounts (129,194).

Mechanism of action

Very little is known about the mechanism of action of either toxin A and toxin B. Recent work by Vesely et al. (213,214) has shown that both toxins stimulated soluble guanylate cyclase activity and that enhancement occured at concentrations at which the respective toxins have been shown to cause increased vascular permeability in rabbits and ileocecitis in hamsters. Toxin A was also found to enhance particulate guanylate cyclase activity while toxin B did not. An interesting result reported by these investigators was that toxin A and B were additive with respect to their maximal stimulation of guanylate cyclase activity. This may suggest that there are separate receptors for C. difficile toxins A and B on the colonic plasma membrane. Furthermore, these results would also suggest that once these receptors are activated they both utilize a common pathway, namely, by activating in turn the guanylate cyclase-cyclic GMP system. Whether this stimulation is relevant to the disease process, however, has not been determined.

Other investigators (176) have reported that both toxins decrease intracellular levels of potassium in HeLa cells and shut down protein synthesis. These findings support the previous results of Thelestam and Florin (204) who found that

crude preparations of C. difficile toxin inhibited protein synthesis. Recently, Lima et al, (126) have reported that the cytotoxic effects of toxin A in CHO cells did not appear to involve inhibitable Ca-dependent or prostaglandin synthesis pathways, and that cytotoxicity was not inhibited by sulfhydryl or lysosomotropic agents. They also found that toxin A toxicity did not involve intact microfilament or microtubule function for its activity. This is in contrast to what has been observed by several investigators for toxin B. Toxin B has been demonstrated to disrupt the microfilament bundles in fibroblasts in a manner similar to what has been observed when fibroblasts are treated with cytochalsin B (204,205,206).

Evidence presented by Thelestam and coworkers indicates that toxin B is internalized via endocytosis prior to disruption of the microfilament bundles and that intoxication can be inhibited by lysosomotropic agents (205,206). These results are similar to those of Wedel et al. (217) who recently showed that purified toxin B induced disorganization of the actin-containing myofilament bundles in rabbit smooth muscle cells. With transmission electron microscopy, they observed that the effect occurred concomitantly with the appearance of the cytopathic effect, while all other cell organelles still appeared unaffected. In addition, Pothoulakis et al. (160) recently reported that highly purified preparations of toxin

Table 2. Comparison of the two C. difficile toxins[1]

	Toxin A	Toxin B
Molecular weight	440-600,000	360-470,000
Isoelectric point	5.4-5.6	4.1-4.5
Hydrophobic	+	+
Cytotoxic	+	++++
Enterotoxic	+	-
Positive fluid response (infant mice)	+	-
Lethality on i.p. injection (mice)	+	+
Lethality on intracecal injection (hamster)	+	+
Increases vascular permeability	+	+
Neutralization by C. sordellii antitoxin	+	+
Stimulation of adenylate cyclase	-	-
Stimulation of guanylate cyclase	+	+
Inactivated by:		
Heat	+	+
Oxidation	+	+
pH 4	-	+
pH 10	-	+
Proteolytic enzymes	+	+
Lipase	-	-
Presence of:		
Hexose/pentose	-	+
Amino sugars	-	-
Phosphorus	-	-

[1]Details and references are given in the text

B disaggregated actin and caused a dose dependent increase on cytoplasmic or G-actin in tissue cultured cells. This supports the findings of Thelestam et al. that the action of toxin B resembles that of cytochalasin B. Pothoulakis et al. (160) suggest that this effect on actin may be enzymatic in nature.

In light of these data it has been proposed that the microfilaments of the cell are likely to be the primary intracellular target for the action of toxin B (205,206). For comparison it may be interesting to note that toxin A which reportedly does not interact with the microfilaments of the cell (126) has been observed to cause actinomorphic changes in fibroblast cells identical to those noted for toxin B. This suggests that the mechanism for the cytopathic effect of toxin A and toxin B may be different; however, this hypothesis has yet to be proven.

Clostridium difficile toxin receptors

There is very little information available concerning the cell surface binding sites for either toxin A or toxin B; in fact, a receptor for either toxin has not been identified. Only a few preliminary reports have focused on this problem, and none of the studies have analyzed binding to tissue or cells from the gastrointestinal tract of humans or animals.

Early studies by Chang and coworkers (26) determined binding of crude preparations of toxin B to human erythrocyte

ghosts indirectly, by measuring loss of cytotoxic activity after contact with the membranes. They reported that binding was inhibited by certain sterols and bile acids, whereas monosaccharides, amino sugars, and treatment of membranes with neuraminidase had no effect on binding activity. Thus a lipid component was suggested to play a role in toxin binding to erythrocytes. About this same time other investigators reported that preincubation of crude toxin with high concentrations of cholesterol, gangliosides, or lecithin did not abolish cytotoxic activity on fibroblasts, and incubation of cells with phospholipase C also did not alter the cytotoxic response (204). Donta and Shaffer (46) also could not inhibit intoxication of epithelial-like cells with gangliosides. These investigators concluded that a lipid receptor on the surface of cultured cells seemed less likely.

Donta and Schaffer (46) found that concanavalin A (con A) was not protective for epithelial-like cells. These findings, however, were contrary to that of Florin and Thelestam (60) who later reported that con A and ricin caused a delay in the intoxication response. Levy and Onderdonk (123) found that wheat germ agglutinin (WGA) also reduced the cytotoxic effect for monkey kidney fibroblasts, whereas phytohemagglutinin (PHA) was without effect. The results obtained in these studies, however, should be interpreted with caution for two reasons: (i) all investigations published before 1981, and also some more recent ones, were performed with crude toxin

preparations probably containing both toxin A and B and other unknown factors; and (ii) it has been reported that some lectins, in particular con A, interfere with endocytosis in various cells (42,144,222).

Recently Florin and Thelestam (60,62) have proposed that purified toxin B must be internalized by endocytosis for expression of the cytotoxic effect to occur. This conclusion was based on the fact that intoxication by toxin B was prevented by the lysosomotropic agents ammonium chloride and cloroquine at concentrations inhibiting endocytosis. The development of the cytotoxic effect was also prevented in the presence of inhibitors of cellular energy metabolism or by transfer of cells to $0^{o}C$ (59,60). These findings are consistent with the view that toxin B must be internalized by endocytosis to exert a toxic response.

More recently Florin and Thelestam (61) reported that ATP and other polyphosphorylated compounds also inhibit the cytotoxic effect of toxin B on human lung fibroblasts. They suggested that these compounds bind to a site on the toxin which is distinct from the receptor-binding site. This site may be analagous to the P-site which has been described for diphtheria toxin (162). Presence of ATP interferes with some step in the intoxication process but does not prevent binding of the toxin to the cell. Florin and Thelestam (61) have based their conclusions on the observation that toxin B remained accessible to neutralization with externally added

antitoxin in the presence of ATP, indicating that ATP blocked the intoxication process at a stage when the toxin was still located outside the cytoplasmic membrane.

Thelestam and Florin (206) have proposed a model of the intoxication process by toxin B in cultured cells. Although the receptor structure remains unidentified, they propose that the toxin binds rapidly and firmly to the cell surface independent of temperature and metabolic energy. The actual binding of the toxin may be stabilized via the polyphosphate binding site. The toxin is internalized via endocytosis into acid endosomes, which ultimately fuse with lysosomes where the toxin is proteolytically activated. Membrane channels are formed in the lysosomal membrane permitting the transfer of the toxin to the cytosol. Once in the cytosol, the toxin may act on the microfilament bundles in the same way as the cytochalasins, which bind to actin and prevent polymerization of microfilaments. These effects, they concluded, result in the actinomorphic changes in the cell shape before the cells deteriorate.

Only one report has been published on the binding of toxin A to tissue cultured cells. Ferritin-conjugated toxin A has been shown to attach to the cell membrane of L-cells as aggregates (179), which is suggestive of a clustering process. These investigators showed that the ferritin-labeled toxin A was later found intracellularly inside cytoplasmic vesicles (179). They also reported that tunicamycin treatment of

cells, which blocks the synthesis of both high mannose type and complex type oligosaccharide chains of asparagine-linked glycoproteins, reduced the cell-binding efficiency of toxin A to 50%, suggesting the receptor is glycoprotein in nature.

Chang et al. (27) compared the susceptibility of fetal intestinal mucosal cells and adult intestinal mucosal cells to both toxins A and B and found that the fetal tissue was more resistant to both toxins. These investigators concluded that toxin resistance was due to the nature of the intestinal cells and not due to factors in the lumen. The reason why fetal tissue is more resistant is not known; however, it is interesting that these findings agree with what is currently known about the lack of C. difficile disease in infants as discussed previously. These observations are further supported by the findings of Rolfe and Iaconis (175a), who showed that infant hamsters are more resistant to C. difficile disease than adult hamsters.

Overview of known bacterial toxin receptors

The word receptor is now used by many scientists with so many different meanings that it is practically impossible to cover in one definition all the biological processes in which the term has been applied. A wide variety of molecules, including hormones, drugs, neurotransmitters, growth factors, chemotactic agents, antigens, viruses, plasma lipoproteins, glycoproteins, lectins and toxins, elicit their biological

effects by virtue of their binding to cellular receptors. For the purpose of this discussion the word receptor will be used to describe a binding component on the cell surface that is involved in the initial step which ultimately leads to the expression of toxicity of a particular toxin. The most important hallmark of a receptor binding site is its specificity.

A. The plasma membrane. The plasma membrane is known to be a fluid, mobile, lipid bilayer which is functionally and structurally asymmetric (188). The outer surface of the membrane carries the molecular markers of cellular uniqueness in the form of glycoproteins and glycolipids. Because the integral glycoproteins and glycolipids have both buried hydrophobic and exposed hydrophilic groupings, such membrane components are termed amphipathic (87). The presence of carbohydrate sequences on these amphipathic glycolipids and glycoproteins forms a network of outer surface projections that constitute a first line of interaction between the cell and its environment. In addition, carbohydrate sequences provide a tremendous amount of structural diversity. A simple glucose disaccharide can exist in 11 distinct forms, and a glucose trisaccharide yields 176 anomeric configurations (87). Thus a mixed trisaccharide would, obviously, yield many more possible structural variants.

Typically, oligosaccharides of mammalian cell membranes

are composed of between 4 and 20 monosaccharide residues, most commonly of glucose, galactose, mannose, fucose, N-acetyl-glucosamine, N-acetylgalactosamine, and N-acetylneuraminic acid (181). The specific sequence and anomeric configuration of these sugars, then, make cell surface oligosaccharides likely candidates as receptors. In fact, well documented examples exist of the critical involvement of carbohydrate on glycoproteins and glycolipids as receptors for agents of the immune system, viruses, lectins, hormones and toxins (36,48,73,88,92,154,223). With this concept in mind, a brief overview of those bacterial toxins for which a receptor has been identified will be discussed.

B. **Receptor for cholera toxin.** Cholera toxin is probably the best studied of the bacterial toxins in that its structure, mechanism of action and toxin receptor have all been elucidated. The purified toxin consists of three subunits: A_1 (MW 23,000), A_2 (MW 6000), and B (MW 11,500) (79,143). Subunit A_1 has been shown to have enzymatic activity and can activate the adenylate cyclase system by ADP-ribosylating the GTP regulatory protein (22,67). Electron microscopic studies have shown that there are five B subunits per toxin molecule arranged in a ring around a central core which contains the enzyme A_1. A_2 connects A_1 to the ring through a single disulfide bond, which must be severed for expression of maximum enzymatic activity (66,143).

In 1971 van Heyningen and coworkers (211) suggested that the receptor is a ganglioside since the effect of cholera toxin on isolated fat cells and on intestinal loops of rabbits was blocked by incubation of the toxin with a crude preparation of gangliosides. These results were later confirmed by Cuatrecasas (40) who showed that extraction of glycolipids from cell membranes resulted in a loss of toxin binding that could be recovered in the ganglioside fraction of the extracts. By using purified gangliosides, it has since been demonstrated in a variety of systems that the monosialo-ganglioside G_{M1} (Table 3) is the component that most strongly interacts with cholera toxin.

G_{M1} inhibits the binding of ^{125}I-labeled cholera toxin to liver membranes and fat cells (39), inhibits the toxin-induced effects on fat cells (40), and on mouse thymocytes (224), and also inhibits accumulation of fluid into rabbit ileal loops (77,78,158). The role of G_{M1} as receptor for cholera toxin is even further strengthened by the observation that toxin-resistant cell lines lack G_{M1} in the cell membrane and can be made sensitive to the toxin by the incorporation of G_{M1} into the cell membrane (57,149). This reconstitution experiment with G_{M1}-deficient cells demonstrates that G_{M1} can serve as a functional receptor for cholera toxin. Nevertheless, the possibility remains that other molecules exist that can bind cholera toxin and could also act as receptors. Critchley et al. (36) have shown that

Table 3. Structures of gangliosides related to receptors for bacterial toxins[1].

Ganglioside	Structure	Reference
G_{M1}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 | 2αNeuAc	112,113
G_{D1a}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 3 | | 2αNeuAc 2αNeuAc	108,110, 112,114
G_{D1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 | 2αNeuAc8-2 NeuAc	94,109,113,
G_{T1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer 3 3 | | 2αNeuAc 2αNeuAc8-2αNeuAc	109,113

[1]Nomenclature of gangliosides from Svennerholm (196). Abbreviations: Cer, ceramide; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; NeuAc, N-acetyl-neuraminic acid

cholera toxin binds mostly to G_{M1} but also binds in minor amounts to glycoproteins from mouse fibroblasts. These investigators have suggested that these glycoproteins ("galactoproteins") may possess a carbohydrate sequence similar to what is contained in G_{M1}. Morita et al., (148) using rat small intestine epithelial cells identified cholera toxin-binding glycoproteins with molecular weights of 69,000, 90,000, 100,000, 114,000, and 132,000. Subsequent work by Critchely et al. (37,38) in which they analyzed cholera toxin-binding components of rat intestinal brush border membranes and mouse fibroblasts confirmed their original observations that most of the cholera toxin bound to G_{M1} but also in small amounts to galactoproteins.

There appears at this time little doubt that the effects of cholera toxin are mediated via the cell surface receptor and that G_{M1} ganglioside is the major membrane component which binds cholera toxin, although some binding may also occur to glycoproteins.

The interaction of toxin with G_{M1} occurs between the toxin B subunit and the oligosaccharide component of the ganglioside and the binding is multivalent (78,84,168). It is thought that initially a single B moiety binds to a single GM_1 molecule. Then lateral diffusion in the plane of the membrane occurs until all five B subunits are associated with five corresponding GM_1 gangliosides. Craig and Cuatrecasas (34) have shown using fluorescein-labeled toxin conjugates,

that the cholera toxin-receptor complex is mobile in the plane of the membrane, redistributing in a temperature-dependent manner to form "patches and caps". Cuatrecasas and coworkers (15,34,177) have proposed that multivalent binding of the toxin to cells with few receptors requires lateral movement of the receptors. Multivalent binding of the toxin to cells with many receptors is rapid and can occur at low temperatures where lateral diffusion is minimal. Thus, multivalent binding appears to be essential for toxin action but lateral distribution may only be required to achieve multivalent binding of cholera toxin to cells with few receptors.

C. **Receptor for Escherichia coli LT toxin.** There is now substantial evidence that most cases of Traveler's diarrhea are caused by infection with certain toxigenic strains of E. coli. This enterotoxin is structually, functionally, and immunologically similar to cholera toxin (122). E. coli enterotoxin (LT) activates adenylate cyclase by the same mechanism as cholera toxin (51). The LT A subunit which has sequence homologies with the A subunit of cholera toxin (191) activates adenylate cyclase and catalyzes the ADP-ribosylation of the regulatory component in the membranes (68). The B subunit structures also show marked similarities (28,29) and consequently share antigenic determinants, but possess unique ones as well.

LT can be inactivated by G_{M1} ganglioside (44,77,158,224)

and its binding to G_{M1}-deficient cells is enhanced by treating the cells with G_{M1} (151); other gangliosides (shown in Table 3) added to these cells did not facilitate binding. Other investigators have shown that addition of G_{M1} to a mouse fibroblast cell line unresponsive to both cholera and E. coli LT, and which can not synthesize G_{M1}, resulted in a significant increase in cyclic AMP content of the these cells follwoing exposure to LT (150). Moss and coworkers (151) have shown that each LT molecule binds several oligosaccharide moities and thus appears to bind in a multivalent fashion similar to cholera toxin.

It is unclear as to whether G_{M1} is the only receptor for LT in the intestine. E. coli LT strongly binds to galactose-containing supports (agarose) (30,115) and, in fact, the toxin has been affinity purified in this manner using galactose to elute the toxin from an agarose column (30). These investigators also reported that under similar chromatographic conditions cholera toxin does not bind to agarose columns, although it is slightly retarded. Recently Holmgren et al. (81) reported that cholera toxin bound selectively to G_{M1} ganglioside receptor sites from rabbit small intestine but that E. coli LT bound both to G_{M1} ganglioside and to a main glycoprotein receptor for which cholera toxin lacked affinity. Furthermore, Holmgren et al. (81) showed that blocking of G_{M1} by cholera toxin B subunits did not give any detectable inhibition of the LT effect

mainly because the glycoprotein receptors predominated more than 10-fold over ganglioside binding sites. Recently, Holmgren et al. (82) confirmed these results using human intestine. LT binds to both ganglioside and glycoprotein receptor sites in the human small intestinal epithelium, whereas cholera toxin binding was restricted to the gangliosides receptors. It is interesting to note that the "galactoproteins" reported by Critchley et al (36,37,38 and Morita (148) (as discussed in the section on cholera toxin) which were described as binding to cholera toxin may conceivably be the main LT receptor.

It is clear from the data on both cholera and LT enterotoxins that the definition of receptor is an ambiguous one. The recurring theme seems to be that the carbohydrate component itself is intimately involved in toxin binding and that the rest of the macromolecule, protein or lipid, serves merely as an anchor in the cell membrane.

D. Shiga toxin receptor. Shiga toxin is a protein produced by Shigella dysenteriae and other organisms of the genus Shigella. This potent toxin has been shown to be responsible for the severe diarrheic and dysenteric syndrome observed with patients with shigellosis (102). The toxin consists of two functionally different moieties, an enzymatically active A subunit (MW 30,800) and a B subunit (MW 5000). The A chain, like cholera and E. coli enterotoxins, can be split

by trypsin into two disulfide-linked fragments, A_1 and A_2, whereas the B moiety consists of six to seven identical B chains (156). An interesting feature of the toxin is that it possesses multiple biological effects. The toxin is cytotoxic against HeLa cells, elicits a positive enterotoxic response in rabbit ileal loops, and is a strong neurotoxin, causing death in monkeys, rabbits and mice (100,155). These results were later confirmed by Eiklid and Olsnes (50) who showed that these toxic activities were all caused by one protein. The primary biological activity, however, appears to be inhibition of protein synthesis (207) brought about by inactivation of the 60S ribosomal subunit of the eucaryotic ribosome (167).

The biochemical nature of the receptor for shiga toxin has been investigated by Keusch and co-workers (99,100,103, 104). These investigators have proposed that Shiga toxin receptors on HeLa cells and rat liver membranes are glycoproteins which contain short chain B-1,4-linked N-acetyl-D-glucosamine oligomers (GlcNAc) (103,104). Lethal effects of the toxin to both cell types were inhibited by pretreating cells with trypsin, lysozyme, and wheat germ agglutinin. The binding of the toxin to HeLa cells was also inhibited by the chitin-derived short chain oligosaccharides containing B-1,4-linked GlcNAc, chitotriose and chitotetrose being the best inhibitors. They have also reported (98,101) that some of these receptors might be masked since treatment

of toxin-sensitive HeLa cells with B-galactosidase resulted in enhancement of sensitivity to toxin.

Recently, Keusch et al. (101) have reported that tunicamycin treatment, which blocks asparagine-linked oligosaccharide synthesis, rendered cells completely resistant to toxin within 24 hours. Although the structure of the carbohydrate is still not known, evidence does suggest that a glycoprotein receptor is involved.

E. Receptors for tetanus and botulinum toxins. Tetanus and botulinum toxins are the two most potent poisons known, being about a million times as toxic as strychnine (209). They are neurotoxins produced by Clostridium tetani and Clostridium botulinum respectively and have been suggested to have identical (or very similar) modes of action at the molecular level (17,184). Their action results in an impairment of neurotransmission due to prevention of the exocytosis of transmitter-containing vesicles. It is believed that both neurotoxins bind to nerve cells which contain substantial amounts of gangliosides and are internalized, but there is no known enzymatic activity associated with either one of the toxins (145,185). Tetanus and botulinum toxins exhibit high affinity for gangliosides of the G_{1b} and G_{T1} series respectively (80,186,187,210) and are shown in Table 3. Tetanus toxin has highest affinity for the disialosyl (G_{D1b}) and trisialosyl (G_{T1b}) gangliosides (210) while the trisialosyl

ganglioside binds botulinum toxin best followed by the disialosyl gangliosides (G_{D1A} and G_{D1b}) (187). Like cholera and E. coli enterotoxins, it has been suggested that both tetanus and botulinum toxin receptors may be either a ganglioside or a glycoprotein with ganglioside-like oligosaccharide structures (49), this again reinforces the idea that the carbohydrate moiety of a particular glycoconjugate functions as the receptor rather than the whole macromolecule.

F. Receptor for Clostridium perfringens enterotoxin.

C. perfringens produces a sporulation-specific enterotoxin that is responsible for one of the most common forms of food poisoning in the United States (180). The enterotoxin has been purified and characterized as a single protein with an estimated molecular weight of 35,000. The toxin appears to have multiple biological effects which distinquishes it from cholera and E. coli LT toxins. McDonel and coworkers (135,139,140) have found C. perfringens enterotoxin to be cytotoxic causing inhibition of glucose uptake, energy production and macromolecular synthesis within the intestinal epithelium. Although the mechanism of action is unknown, the toxin is thought to act directly on the plasma membrane producing membrane blebs in intestinal epithelial and Vero cells.

Recently Wnek and McClane (221) partially purified a protein (MW 50,000) from rabbit brush border membranes by

affinity chromatography on a C. perfringens enterotoxin-affinity column. The protein was found to inhibit biological activity of the enterotoxin on Vero cells. It is not known if this protein contains carbohydrate and further work will have to be done before it is known if it is the receptor for C. perfringens enterotoxin.

G. **Receptors for other bacterial toxins.** Very little information is available concerning the cell surface binding sites for other bacterial toxins. Diphtheria toxin and Pseudomonas aeruginosa exotoxin A have been studied in great detail. The intracellular action of both toxins are indistinguishable, inhibiting protein synthesis by the ADP-ribosylation of elongation factor 2 (33). The experimental evidence for the involvement of carbohydrates in the binding of both toxins to receptors in not conclusive. The best studied candidate for a diphtheria toxin receptor comes from the studies by Proia et al. (162,164). These investigators have identified and characterized specific diphtheria toxin-binding cell surface glycoproteins from guinea pig lymph node cells and from hamster lymph node and thymus cells. The glycoprotein was isolated by affinity chromatography on a diphtheria toxin column and is thought to be an integral membrane protein with an estimated molecular weight of 153,000. Robles et al. (174) have also found that some glycoproteins present in fetal calf serum may also adsorb to

cells and bind the toxin. Recent studies by Hranitzky et al. (86) have confirmed that the diphtheria toxin receptor is a glycoprotein but have hypothesized that the toxin does not bind a carbohydrate moiety but instead binds to the peptide backbone of the glycoprotein receptor. These investigators have based their conclusions on the fact that CHO-K1 mutants, defective in the synthesis of either high-mannose-type or complex type oligosaccharides, showed no difference in toxin sensitivity compared with that of their parental cell lines. Other workers have reported that treatment of cells with neuraminidase sensitized the cells approximately three-fold to diphtheria toxin (142), and that once sialic acid is removed the unmasked receptor bound more toxin. In light of all the data, further studies are needed to identify the binding site for diphtheria toxin.

Treatment of cells with neuraminidase has also been reported to increase the sensitivity to Pseudomonas exotoxin (142) and Fitzgerald et al. (58) found that the binding was inhibited by conconavalin A. Thus there is some indirect evidence that the receptors for pseudomonas exotoxin A may have a carbohydrate component.

Essentially nothing is known about the nature of the receptor for pertussis toxin, a protein produced by Bordetella pertussis which is believed to be of major importance in the pathogenesis of whooping cough (216). The toxin has a molecular weight of 117,000 (197) and has been demonstrated to exert its

effects on the adenylate cyclase system (96). Preliminary data from two laboratories infer that the receptor for pertussis toxin may involve sialic acid-containing glycoconjugates, possibly a ganglioside (91,197).

H. Conclusions. All of the bacterial toxin-receptors for which structures have been elucidated have one feature in common--all are carbohydrate-containing macromolecules and most are gangliosides. There are many toxins produced by many different species of bacteria which go far beyond the scope of this dissertation. In general, bacterial toxins are generally classified as either membrane damaging (hemolysins, phopholipases, lysins) or intracellular acting, and it is thought that the former act nonspecifically on the cell membrane. The intracellular acting toxins (Table 4) share a common mechanism of action involving (i) binding to specific cell surface receptors; (ii) internalization by endocytosis across the cell membrane; and (iii) interaction with an intracellular target. Obvious examples include diphtheria and *Pseudomonas* A toxins, which inhibit protein synthesis (32,90) by apparently the same mechanism; cholera and *E. coli* LT enterotoxins which stimulate adenylate cyclase (22,68) and shiga toxin which acts upon the 60S ribosomal subunit (167).

This process of toxin binding and internalization is very similar to what has been observed for many hormones.

Table 4. Summary of features of bacterial toxins and receptors[1]

Toxin	Mol. wt.	Intracellular Target	Receptor
Cholera	84,000	Adenylate cyclase	G_{M1}
E. coli LT	91,000	Adenylate cyclase	G_{M1}, Galacto-protein
Shiga	68,000	60S ribosome	Glycoprotein (β-1,4-GlcNAc)
Tetanus	160,000	?[2]	G_{D1b}, G_{T1b}
Botulinum	150,000	?	G_{T1b}
Diphtheria	62,000	EF-2	Glycoprotein (150,000 MW)
Pseudomonas	66,000	EF-2	?
Pertussis	117,000	Adenylate cyclase	SA-containing molecule?
C. perfringens	50,000	?	Protein?
C. difficle			
Toxin A	440-666,000	?	Galα1-3Galβ1-4GlcNAc Glycoconjugate
Toxin B	107-550,000	Microfilaments	?

[1]Details and references are given in the text.
[2]?, No available information.

Since it seems unlikely that mammalian cells possess "suicide receptors" for bacterial toxins, a reasonable explanation may be that bacterial toxins parasitize pathways for molecules normally taken up by the cell.

Purpose of dissertation research

The normal gut flora is thought to be critical in forming a protective barrier against the establishment and subsequent infection of pathogens. When patients receive antibiotics the normal ecology of the colon is disrupted and can permit the overgrowth of Clostridium difficle. Extensive research has implicated this anaerobe and its toxins as the cause of both pseudomembranous colitis in humans and antibiotic-associated enterocolitis in hamsters. More than 90% of the patients with psudomembranous colitis are associated with toxigenic C. difficile (5,15,40).

The Golden Syrian hamster has provided a tremendous increase in our understanding of C. difficile mediated gastrointestinal disease over the last few years. The hamster model has been shown to reproduce important aspects of the human disease, including induction by antimicrobial agents, occurrence of C. difficile and its toxins as the major etiology, and successful treatment with vancomycin. Indeed, previous work from this laboratory has shown that toxin A can act alone in causing enteric disease in hamsters, whereas toxin B requires either damaged mucosa or the prior

action of toxin A before a toxic response is observed. These results have recently been confirmed by other investigators (146).

The recent success in purification of both toxin A and B has allowed many investigators to study different aspects of the toxins and their role in the pathogenesis of C. difficile disease. Much knowledge has been gained about the biochemical and immunological properties of the toxins; however, very little information is available concerning the cell surface binding sites. The identity of a receptor for either toxin A or B was unknown when this research was started. It was therefore the purpose of my dissertation research to determine whether a cell surface binding site existed for C. difficile toxin A, an enterotoxin which is believed to be an important determinant of pathogenicity in the disease.

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SECTION II

Cell Surface Binding Site for *Clostridium difficile* Enterotoxin: Evidence for a Cell Binding Site Containing the Sequence Galα1-3Galβ1-4GlcNAc

SUMMARY

This study was undertaken to determine whether a binding site for *Clostridium difficile* enterotoxin (toxin A) exists in the brush border membranes (BBMs) of hamsters, an animal known to be extremely sensitive to the action of the toxin. Toxin A was the only antigen adsorbed by the BBMs from the culture filtrate of *C. difficile*. The finding that binding activity could not be destroyed by heat indicated that a carbohydrate moiety might be involved. We therefore examined erythrocytes from various animal species for binding activity since erythrocytes provide a variety of carbohydrate sequences on their cell surfaces. Only rabbit erythrocytes could bind the toxin, and the cells agglutinated. A binding assay based on an enzyme-linked immunosorbent assay method for quantifying *C. difficile* toxin A was used to compare binding of the toxin to hamster BBMs, rabbit erythrocytes, and to BBMs from rats, which are less susceptible to the action of *C. difficile* toxin A than hamsters. Results of this comparison indicated the following order of toxin binding frequency: rabbit erythrocytes > hamster BBMs > rat BBMs. Binding of toxin A to BBMs from hamsters at 37°C was comparable to what has been observed with cholera toxin, but binding was enhanced at

4°C. A similar binding phenomenon was observed with rabbit erythrocytes. Examination of the cell surfaces of BBMs from hamsters and rabbit erythrocytes with lectins and specific glycosidases revealed a high concentration of terminal α-linked galactose. Treatment of both membrane types with α-galactosidase destroyed the binding activity. The glycoprotein, calf thyroglobulin, also bound the toxin and inhibited toxin binding to cells. Toxin A did not bind to human erythrocytes from blood group A, B, or O donors. However, after fucosidase treatment of human erythrocytes, only blood group B erythrocytes which possess the blood group B structure Galα1-3[Fucα1-2]Galβ1-4GlcNAc-R could bind the toxin. This indicated that toxin A was likely binding to Galα1-3Galβ1-4GlcNAc, a carbohydrate sequence also found on calf thyroglobulin and rabbit erythrocytes. All of the results indicate that BBMs from hamsters contain a carbohydrate binding site for toxin A that has at least a Galα1-3Galβ1-4GlcNAc nonreducing terminal sequence.

INTRODUCTION

Toxigenic strains of Clostridium difficile are recognized as the major cause of antibiotic-associated pseudomembranous colitis in humans (5,6,17,18,25,35). The toxins, designated toxin A and toxin B, are large, heat-labile, cytotoxic proteins that are lethal in animals (4,28,38). Toxin A elicits a positive fluid response when injected into rabbit ileal loops and presumably causes the severe diarrhea seen in humans with C. difficile disease. Toxin B does not cause fluid accumulation in intestinal loops but does display a strong cytopathic activity such that the morphology of monolayer cultured fibroblasts has been termed actinomorphic (8,39,40). The mechanism of action of the toxins is unknown.

There is a growing body of evidence that carbohydrate sequences on the surface of cell membranes serve as receptors for infective agents and their toxins (14,16,22,23,31). However, to date, a receptor for either toxin A or toxin B has not been identified. This report describes studies designed to explore the interaction of toxin A with brush border membranes (BBMs) from hamsters, an animal highly sensitive to the toxin. We found that toxin A specifically binds to BBMs at physiological temperatures, but that the amount of toxin bound actually increased at lower temperatures. A similar binding phenomenon was also found with rabbit erythrocytes. We present evidence that C. difficile toxin A binds to cell surface glycoconjugates containing the nonreducing

terminal sequence Galα1-3Galβ1-4GlcNAc and that the binding is temperature dependent.

MATERIALS AND METHODS

Reagents. (i) Immunochemicals. Rabbit anti-goat immunoglobulin G-alkaline phosphatase was purchased from Sigma Chemical Co., St. Louis, Mo. Rabbit and goat antisera against crude C. difficile 10460 culture filtrate were produced as previously described (11). Affinity purified goat antibody against toxin A was also prepared as previously described (29). Neutral rabbit antiserum was purchased from Pel-Freez Biologicals, Rogers, Ark. Fetal calf serum was obtained from Flow Laboratories, Inc., McLean, VA. Human serum was obtained from volunteers in our laboratory.

(ii) Enzymes and substrates. Coffee bean α-galactosidase and E. coli α-galactosidase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Jack bean β-galactosidase, α-L-fucosidase (bovine kidney), Clostridium perfringens neuraminidase, trypsin, trypsin soy bean inhibitor, Streptomyces griseus pronase E, p-nitrophenyl-α-D-galacto-pyranoside, p-nitrophenyl-β-D-galactopyranoside, and p-nitro-pheny-α-L-fucoside were purchased from Sigma.

(iii) Carbohydrates and glycoconjugates. The following chemicals were purchased from Fisher Chemical Co., Pittsburgh, Pa.: D-glucose, D-galactose, D-fructose, L-fucose, D-xylose, sucrose, D-glucuronic acid, lactose, maltose, mellibiose,

cellobiose, D-glucosamine, D-galactosamine, and raffinose. The following compounds were purchased from Sigma: N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetylneuraminic acid, N-acetylneuramine-lactose, 1-0-methyl-α-D-galactose, 1-0-methyl-β-D-galactose, stachyose, calf thyroglobulin, asialofetuin, fetuin, orosomucoid, human glycophorin, ovomucoid, ovalbumin, α-lactalbumin, β-lactoglobulin, bovine brain gangliosides (Type III), cerebrosides type I and II (bovine brain), L-galactose, psychosine (DL-sphingosyl-β-D-galactoside), p-aminophenyl-β-D-galactopyranoside, D-galactopyranosyl-β-D-thiogalactopyranoside, and fibronectin. Human thyroglobulin was purchased from DAKO Corp., Santa Barbara, Calif. Galactose α 1-3 galactose was purchase from BioCarb Chemicals AB, Lund, Sweden.

Protein Determination. Protein concentration were estimated by the dye-binding method of Bradford (7) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA), using bovine gamma globulin as the standard.

Preparation of Toxin A. A highly toxigenic strain of C. difficile, VPI strain 10463, was obtained from the collection of the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University (Blacksburg, VA). The organism was grown at 37C for 48h in brain heart infusion (BHI) dialysis flasks as previously described (11). The culture filtrate and homogeneous toxin A were prepared as previously described (37).

Erythrocytes. Rabbit blood was obtained from four sources. Heparinized rabbit blood was purchased from Pel-Freeze Biologicals (Rogers, AR). Defibrinated, sterile rabbit blood was purchased from Brown Laboratories (Topeka, KS) and Hazelton Dutchland, Inc. (Denver, PA). Fresh rabbit blood (drawn in EDTA) was obtained from animals maintained in the laboratory. Human blood (drawn in EDTA) was obtained from volunteers in our laboratory. Blood from other animal species was obtained from Pel-Freeze Biologicals (Rogers, AR) and the Department of Veterinary Medicine, Virginia Polytechnic Institute and State University (Blacksburg, VA). Erythrocytes were sedimented by centrifugation at 1500 x g for 5 min and washed 4 times in isotonic TBS (0.1 M Tris(hydroxymethyl)-aminomethane buffer containing 50 mM NaCl, pH 7.2) at 4 C prior to use.

Preparation of Membranes. (i) Isolation of brush border membranes (BBMs). BBMs were isolated according to the method of Forstner et al. (15) from male golden Syrian hamsters (Engle Laboratory Animals, Inc., Farmersburg, Ind.) weighing about 120 g and from Sprague Dawley outbred rats (Dominion Laboratories, Dublin, Va.) weighing about 450 g. Purity of the BBMs was monitored by phase contrast microscopy.

(ii) Preparation of ghost membranes. Hemoglobin-free rabbit red cell membranes (ghosts) were prepared using a hemolysis procedure similar to the method of Dodge et al. (10) with

5 mM sodium phosphate buffer, pH 8.0 at 4 C. Sedimented membranes were creamy white and were resuspended to the original blood volume in TBS at 4 C prior to use.

Detergent solubilization. Brush border and ghost membranes were suspended in an equal volume of detergent solution to give final concentrations of 1% Triton X-100 (Sigma), in 20 mM sodium phosphate, pH 8.0. Brush borders and ghosts were incubated 60 min. at 37C and 4C respectively and centrifuged at 20,000 x g for 60 min. The clear supernatant, representing the solubilized extract, was aspirated from the gelatinous pellets, filter-sterilized (0.22 µm) and stored at 4 C prior to use.

Toxin Binding Assay. Binding assays were performed in 1.5 ml polypropylene microcentrifuge tubes (American Scientific Products, McGaw Park, Ill.) which had been soaked with 20 mg bovine serum albumin (BSA) per ml before use. Rabbit erythrocytes were washed 4 times in TBS and resuspended in TBS-0.2% BSA (pH 7.2) just before use. Binding of toxin A to membranes was assayed by an enzyme-linked immunosorbent assay (ELISA) similar to the methods used by Holmgren et al. (22,23). Reaction mixtures contained BBMs, erythrocytes or ghosts at various concentrations in TBS-0.2% BSA plus 25 ng of toxin A per ml, in TBS-0.2% BSA in a total volume of 0.3 ml. After incubation for 15 min at 4, 22, or 37C, the cells were sedimented by centrifugation at 2000 rpm for 1 min, and the

supernatant fluid (0.2 ml) was tested for its concentration of unbound toxin with the toxin A-ELISA procedure (see below). All experiments were performed in triplicate.

Toxin A ELISA. Concentrations of toxin A were determined by the toxin A ELISA method, a procedure described previously (30). In short, the wells of polystyrene plates (Immulon type 2 ELISA plates, Dynatech Laboratories, Inc., Alexandria, Va.) were coated with rabbit antiserum against C. difficile 10463 culture filtrate at 37 C for 18 hours. Unoccupied sites were blocked with BSA (10 mg/ml) in TBS, pH 7.2 for 30 min at room temperature. The wells were washed once and then the toxin-containing supernatant fluid was added. Toxin bound to the wells was then assayed immunologically by sequential incubations with affinity purified goat antibody, rabbit antigoat immunoglobulin conjugated to alkaline phosphatase, and alkaline phosphatase substrate. Absorbance readings at 405 nm for unknown reactions were compared with a standard curve for known concentrations of toxin A tested concurrently.

Enzyme Treatments. (i) α and β-galactosidase treatment. Washed rabbit erythrocytes (2.5 x 10^8 cells/ml) and hamster BBMs (2 mg membrane protein/ml) in 0.05 M sodium citrate-0.1 M NaCl containing 0.04% sodium azide (Citrate-buffered saline, CBS) were incubated at room temperature or at 37 C with either 1.5 units of coffee bean α-galactosidase (pH 6.0)

or 2.5 units of either Jack bean β-galactosidase (pH 4.0) or E. coli β-galactosidase (pH 4.0). At various times 0.15 ml aliquots were removed and washed 2 times with excess ice cold TBS at 4 C before use in the binding assay. When whole erythrocytes were used no apparent hemolysis was observed. Triton X-100 solubilized extracts were titrated with 1N HCl to pH 6.0 or 4.0 before incubation (37C, 1.5h) with 1 unit of either enzyme. The efficacy of each enzyme was examined under the conditions described above by the ability to hydrolyze p-nitrophenylgalactopyranoside substrates. Jack bean β-galactosidase was found to be free of α-galactosidase activity as determined by its failure to hydrolyze p-nitrophenyl-α-D-galactopyranoside. The coffee bean α-galactosidase was free of β-galactosidase as determined by its failure to hydrolyze p-nitrophenyl-β-D-galactopyranoside.

(ii) Fucosidase Treatments. Washed human ghosts (10 mg membrane protein/ml) in citrate-buffered saline (pH 5.5) were incubated at 37 C for 12h with 5U units of α-L-fucosidase. The membranes were removed, washed 3 times in excess ice-cold TBS at 4 C before use. The efficacy of α-L-fucosidase was examined by the ability to hydrolyze p-nitrophenyl-α-L-fucoside.

(iii) Proteolytic digestion. Streptomyces griseus Pronase (2mg) was added to Triton X-100 solubilized extracts in 2 ml and incubated at 37C for 3 hours. The incubation was terminated

by placing the reaction tube in a boiling water bath for 5 min. prior to assaying for soluble binding activity. Mild proteolytic treatment of washed erythrocytes (2.5 x 10^8 cells/ml) was carried out in the presence of 0.25% trypsin in TBS (pH 7.2) for 30 min. at 37 C. After addition of soybean trypsin inhibitor (1% final concentration), erythrocytes was washed 3 times in TBS. No apparent hemolysis was encountered under these conditions.

(iv) Neuraminidase treatment. Washed erythrocytes (2.5 x 10^8 cells/ml) and hamster BBMs (2 mg/ml membrane protein) in citrate buffered saline (pH 5.5) were treated with 1.0 units of Clostridium perfringens neuraminidase. After 90 min at either 22C or 37 C, cells were washed 3 times in TBS before use.

Hemagglutination and Hemagglutination Inhibition Assay. Erythrocytes were washed 4 times in 10 volumes of TBS, pH 7.2, and diluted to a 2.5% suspension. Two-fold serial dilutions of toxin A (50 μl) were performed with TBS in V-bottom microtiter plates (Dynatech) and 50 μl of fresh washed erythrocytes was added to each well. The plates were gently tapped and the red blood cells were allowed to settle at either 4, 22, or 37C. Titers were expressed as the reciprocal of the highest dilution of toxin A in which hemagglutination was visible macroscopically.

When C. difficile antiserum was used the assay was slightly modified. Toxin A was diluted in TBS (in two-fold series) in

small 12 x 75 mm glass test tubes and each tube was mixed with an equal volume of a 5% freshly washed rabbit erythrocyte suspension. After 5 min. at room temperature or 4 C, 50 μl aliquots of erythrocyte suspension were added to V-bottom microtiter plates which contained either TBS or C. difficile 10463 antitoxin (1:2000 dilution for room temperature or 1:500 dilution for 4 C in TBS). The suspensions were mixed by tapping the plates and the erythrocytes were allowed to settle.

To test whether hemagglutination was inhibited by specific carbohydrates and glycoconjugates, the agents were serially diluted in microtiter plates in 25 μl of TBS. An equal volume of toxin A, diluted in TBS to a titer of 32, was added to each well and allowed to interact 30 min at 4C or 22C. A 2.5% suspension of fresh, washed rabbit erythrocytes (50 μl in TBS) was then added to each well, the plates were mixed and the erythrocytes were allowed to settle.

Lectin Treatments. Hamster BBMs and rabbit erythrocyte membranes (1 mg/ml membrane protein) were incubated in TBS at room temperature in a final volume of 1 ml with each of the following lectins with and without its specific hapten inhibitor: Bandeirea simplicifolia agglutinin (BS 1) with or without 10 mM 1-O-methyl-α-D-galactose and Dolichos biflorus agglutinin (DBA) with or without N-acetyl-D-galactosamine. After treatment lectins were removed by centrifugation and

membranes were assayed for toxin binding at 22C by the toxin A ELISA. All lectins were purchased from Sigma.

Crossed Immunoelectrophoresis (IEP). Crossed IEP was performed on 5 x 5 cm glass plates in 1.2% low electroendosmotic agarose (Sigma Chemical Co.) in 0.125 M Tris-Tricine buffer, pH 8.6, as described by Axelsen et al. (3). The agarose use for the second dimension contained 0.1 ml goat antiserum produced against crude culture filtrate of C. difficile VPI strain 10463 (11).

RESULTS

Toxin specificity. We examined the ability of BBMs from hamsters to bind the toxins produced by C. difficile. Figure 1 shows that the enterotoxin (toxin A) was the only antigen adsorbed by the BBMs from the culture filtrate as analyzed by crossed immunoelectrophoresis (IEP). Binding of the toxin appeared to be specific because none of the other antigens present in the culture filtrate (including toxin B) showed a measurable reduction in peak height, whereas toxin A was completely removed. Heating the BBMs (100C, 10 min) did not reduce the binding activity.

Erythrocyte specificity. Because the cell surface of erythrocytes are known to contain a variety of carbohydrate sequences on their membranes, erythrocytes from the following animal species were studied for their ability to bind the toxins: human (type A, B, and O), Rhesus monkey, hamster,

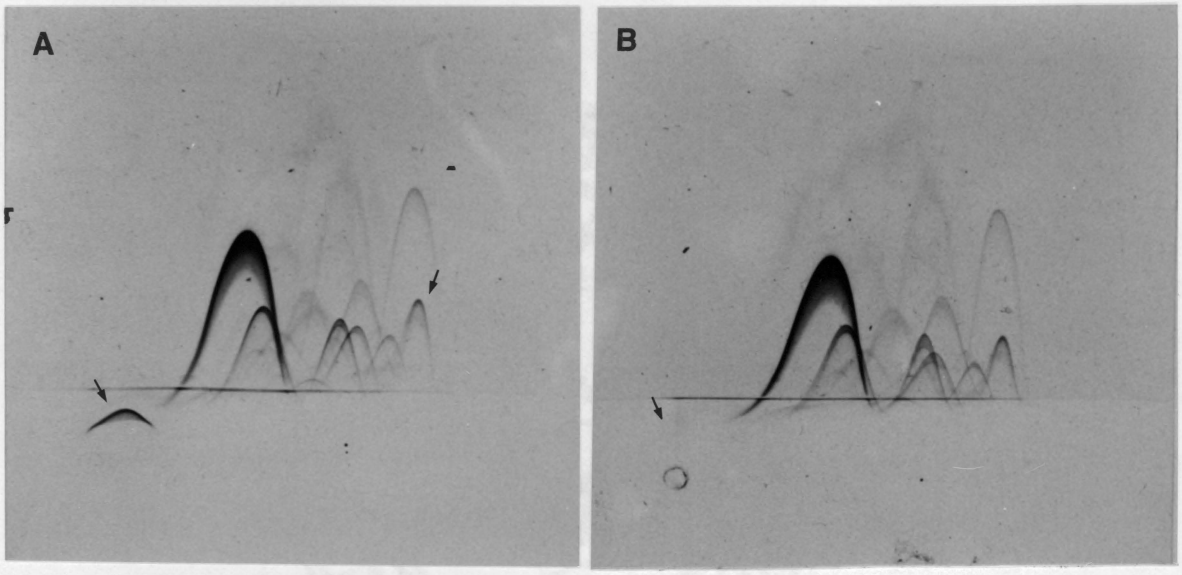

Figure 1. Analysis by crossed immunoelectrophoresis of C. difficile strain 10463 culture filtrate adsorbed with hamster brush border membranes at 4°C. The upper portion of the gel in each plate contained 0.1 ml of goat antiserum against C. difficile VPI strain 10463 culture filtrate (9). (A) The well contained 50 µg of 10463 culture filtrate in 25 µl of TBS. The arrows designate the location of toxins A and B. (B) The well contained 50 µg of strain 10463 culture filtrate adsorbed with 25 µl of packed hamster brush border membranes. Note the absence of toxin A (arrow) in plate (B) demonstrating that hamster brush border membranes specifically bind on toxin A. The toxin B arc in the adsorbed culture filtrate (shown by arrow on right) is still present.

rabbit, guinea pig, rat, mouse, calf, horse, pig, dog, cat, goose, and chicken. Of the 16 types of erythrocytes tested, only those from rabbits bound toxin A. The finding that toxin A could also agglutinate rabbit erythrocytes enabled us to develop a specific hemagglutination assay to detect the toxin (H.C. Krivan, and D.M. Lyerly, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C245, p. 340; 44). Toxin B did not agglutinate rabbit erythrocytes.

The hemagglutination assay was most sensitive for detecting toxin A at 4C and hemagglutination was not observed at 37C. Toxin A agglutinated erythrocytes from rabbits at concentrations as low as 0.8 μg/ml at 4C. When antibody to toxin A was incorporated into the assay, the sensitivity of the agglutination reaction was increased and the erythrocytes agglutinated with as little as 50 ng/ml of toxin A. Agglutination of the erythrocytes by toxin A at 4C disappeared when the cells were warmed to 37C. The possibility that toxin A was enzymatically destroying its binding site at 37C was ruled out because erythrocytes incubated with toxin at 37C for 60 min, then cooled to 4C, agglutinated. Treatment of erythrocytes from the 15 other types with trypsin and neuraminidase did not unmask a potential binding site for either toxin A or toxin B. Similar enzyme treatment of rabbit erythrocytes did not affect toxin binding.

Binding of toxin A to isolated brush border and erythrocyte ghost membranes.

(i) Effect of membrane concentration. The binding of toxin A to hamster and rat BBMs and to ghosts from rabbit erythrocytes was compared (Fig. 2). The order of toxin binding was as follows: rabbit erythrocyte membranes > hamster BBMs > rat BBMs. The binding of toxin A exhibited a linear dose response with all three membrane types at 4C until the toxin became limiting in the reaction medium. In all experiments the amount of unbound toxin was calculated from the linear portion of the ELISA standard curve (Fig. 2, inset). The contribution of nonspecific binding appeared to be very small because we could not detect any binding with high concentrations (25% suspension) of human type A, B, and O erythrocyte membranes.

(ii) Effects of time and temperature. The binding of toxin A to erythrocytes from rabbits and to BBMs from hamsters incubated at 4, 22, and 37C is shown in Fig. 3. Binding was rapid and the amount of toxin bound increased at lower temperatures. We did not detect toxin binding at 37°C to rabbit erythrocytes, whereas binding to BBMs did occur at this temperature (Fig. 3). To test for temperature-dependent dissociation of the toxin, toxin was allowed to bind at 4C, and residual toxin was removed by three cycles of centrifugation and washing at 4C. Bound toxin dissociated from both hamster BBMs and rabbit erythrocytes when warmed to 37C although not

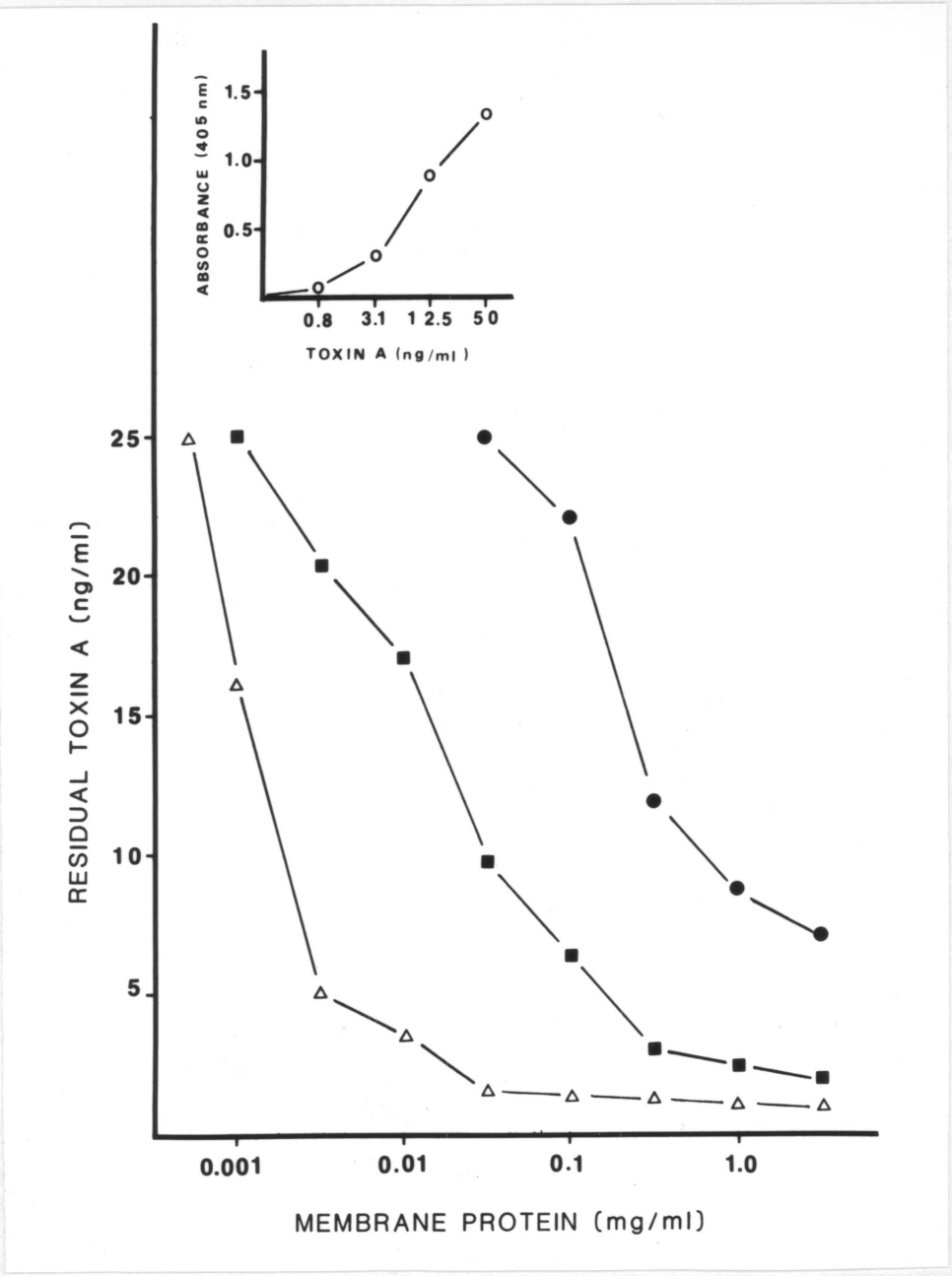

Figure 2. Effect of membrane concentration on the binding of C. difficile toxin A. The indicated concentration of membranes (membrane protein, mg/ml) was incubated 30 min at 4°C in 0.3 ml of TBS containing 0.2% BSA and 25 ng/ml toxin A. Concentrations of toxin A were determined by ELISA as described in the text. △, rabbit ghosts ■, hamster brush border membranes; ●, rat brush border membranes. Inset, ELISA standard curve for known concentrations of toxin A.

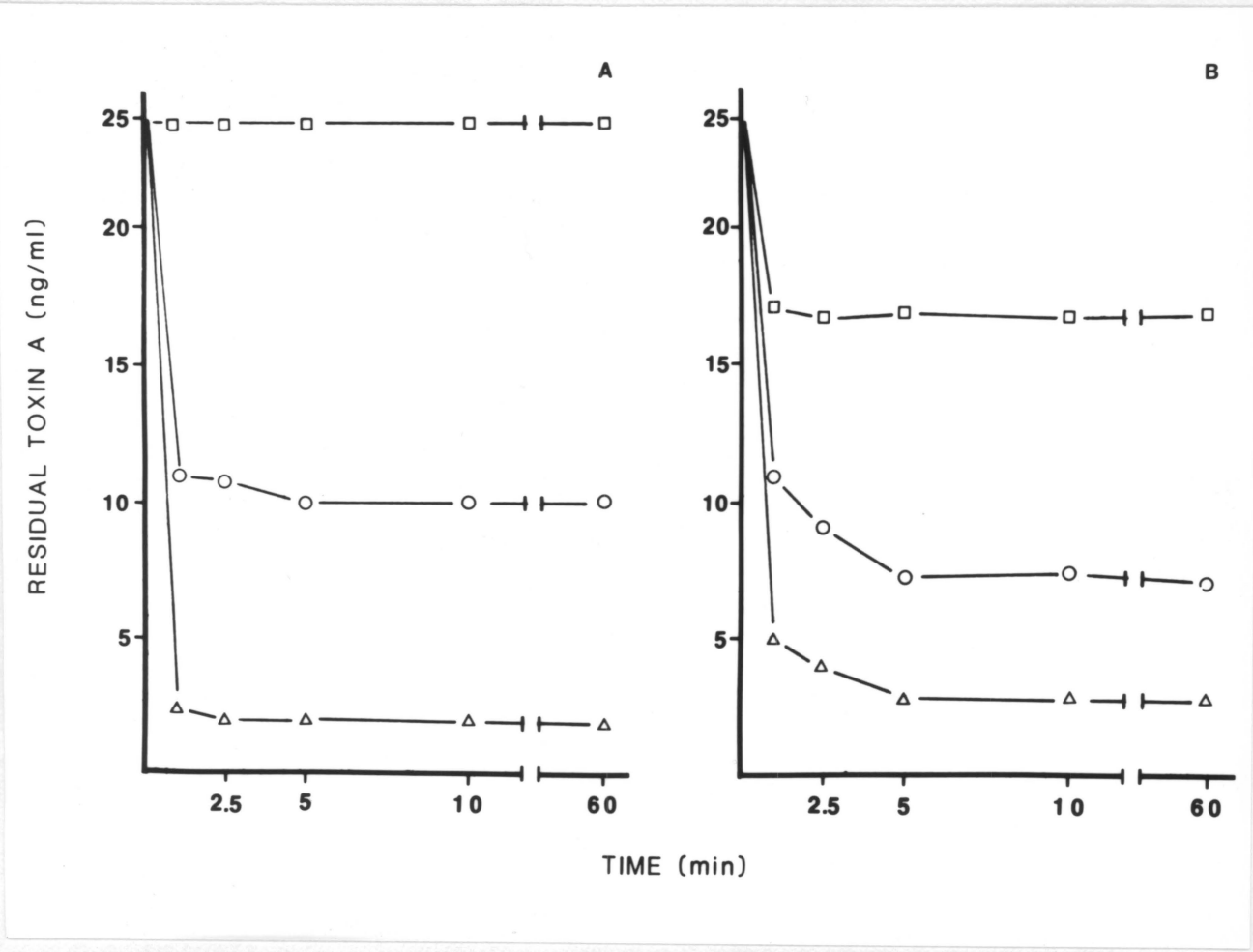

Figure 3. Time course of binding of C. difficile toxin A to (A) rabbit erythrocytes and (B) hamster brush border membranes at 4°C (△), 22°C (○), and (□) 37°C. Reaction mixtures contained 1 x 10^8 rabbit erythrocytes or brush border membranes (1 mg/ml membrane protein) and 25 ng/ml toxin A in 0.3 ml of TBS-0.2% BSA. Concentrations of residual toxin were determined by ELISA as described in the text.

all of the toxin was recovered from the BBMs. These results are consistent with data obtained with the hemagglutination assay. Toxin A does not agglutinate rabbit erythrocytes at 37C, and the sensitivity of the hemagglutination assay is greatest at 4C.

Solubilization studies. In an attempt to study the binding component apart from the membrane, we extracted hamster BBMs and rabbit erythrocyte membranes with the nonionic detergent Triton X-100 and assayed the soluble extract and the residual membrane pellet for binding activity. As detected by ELISA, 95% of the binding activity was removed from the membranes by detergent treatment. The loss of binding activity was accompanied by the appearance of toxin-binding material in the extracted supernatant fluid from both membrane types. Analysis by crossed IEP showed that toxin A, when combined with the extracted supernatant fluid from either membrane type, migrated further than when in the presence of Triton X-100 alone (Fig. 4). The toxin appeared to be complexed with some membrane component which we designated the toxin A binding moiety.

Erythrocyte membranes from each animal species previously tested for toxin A binding were treated with Triton X-100. Of the 15 erythrocyte types examined, only Triton extracts from calf erythrocyte membranes affected toxin A similar to extracts from rabbit erythrocytes; however, only rabbit

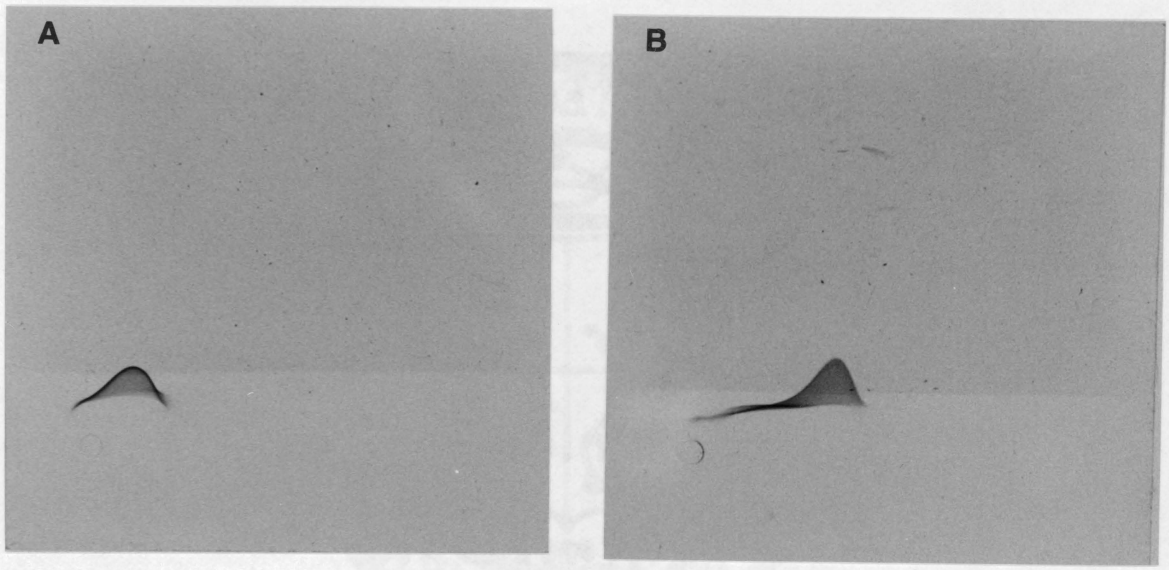

Figure 4. Analysis by crossed immunoelectrophoresis of C. difficile VPI strain 10463 purified toxin A combined with Triton X-100 solubilized extract from rabbit ghost membranes. The upper portion of the gel in each plate contained 0.1 ml of goat antiserum against C. difficile strain 10463 culture filtrate (9). (A) The well contained 15 µg of purified toxin A combined with 20 mM sodium phosphate-1% Triton X-100 buffer. (B) The well contained 15 µg of toxin A combined with 20 mM sodium phosphate-1% Triton X-100-solubilized membrane extract. The altered migration of the toxin immunoprecipitin arc in (B) was also observed when extracts from hamster brush border membranes or thyroglobulin was combined with toxin A and analyzed.

erythrocytes could be agglutinated by the toxin in the hemagglutination assay. This suggests that binding structures exist on calf erythrocytes which could only be detected after Triton X-100 solubilization.

Chemical properties of the toxin A binding moiety.

(i) Enzyme degradation of soluble binding activity. We treated soluble Triton X-100 extracts from hamster BBMs and membranes from calf and rabbit erythrocytes with enzymes in an attempt to abolish binding activity; α-galactosidase (coffee bean) but not β-galactosidase (Jack bean or E. coli) completely destroyed the binding activity of all three membrane extracts. Pretreatment of the solubilized Triton X-100 extract with pronase, neuraminidase or boiling did not abolish binding.

(ii) Enzyme degradation of the membrane-bound binding activity. Pretreatment of rabbit erythrocytes and BBMs from hamsters with trypsin, neuraminidase and β-galactosidase had no effect on the ability of these membranes to bind toxin A (Table 1); however, binding to rabbit erythrocytes was markedly reduced by pretreatment with α-galactosidase. Similar results were obtained with hamster BBMs. Pretreatment of hamster brush borders with α-galactosidase greatly decreased toxin binding at both 4C and 37C.

Hemagglutination inhibition. We tested a large number of saccharides, α- and β-substituted galactosides, neutral serum,

Table 1. Effect of enzymatic treatment of rabbit erythrocytes and hamster brush border membranes on binding of C. difficile toxin A.[a]

Enzyme	Activity	Rabbit RBC HA Titer	Hamster BBMs % Inhibition
None	-	512	0
α-Galactosidase	1.5 U	8	42
β-Galactosidase	2.5 U	512	0
Neuraminidase	1.0 U	512	0

[a]Washed rabbit erythrocytes (5% suspension RBC) and hamster brush (BBMs, 2 mg/ml membrane protein) were incubated in CBS 90 min at 22C and 37C respectively with each enzyme. After incubation RBCs and BBMs were washed 2 times at 4C with ice-cold TBS to remove residual enzyme. Toxin hemagglutination of RBCs and binding assays with BBMs were performed as described in Materials and Methods.

Table 2. Carbohydrate compounds which did not inhibit toxin hemagglutination of rabbit erythrocytes.[a]

Sera (undiluted)	Other Saccharides (10mg/ml)
Neutral rabbit serum	D-Glucose
Fetal calf serum	D-Mannose
Human serum type A	D-Galactose
Human serum type B	L-Galactose
	L-Fucose
Glycoproteins (1 mg/ml)	D-Xylose
	D-Glucuronic acid
Asialofetuin	D-Glucosamine
Fetuin	D-Galactosamine
Orosomucoid	N-Acetyl-D-glucosamine
Ovomucoid	N-Acetyl-D-galactosamine
α-macroglobulin	N-Acetylneuraminic acid
β-lactoglobulin	Lactose
Fibronectin	Sucrose
Human thyroglobulin	Maltose
	Lactose
	N-Acetylneuramin-lactose
Glycolipids (1 mg/ml)	N,N'-diacetylchitobiose
Gangliosides	
Cerebrosides type I	
Cerebrosides type II	
Psychosine (Galβ 1-sphingosine)	

α- and β-Substituted Galactosides (10 mg/ml)

1-O-Methyl-α-D-galactopyranoside
1-O-Methyl-β-D-galactopyranoside
p-Aminophenyl-β-D-galactopyranoside
D-Galactopyranosyl-β-D-thiogalactopyranoside
α-D-Galactose-(1-3)-D-galactose
Melibiose (α-D-Gal-(1-6)-D-Glc)
Stachyose (α-D-Gal-(1-6)-α-D-Gal-(1-6)-α-D-Glc-(1-2)-β-D-Fru)
Raffinose (α-D-Gal-(1-6)-α-D-Glc-(1-2)-β-D-Fru)

[a]Each compound was tested as a potential inhibitor in the toxin hemagglutination assay by serial dilution in 25 µl volumes in microtiter plates. Toxin A, diluted to a titer of 32, was added to each well and plates were incubated at 4 or 22C for 30 min. A 2.5% suspension of fresh, washed rabbit erythrocytes in 50 µl was added to each well and plates were incubated at either 4 or 22C. Gal, Galactose; Glc, Glucose; Fru, Fructose

and various glycoproteins and glycolipids for their ability to inhibit toxin hemagglutination. We did not find inhibitory activity with any of the compounds listed in Table 2; however, one glycoprotein tested, calf thyroglobulin, inhibited hemagglutination at concentrations of 0.5 μg/ml and higher. To determine whether thyroglobulin inhibited hemagglutination by binding the toxin, thyroglobulin was combined with toxin A and analyzed by crossed IEP. The results showed an increased electrophoretic migration of the toxin similar to what is shown in Fig. 4 for solubilized rabbit erythrocytes membranes. Treatment of calf thyroglobulin with α-galactosidase abolished its inhibitory activity in the hemagglutination assay and enzyme-treated thyroglobulin could not bind the toxin as detected by crossed IEP.

Effect of lectins. Bandeirea simplicifolia (BS 1) agglutinin blocked binding of the toxin to both hamster BBMs and rabbit erythrocytes (Table 3). BS 1 agglutinin did not block binding of the toxin when 10 mM 1-O-methyl-α-D-galactose was incubated with BS 1 in the reaction mixture. Dolichos biflorus agglutinin did not block binding of the toxin to either membrane type.

Unmasking the binding site on human type B erythrocytes. We were unable to demonstrate toxin binding to human type B erythrocytes which are known to contain terminal alpha-linked galactose. Because blood-group B substance

Table 3. Effect of lectin blockade on binding of C. difficile toxin A to hamster brush border membranes and rabbit erythrocytes.[a]

Lectin[b]	Specificity	Hamster BBMs	Rabbit Ghost
BS 1	α-D-Gal	31.5% (25)	51.0% (0.5)
DBA	α-D-GalNAc	0% (25)	0% (25)

[a]Hamster brush border membranes (BBMs) and rabbit ghosts were incubated in TBS at room temperature in a final volume of 1 ml with each lectin. After 60 min lectins were removed by centrifugation and membranes were assayed for toxin binding activity as described in Materials and Methods.
[b]The final concentrations (μg/ml) of Bandeirea simplicifolia (BS 1) and Dolichos biflorus agglutinin (DBA) incubated with hamster BBMs and rabbit ghosts are indicated in parentheses.

(Galα1-3[Fucα1-2]Galβ1-4GlcNAc...) contains an α-1-2 fucosyl unit linked to the penultimate galactose, experiments were designed to test if enzymatic removal of fucose would allow the toxin to bind membranes from human type B erythrocytes. Human type B membranes pretreated with fucosidase bound toxin A in a dose-dependent manner (1.0 ng toxin A/ mg membrane protein). Toxin A did not bind to membranes from human type A or O erythrocytes which had been pretreated with fucosidase.

DISCUSSION

This study was undertaken to determine whether receptors for Clostridium difficile enterotoxin (toxin A) exist in the BBMs of hamsters, an animal known to be extremely sensitive to the action of the toxin. Our initial experiments showed that toxin A was adsorbed from the culture filtrate of C. difficile and that binding to BBMs from hamsters was specific. The technique of crossed IEP allowed us to detect the removal of toxin from the culture filtrate by the membranes, but this method was not quantitative enough to adequately study the binding properties of the toxin. We therefore attempted to label purified preparations of toxin A with iodine-125 so that a direct binding assay could be developed; however, iodination of the toxin by several labelling procedures (Bolton-Hunter, lactoperoxidase, Chloramine T, and Iodogen) rendered the toxin biologically

inactive and destroyed the hemagglutinating activity of the toxin (unpublished results). An ELISA procedure had been previously been used to quantitate binding of E. coli LT and cholera toxin to rabbit intestinal cells and BBMs (22) and more recently to human intestine (23). We therefore used an ELISA technique we had previously developed for toxin A (30) to quantitate toxin binding.

Previous work from this laboratory had shown that toxin A can act alone on the hamster intestinal tract whereas toxin B, the potent cytotoxin, requires either damaged mucosa or the prior action of toxin A before it can exert its lethal effect(s) (29). The data presented herein has shown that BBMs from hamsters bound toxin A and that binding at 37°C was comparable to what has been observed with cholera toxin. An unusual characteristic was that binding was enhanced at 4°C. None of the other extracellular antigens produced by C. difficile (including toxin B) interacted with the membranes. The finding that binding activity could not be destroyed by heat indicated that a carbohydrate moiety might be involved. We therefore examined erythrocytes from various animal species (including hamster) for binding activity since erythrocytes provide a variety of cell surface carbohydrate sequences from species to species. Only rabbit erythrocytes bound the toxin and again we found that binding was enhanced at cold temperatures. The hemagglutinating activity of toxin A indicates that the toxin must contain repeating subunits

because agglutination would require at least two binding sites per molecule of toxin.

All of the evidence we have accumulated indicates that the binding site is a cell surface glycoconjugate containing the nonreducing terminal sequence Galα1-3Galβ1-4GlcNAc. Enzyme studies showed that pretreatment of both hamster BBMs and rabbit erythrocytes with α-galactosidase greatly decreased toxin binding activity at all temperatures. Rabbit and calf erythrocytes are known to have very high concentrations of terminal α-linked galactose on their cell surfaces (9,12,20), and their structures have been recently elucidated and are compared in Table 4. We demonstrated that the binding site was present in solubilized membrane extracts from only calf and rabbit erythrocytes and that binding activity was abolished when these extracts were treated with α-galactosidase. The only other similar structure known to occur on erythrocytes is the human blood group B substance which differs only by an α-L-fucosyl unit linked to the penultimate galactose (Table 4). Human type B erythrocyte membranes did not bind toxin A until the fucose was removed by fucosidase. These results indicated that the Galα1-3Galβ1-4GlcNAc sequence is involved in binding the toxin.

Calf thyroglobulin was the only compound we tested that bound the toxin and inhibited hemagglutination. The binding of thyroglobulin to the toxin appeared to be due to carbohydrate units on thyroglobulin because binding of the glycoprotein to

Table 4. Glycoconjugates containing terminal α-galactoside linkages

Structure[a]	Source	Reference
Galα1-3Galβ1-4GlcNAcβ1-3Gal... α1-2 Fuc	Human RBC	42
Galα1-3Galβ1-4GlcNAcβ1 → 3 Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Ceramide Galα1-3Galβ1-4GlcNAcβ1 → 6	Rabbit RBC	20
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Ceramide	Calf & Rabbit RBC	9,12
(Galα1-3Galβ1-4GlcNAc...)$_n$	Calf Thyroglobulin	36

[a]Gal, Galactose; GlcNAc, N-Acetylglucosamine; Glc, Glucose; Fuc, Fucose; Man, Mannose

the toxin was abolished only after treatment with α-galactosidase. Human thyroglobulin did not bind the toxin. The carbohydrate units on calf thyroglobulin have been extensively studied (1,2,36). Recently, Spiro and Bhoyroo (36) demonstrated that α-D-galactosyl residues occur in thyroglobulins from several species. The concentration was highest in calf and there was a complete absence in man. This latter observation is consistent with the inability of human thyroglobulin to bind toxin A. Spiro and Bhoyroo (36) further showed that about 20% of the galactose residues in calf thyroglobulin occur in α-linked terminal positions as constituents of a Galα1-3Galβ1-4GlcNAc sequence. Furthermore, they reported that these residues are located on oligosaccharide branches similar to what is shown in Table 4 for rabbit erythrocyte glycolipid.

The structures shown in Table 4 all have one feature in common: all of the structures contain a Galα1-3Galβ1-4GlcNAc nonreducing terminal sequence. We were able to show that binding of the toxin to hamster BBMs and rabbit erythrocytes could be blocked by BS 1 agglutinin, a highly specific probe for terminal alpha-linked galactose (21). We were not able to inhibit binding of the toxin with several glycoconjugates including D- or L-galactose, methyl-α-D-galactoside or the disaccharide galactose α 1-3 galactose. Stachyose and raffinose, both of which contain galactose α1-6 galactosyl linkages, also did not inhibit binding. These results

suggest that toxin binding is extremely specific and that at least the trisaccharide Galα1-3Galβ1-4GlcNAc may be required for the toxin to bind since no inhibition was observed with Galα1-3Gal.

When we solubilized rabbit and calf erythrocytes or brush border membranes with Triton X-100 most of the binding activity was recovered in the supernatant fluid of the detergent extract. The binding moiety could be easily detected because it altered the migration of toxin A in agarose IEP. The addition of thyroglobulin to toxin A preparations caused the same phenomenon. Pretreatment of the detergent extracts and thyroglobulin with α-galactosidase completely abolished this binding activity. These results suggest that toxin A interacts with agarose; the toxin has a pI of 5.5 (37) yet does not migrate well in the agarose gel in an electric field unless complexed with its receptor. This retention of the toxin in agarose may be due to the presence of α-linked anhydrogalactose which is known to be a chemical constituent of agarose (34). The possibility that toxin A might interact with agarose has also been investigated by others. Lonnroth and Lange (27) reported that toxin A bound to Biorad agarose A columns and could be eluted with galactose (like cholera and E. coli LT enterotoxins). We have not been able to reproduce these results (unpublished data).

We have been able to purify large amounts of toxin A based on affinity chromatography using rabbit erythrocyte membranes

or immobilized thyroglobulin (H.C. Krivan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-60, p. 34; Krivan and Wilkins, manuscript in preparation). Toxin A purified in this manner has the same biochemical characteristics as toxin A purified by conventional methods.

Toxin binding to both hamster BBMs and rabbit erythrocytes increased with decreasing temperature, although there was significant binding to hamster BBMs at 37C. The enhancement and stability of proteins binding at lower temperatures is not unique since it is well known that antibodies which react with the I,i blood group determinants often are cold agglutinins associated with cold agglutinin disease (33). The behavior of toxin A in our studies thus resembles that of a cold agglutinin. The explanation as to why toxin A bound to BBMs from hamsters and not to rabbit erythrocytes at 37^{o}C may be related to receptor density on the cell membrane surface. Tsai et al. (41) have shown that a cold agglutinin isolated from the serum of a patient with Waldenstrom's macroglobulinemia agglutinated human erythrocytes only in the cold by binding N-acetylneuraminosyl-containing carbohydrate chains on the cell surface while dog erythrocytes also agglutinated at 37^{o}C. These investigators showed that their was a quantitative relationship between receptor density and agglutinability of erythrocytes by the cold agglutinin.

We do not know that the binding moiety described in this report is the receptor which allows toxin A to cause

C. difficile disease. We have described a carbohydrate binding site to which toxin A specifically binds and demonstrated its presence in the intestinal BBMs of hamsters and on the cell surface of rabbit erythrocytes. Binding activity is found in lower amounts in the intestinal brush borders from rats and rats are much less susceptible to toxin A than hamsters (29). These observations could imply that fewer receptors exist on the intestinal cell surface of rats. The major complex carbohydrate of rabbit erythrocyte membranes is the decasaccharide-ceramide depicted in Table 4, and this structure has been shown to be reactive with several I antibodies from patients suffering from cold agglutinin disease (20). Branched structures of this kind are believed to be developmentally regulated antigens that are found on certain adult but not fetal cells. The possibility that such carbohydrate sequences could be developmentally regulated also could explain why many human infants have high levels of toxin A in their colons but lack any disease symptoms (19,26,32; P.J.G.M. Rietra, K.W. Slaterus, H.C. Zonen, and S.G.M. Meuwissen, Letter. Lancet ii:319. 1978). We are currently investigating this possibility.

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Material described herein will be submitted as part of a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology in the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University.

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SECTION III

Purification of Clostridium difficile Toxin A by Thermal Affinity Chromatography On Immobilized Thyroglobulin

SUMMARY

An efficient, single-step method for isolation of highly purified toxin A from Clostridium difficile culture filtrates is described. The purification procedure is based on the affinity binding and release of toxin A to calf thyroglobulin conjugated to agarose beads. The toxin strongly interacts at 4°C with Galα1-3Galβ1-4GlcNAc, a carbohydrate sequence which occurs on calf thyroglobulin. Toxin associated with thyroglobulin at 4°C and this allowed its separation from the contaminating proteins during the purification scheme. The toxin was eluted by increasing the temperature to 37°C. The toxin binding capacity was proportional to the amount of thyroglobulin in the column: an affinity column containing 15 mg calf thyroglobulin per ml of gel bound 0.52 mg toxin A per ml gel. The percent recovery of purified toxin ranged from 56-80% depending on the amount of thyroglobulin coupled to the gel. The affinity purified toxin was homogeneous as judged by crossed immunoelectrophoresis and gradient polyacrylamide gel electrophoresis, and was immunologically identical to toxin A purified by conventional methods as determined by immunodiffusion analysis. The biochemical, hemagglutinating and toxic properties of the toxin were

preserved after affinity chromatography and were comparable with toxin A purified by conventional methods.

INTRODUCTION

Two toxins produced by Clostridium difficile, designated toxin A and toxin B, are implicated in antibiotic-associated psuedomembranous colitis in humans (3,7,10,19). Both toxins are large, heat-labile, cytotoxic proteins that are lethal in animals. Toxin A is an enterotoxin which elicits a hemorrhagic fluid response when injected into rabbit ileal loops (2,12,19 20). Toxin B does not cause fluid accumulation in intestinal loops but is at least 1,000-fold more cytotoxic than toxin A (5,15,19,20).

Toxin A can be separated from toxin B by ion exchange chromatography or $(NH_4)_2SO_4$ precipitation and batch ion exchange and further purified to homogeneity by isoelectric precipitation at pH 5.6 (13,19). Other methods for purifying toxin A have included gel filtration, hydrophobic interaction chromatography, preparative electrophoresis and tangential flow filtration followed by fast protein liquid chromatography (2,13,16,18,19). These methods, however, either require two to three days to obtain pure toxin A or involve expensive equipment.

We have previously demonstrated that the specific binding of toxin A to rabbit erythrocytes and to brush border membranes from hamsters was greatly enhanced at cold temperatures (9). The toxin also agglutinates rabbit

erythrocytes, and we showed that rabbit erythrocytes contained a carbohydrate binding site for toxin A that had a Galα1-3Galβ1-4GlcNAc nonreducing terminal sequence (9). This sequence also occurs as several branched copies in calf thyroglobulin (17). This glycoprotein also binds toxin A at low temperatures and inhibits agglutination of rabbit erythrocytes (9). We concluded that the behavior of toxin A was similar to what has been observed with cold agglutinating antibodies.

Studies on cold agglutinins and their carbohydrate receptors have indicated that binding is dependent on temperature and receptor density (21,23). We have taken advantage of the binding of toxin A at cold temperatures to its carbohydrate binding site to purify large amounts of toxin using a thermal affinity chromatographic procedure. In this report a method is described using immobilized calf thyroglobulin for the purification of C. difficile toxin A from crude culture filtrate in one step.

MATERIALS AND METHODS

Bacterial strain and culture filtrate. A highly toxigenic strain of C. difficile, VPI strain 10463, was obtained from the culture collection of the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University (Blacksburg, VA). The organism was grown in brain heart infusion (BHI) dialysis flasks as prevoiusly described

(6). After incubation at 37°C for 72h, cells were removed by centrifugation. Culture filtrate was obtained by filtration of the culture supernatant fluid through 0.45 µm membranes. Toxin A was purified by DEAE-Sepharose chromatography and acetic acid precipitation as described by Sullivan et al. (17). This preparation was designated as reference toxin A.

Preparation of immobilized thyroglobulin. Calf thyroglobulin (500 mg, Sigma Chemical Co.) was dissolved in 100 ml of 0.1 M MOPS buffer (3-[N-Morpholino]propanesulfonic acid), pH 7.0, centrifuged (8000 x g) to remove insoluble particulate matter and filtered through a 0.22 µm filter. The glycoprotein solution was then reacted with 20 ml of activiated Affi-Gel 15 (Bio-Rad Laboratories, Richmond, CA) overnight at 4°C on a shaker. The remaining active sites on the gel were blocked with 0.1 M ethanolamine for 30 min at 4°C. Approximately 90% of the calf thyroglobulin coupled to the gel as judged by estimating the protein content of washings obtained during the preparation procedures.

The coupled beads (ca. 20 mg thyroglobulin per ml gel) were packed in a glass column (15x50 mm) and washed at room temperature with 10 bed volumes each of 0.1 M Glycine-NaOH buffer containing 0.5 M NaCl, pH 10.0 (basic buffer) and 0.1 M Glycine-HCl buffer containing 0.5 M NaCl, pH 2.0 (acid buffer) to ensure that free ligand did not remain ionically

bound to the immobilized ligand. The washing cycle was then repeated at 4°C with TBS (0.05 M Tris(hydroxymethyl)amino-methane buffer containing 0.15 M NaCl, pH 7.0) to equlibrate the column prior to affinity chromatography. For binding capacity experiments various amounts of calf thyroglobulin, bovine serum albumin (BSA), and human orosomucoid (Sigma Chemical Co.) were coupled to Aff-gel 15 by the same procedure.

Affinity chromatography. Crude C. difficile culture filtrate cooled to 4°C was applied to the column at 4°C. The unbound protein (effluent) was monitored for absorption at 280 nm and cold hemagglutinating activity (see below). After washing the column with at least seven bed volumes of cold TBS, the column was warmed to 37°C and toxin A was eluted with about three bed volumes of pre-warmed TBS at 37°C. Eluted toxin was concentrated by ultrafiltration through an Amicon YM-10 membrane (Amicon Corp.) and filtered through a 0.22 μm filter (Millipore). To reuse the column the gel was washed with ten bed volumes of acid buffer at room temperature followed by ten bed volumes of TBS, pH 7.0 at 4°C to equlibrate the column.

Assays.

(i) **Protein assay.** Protein concentration was estimated as described by the method of Bradford (4) using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Bovine gamma globulin was the standard.

(ii) Cytotoxicity assay. The cytotoxic titer (TCD_{100}) was determined using Chinese hamster ovary cells (CHO-K1) as described previously (17). Units of cytotoxicity were expressed as the minimum amount of toxin causing cell rounding in 100% of the cell population after 24h of incubation.

(iii) Enterotoxicity assay. Samples of thermal eluted toxin preparations were given intragastrically to hamsters as previously described (14). A positive reaction was the development of diarrhea and the accumulation of fluid in the small intestine and cecum, as detected at necropsy.

(iv) Mouse lethality assay. Toxin preparations were diluted in TBS and 0.5 ml aliquots were injected intraperitoneally into ICR outbred mice 8-10 weeks old (Dominion Laboratories, Dublin, VA). The LD_{100} was the minimum amount of toxin that killed 100% of the mice by 18h.

(v) Hemagglutination assay. Cold hemagglutinating activity of toxin A with rabbit erythrocytes (Hazelton Dutchland, Inc., Denver, PA) was determined as described previously (9). Titers were expressed as the reciprocal of the highest dilution of toxin A which caused agglutination macroscopically.

Immunological methods. Crossed immunoelectrophoresis was performed on 5 x 5 cm glass plates in 1.2% low electroendosmotic agarose (Sigma Chemical Co.) in 125 mM Tris-Tricine buffer, pH 8.6, following the general methodology as described by

Axelsen et al. (1). The agarose used for the second dimension contained 0.1 ml goat antiserum produced against crude culture filtrate of C. difficile VPI strain 10463 (6). Ouchterlony double immunodiffusion analysis was done in 1% low electroendosmotic agarose in Borate-EDTA buffer (20 mM sodium borate-150 mM NaCl-5mM EDTA-15 mM NaN_3), pH 8.2. The gels were incubated 48h in a moist chamber at 4°C.

Gradient PAGE. Gradient PAGE was done in 4-30% concave precast gels (Isolab Inc., Akron) in Tris-Borate buffer (90 mM Tris-80 mM-boric acid-2.5 mM Na_2EDTA), pH 8.4. Gels were stained with Coomassie Blue R-250. High molecular weight standards were purchased from Pharmacia Fine Chemicals, Pistcataway, N.J.

RESULTS

Affinity chromatography on immobilized thyroglobulin.

Culture filtrate (100 ml, 1.2 mg protein/ml) was passed at 4°C through the thyroglobulin affinity column (15 x 50 mm) and then the column was washed thoroughly with cold buffer (TBS, pH 7.0). The column was subsequently warmed to 37°C and eluted with buffer (TBS, pH 7.0) at the same temperature. Under these conditions toxin A did not saturate the column, and there was no leakage of the toxin prior to elution at 37°C as indicated by the absence of hemagglutination activity in the unbound column effluent (Fig. 1).

The majority of the toxin was eluted at 37°C (remaining $A_{280\ nm} < 0.01$) with approximately three bed volumes of

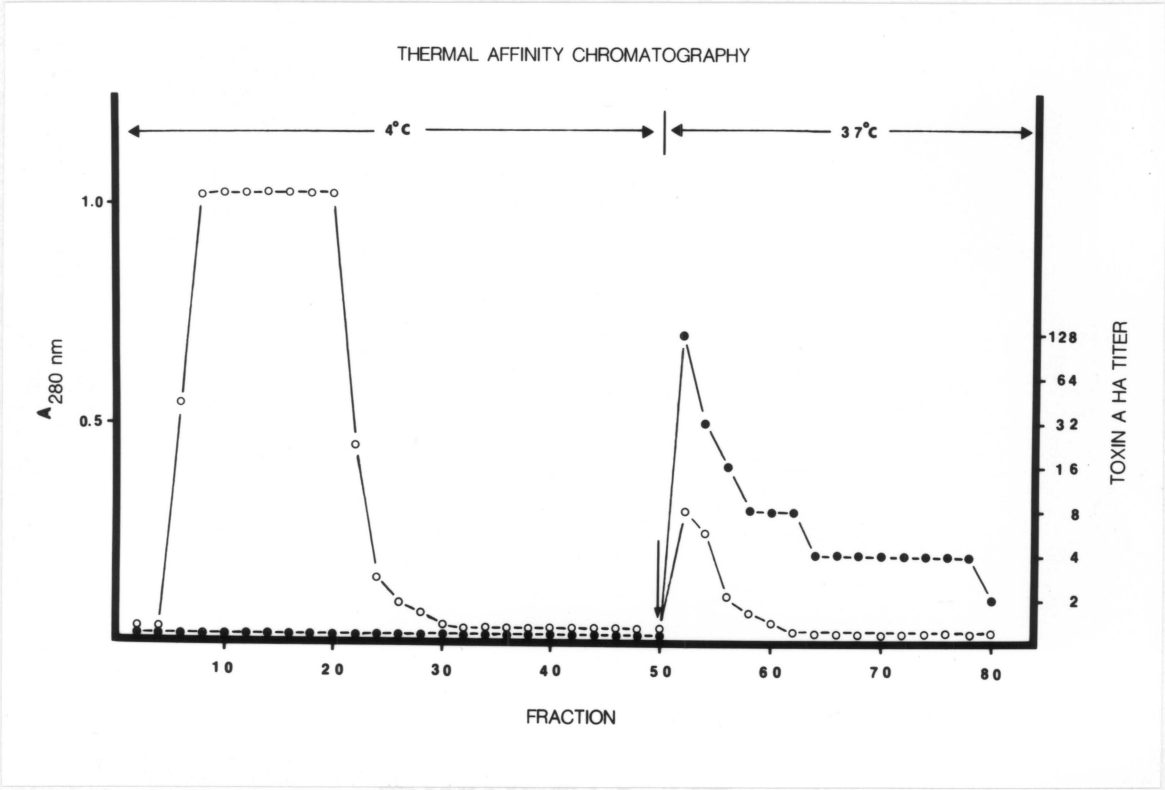

Figure 1. Elution profile obtained after thermal affinity chromatography of toxin A from C. difficile culture filtrate. Culture filtrate was passed through a calf thyroglobulin-Affi-gel 15 column (15 x 50 mm) prepared as described in Materials and Methods. Elution was effected by increasing the temperature to 37°C. Fractions (5 ml) were collected and monitored for absorbtion at 280 nm (o) and cold hemagglutinating activity (●).

pre-warmed TBS (Fig. 1). A small amount of residual toxin continued to be eluted from the column (hemagglutination titer of 4) and this was removed by treating the column with acid buffer (as described in Materials and Methods). Repeated cycles on the affinity thyroglobulin column gave very similar results and the toxin binding capacity of the column was not influenced by multiple acid washings.

Binding capacity and toxin recovery.

Homogeneous preparations of toxin A (0.1 mg/ml) were passed through columns containing varying amounts of bound calf thyroglobulin bound per ml of gel to determine the relationship between the ligand concentration and the toxin binding capacity of the column (Table 1). Toxin A did not bind to the gel in the absence of thyroglobulin or to gels coupled with other proteins. After saturation, each column was washed with TBS and eluted at 37^{o}C. The amounts of purified toxin recovered were estimated by protein determination and confirmed by the hemagglutination assay. The binding capacity of the columns was related to the concentration of calf thyroglobulin and reached a maximum of 1.4 mg/ml of gel. The estimated recovery varied between 55-80%. The percent recovery was inversely proportional to the concentration of calf thyroglobulin coupled to the gel.

Characterization of affinity purified toxin A

Unbound and eluted fractions were pooled separately and

Table 1. Binding capacity and recovery of C. difficile toxin A on Affi-gel 15 columns containing different amounts of calf thyroglobulin or other proteins.

Column Type[a]	Amount Coupled	Binding Capacity[b]	Amount Eluted	Recovery
	mg/ml gel	mg/ml gel	mg	%
No protein	0	0	0	0
Calf TG	1.5	0.15	0.48	80
Calf TG	15	0.52	1.49	71
Calf TG	35	1.40	3.20	56
Orosomucoid	25	0	0	0
BSA	25	0	0	0

[a]Each column contained 4 ml of gel

[b]mg toxin A bound/ml gel calculated by analyzing effluent by hemagglutination

concentrated before analysis by crossed immunoelectrophoresis and gradient gel electrophoresis. These analyses showed that only toxin A bound to the column and that it was eluted at 37°C in pure form (Fig. 2 & 3). This was also confirmed by the absence of hemagglutination activity before thermal elution (Fig. 1).

To determine whether the affinity-purified toxin A was identical to toxin A purified by conventional methods, we analyzed a reference toxin A preparation (17) with our thermal eluted toxin A. Ouchterlony double immunodiffusion analysis (Fig. 4) showed a reaction of identity.

The enterotoxic, cytotoxic and lethal activities of the thermal eluted toxin were within the range of values previously reported for this toxin (Table 2). The affinity purified toxin A also showed no difference in the titer of agglutination of rabbit erythrocytes when compared with the reference toxin A preparation.

DISCUSSION

A simple one-step purification of C. difficile toxin A was achieved on an immobilized calf thyroglobulin column by thermal affinity chromatography. The procedure is based on the presence of terminal Galα1-3Galβ1-4GlcNAc, a carbohydrate sequence that occurs as a highly branched structure in calf thyroglobulin, and which also is found on the cell surface of rabbit erythrocytes (8,17). We have previously shown that toxin A

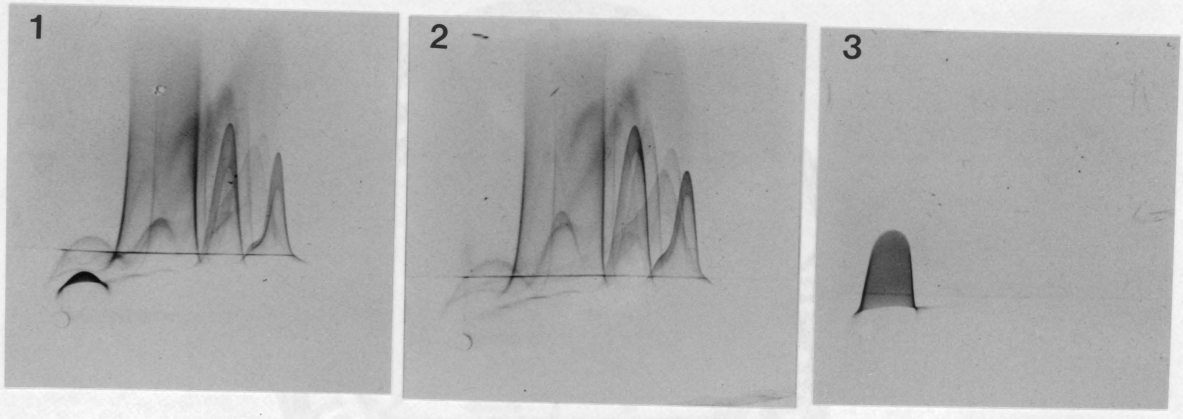

Figure 2. Analysis of toxin preparations by crossed immuno-electrophoresis . The upper portion of the gel in each plate contained 0.1 ml of goat antiserum against C. difficile strain 10463 culture filtrate. (1) the well contained 60 µg of 10463 culture filtrate. The toxin A immunoprecipitin arc is indicated by the arrow. (2) The well contained 50 µg of effluent from the thyroglobulin Affi-gel 15 column. (3) The well contained 50 µg of eluted toxin A.

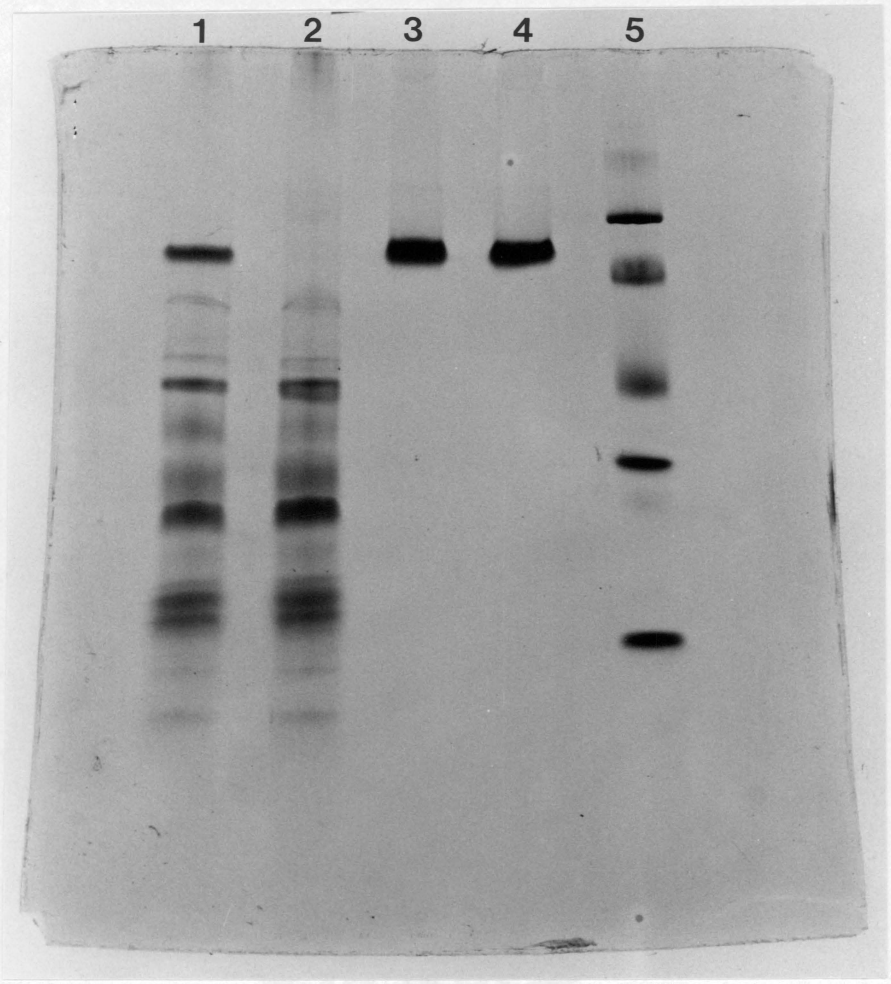

Figure 3. Analysis of toxin preparations by gradient polyacrylamide gel (4-30%) in Tris-Borate buffer, pH 8.2. Samples included (1) strain 10463 culture filtrate (120 µg); (2) culture filtrate effluent (110 µg); (3) affinity purified toxin A (15 µg); (4) reference toxin purified by acetic acid precipitation (15 µg); and (5) high molecular weight markers (12.5 µg): thyroglobulin (MW 669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase, (MW 140,000), and bovine serum albumin (67,000).

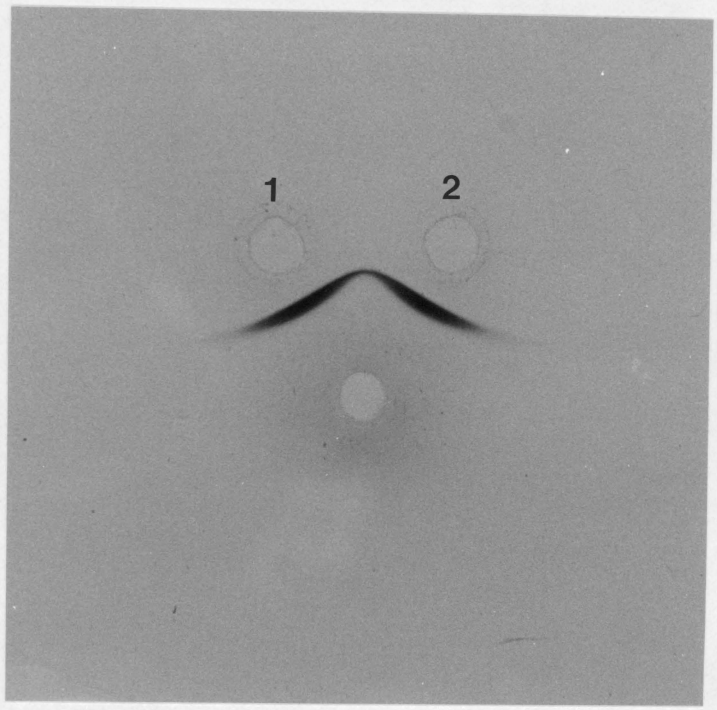

Figure 4. Analysis of toxin preparations by Ouchterlony double diffusion. The center well contained 20 µl of antiserum against C. difficile strain 10463 culture filtrate. Well (1) contained 30 µg of reference toxin A purified by acetic acid precipitation; well (2) contained 30 µg of toxin A purified by thermal affinity chromatography.

Table 2. Comparison of biological activities of toxin A

Method	Fluid Accumulation		TCD_{100}[b]	LD_{100}[c] (mice)	Ref.
	rabbit ileal loop	hamster[a] model			
Thermal elution	30 µg	75 µg/kg body wt.	10 ng	75 ng	-
Banno *et al.*	3 µg	ND	"low"	26 ng	2
Lyerly *et al.*	50 µg	100 µg/kg body wt.	10 ng	50 ng	12,13
Sullivan *et al.*	50 µg	ND	10 ng	90 ng	19
Taylor *et al.*	1 µg	ND	2 ng	50 ng	20

[a]Hemorrhagic fluid in the intestine and cecum

[b]TCD = Tissue culture dose

[c]LD = Lethal dose

has a temperature-dependent affinity for this carbohydate sequence in calf thyroglobulin and on rabbit erythrocytes and that the toxin could also agglutinate these cells (9). Toxin binding is enhanced and stable at 4°C but the toxin dissociates from its binding site at 37°C in a manner similar to what has been observed with cold agglutinins. The unusual behavior of the toxin at 4 and 37°C had allowed us to purify the toxin by a similar procedure using rabbit ghost membranes (H.C. Krivan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-60, p. 34).

It has been previously reported that toxin A could be affinity purified using agarose beads and that the toxin could be eluted with galactose (11). Although these investigators made no reference to temperature, we have not been able to confirm these experiments at any temperature. Our findings indicate that toxin A does not bind to agarose beads or other proteins (BSA, orosmucoid) coupled to Affi-gel 15 agarose unless the glycoconjugate contains the carbohydrate sequence Galα1-3Galβ1-4GlcNAc.

The thyroglobulin thermal affinity purification procedure offers several advantages over other purification methods for toxin A: (i) it is an efficient, single step method for isolation of highly purified toxin A; (ii) the column is reusable; (iii) thermal elution is a gentle process, all of the biochemical, immunological, and toxic properties of the toxin are preserved; and (iv) the thyroglobulin column offers an advantage over the use of rabbit erythrocytes or

ghosts in that there is no possibility that the final product will be contaminated with hemoglobin or membrane components.

There are two important factors to be considered when preparing the affinity column. First, the source of the thyroglobulin is critical and must be of bovine origin. We have previously shown that human thyroglobulin does not bind toxin A (9) because of the complete absence of terminal alpha-galactosyl residues on this glycoprotein (17). Thyroglobulin from other species contain varying amounts of terminal alpha-galactosyl residues (17) but calf thyroglobulin contains the highest amount. And second, the concentration of calf thyroglobulin immobilized on the agarose beads can influence the percent recovery of toxin A. Although the toxin binding capacity is related to the amount of coupled thyroglobulin in the gel, the percent recovery appears to be inversely related (Table 1). This may be related to the density of the toxin binding sites in the gel. It has been postulated for cold agglutinins that receptor density and multivalency influence their thermal amplitude (23), i.e., when the receptor density is high enough cold agglutinins can bind at 37^{o}C. The lower percent recovery of toxin A from our columns containing a high concentration of thyroglobulin may be due to interaction of the toxin with its binding site at 37^{o}C and hence less toxin is eluted off the column at this temperature. We therefore recommend the use of 15-20 mg calf thyroglobulin immobilized per ml of gel. With such columns

containing 10 ml of gel we can purify approximately 3.5 mg of toxin A from 50 ml of culture filtrate.

ACKNOWLEDGMENTS

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SECTION IV. OVERALL DISCUSSION AND SUMMARY

Biological relevance of the toxin A binding site

Probably the most striking result to come from this research was the discovery that the binding of toxin A to brush border membranes from hamsters and to rabbit erythrocytes had the unusual characteristic of being greatly enhanced at lower temperatures (Section II). This phenomenon was most pronounced with rabbit erythrocytes which did not bind toxin A at 37°C. The phenomenon is useful for affinity purification of large amounts of toxin A but the question remains as to whether the binding site described in this dissertation is of biological relevance.

There are two important findings that relate to this question. First, the brush border membranes from hamsters did bind toxin A at 37°C in amounts comparable to what has been observed with cholera toxin. Second, binding activity was found in lower amounts in the intestinal brush border membranes of rats, and rats are much less susceptible to the action of toxin A than hamsters. Although these results are indirect, they do indicate that at least the binding levels observed in these experiments are in an acceptable physiological range.

Toxin A: bacterial lectin or cold agglutinin

Another interesting finding was the discovery that C. difficile toxin A is a bacterial lectin. A lectin (from

the Latin legere: to chose), by definition (18), is a carbohydrate-binding protein of non-immune origin that agglutinates cells. Toxin A has been shown to bind to a specific carbohydrate sequence on rabbit erythrocytes and cause these cells to agglutinate. For this to occur the toxin must bear at least two carbohydrate-binding sites. As has been previously discussed in Section I, toxin A is thought to be an aggregate of repeating subunits and it is probably this feature which allows toxin A to bind to rabbit erythrocytes in a multivalent fashion.

Although toxin A is by definition a bacterial lectin, it only functions as a lectin at temperatures below 37°C. The nature of this temperature-dependent interaction is not entirely understood. A similar phenomenon, however, has been described with a completely different class of proteins, called cold agglutinins. Cold agglutinins are carbohydrate-binding antibodies that agglutinate cells but only below 37°C. Cold agglutinins are not by definition lectins because they are immunoglobulins. In most instances they represent examples of homogeneous monoclonal IgM antibodies (27), although IgA and IgG cold agglutinins have also been characterized (1,2). Because of the similarity of toxin A with cold agglutinins, an understanding of the molecular basis of cold agglutination may provide some insight about how toxin A functions as a cold agglutinating toxin.

Cold agglutinins

The first published report on cold agglutinin disease is thought to be by Elliotson (9) who in 1832 described a patient with heart disease and "cold fits" who passed bloody urine "whenever the east wind blew." It was not until the late 1950's, however, before cold agglutinins were shown to be antibodies (4) which were directed against the I antigen or related antigens on erythrocytes (39). Today, several diseases are known to be associated with cold agglutinins (Table 1) and some of these diseases are incited by bacteria, like Mycoplasma pneumoniae. Infection with this agent in man is associated with atypical pneumonia and in the convalescent period, high titer cold agglutinins are produced which are usually monoclonal and directed to the I antigen (9a). Although the mechanism awaits elucidation, it is thought that the organism adheres to long chain oligosaccharides of Ii antigen type which eventually results in the formation of an autoimmunogenic complex. The resulting autoantibodies (anti-I cold agglutinins) are directed against the carbohydrate backbones of the host-cell receptors (10).

The I,i receptors for cold agglutinins are carbohydrate antigens found on the surface of many types of cells. These carbohydrate determinants have been termed the I,i antigens by Wiener et al. (39) who proposed the term I to emphasize the Individuality of donors whose cells reacted with high-titer cold agglutinins. The cell surface i antigen has also been

Table 1. Diseases which have been associated with cold agglutinins[a].

Monoclonal Cold Agglutinins	Polyclonal Cold Agglutinins
Chronic cold-hemagglutinin disease	Mycoplasma pneumonia
Walenstrom's macroglobulinemia	Infectious mononucleosis
Hodgkin & non-Hodgkin lymphoma	Cytomegalovirus infection
Chronic lymphocytic leukemia	Listeriosis
Chronic myelogeneous leukemia	Munps orchitis
Plasmacytoma & multiple myeloma	Subacute bacterial endocarditis
Severe combined immuno-deficiency	Angioimmunoblastic lymphadenopathy
Karposi's sarcoma	Syphilis
Mycoplasma pneumonia (rare)	Tropical diseases (trypanosomiasis,
malaria,	tropical eosinophilia)
	Collagen-vascular & immune complex diseases

[a]Modified from Pruzanski and Shumak (26).

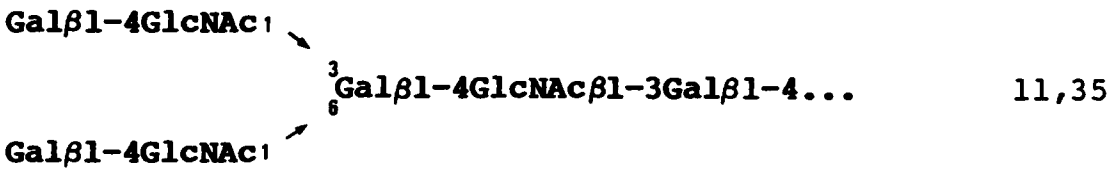
called fetal i antigen because it eventually changes into adult I antigen during cellular development (16). The structural distinction between fetal i and adult I is shown in Fig. 1. In fetal cells, a linear unbranched polylactosamine, $(Gal\text{-}GlcNAc)_n$ is present. When these cells develop into adult cells, the linear chains are converted to those having Gal-GlcNAc branchings (10). Therefore, an anti I cold agglutinin can react with erythrocytes of normal adults but not with fetal or neonatal erythrocytes.

The I,i antigens may occur as glycoproteins (21) or glycolipids (36), and appear to be ubiquitous having been found on many non-erythroid cells and in body fluids (Table 2). The wide distribution of I,i antigens is not limited to human cells; human anti I and anti i react with certain bacteria and with erythrocytes and lymphocytes from many species (25,38). For example, rabbit erythrocytes contain large amounts of I antigen on their cell surfaces. The major complex carbohydrate found on rabbit erythrocytes is a decasaccharide ceramide, a branched glycolipid containing the sequence Galα1-3Galβ1-4GlcNAc. This structure has been shown to be reactive with several anti-I antibodies from patients suffering from cold agglutinin disease (19), and is the structure to which toxin A binds (Section II). This I reactive sequence is also found in multiple copies on certain glycoproteins such as calf thyroglobulin and laminin (29).

Fetal i antigen — Reference

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4... 24,35

Adult I antigen



11,35

Figure 1. Structures of fetal i and adult I antigens. I- and i-antigenic activities are expressed by branched polylactosamine I and linear repeating lactosaminyl structure i.

Table 2. Some non-erythroid cells and body fluids which contain I,i carbohydrate determinants.

	Reference
Non-erythroid cells with I,i activity	
Lymphocytes (B and T cells)	17
Polymorphonuclear leukocytes	22
Monocytes and macrophages	25
Platlets	37
Cultured fibroblasts and HeLa cells	17,30
Gastrointestinal mucosa cells	10,13
Mycoplasma pneumoniae	9a,23
Listeria monocytogenes	23
Streptococcus sp.	23
Body fluids containing I,i activity	
Plasma	3
Saliva	8,20
Milk	20
Amniotic fluid	5
Ovarian cyst fluid	34

It is clear that toxin A and cold agglutinins have much in common. Both require cold temperatures to agglutinate cells and both appear to bind to the same kind of receptors, the I,i antigens. But what is the explanation for their thermal dependency?

Molecular basis of cold agglutination

The nature of the temperature dependency of cold agglutinins is not yet fully understood. Clearly, either the cold agglutinin or its receptor or water structure must be affected by the changes in temperature. If the receptor changes, it may either be a property of the receptor itself or the membrane that bears it. There have been conflicting reports in this regard. One group of investigators postulated that temperature-dependent changes in the membrane were responsible for thermal variation (28) and that the I antigen became hidden in the membrane at 37^{o}C and therefore could not react with the cold agglutinin. Other investigators have suggested that the thermal characteristics of the reaction of cold agglutinins with I,i antigens does not always depend on changes in membrane conformation, but that it is either the I antigen or the cold agglutinin that changes in conformation (14).

Tsai and coworkers (33) have proposed a completely different hypothesis to explain thermal variation in cold agglutinins. These investigators isolated and characterized a monoclonal IgM

isolated from a patient with Waldenstrom's macroglobulinemia (31,32). The membrane receptors for this Waldenstrom's macroglobulin are sialic acid-containing glycoproteins in human erythrocytes and sialic acid-containing glycolipids in dog cells. These investigators reported that human erythrocytes are agglutinated only in the cold while dog erythrocytes are also agglutinated at 37°C, though less strongly than at 0°C. Their explanation for this "thermal amplitude" was attributed to cooperative binding due to the multivalency of the cold agglutinin and the density of the receptors on the cell surface. A subsequent paper by this same group confirmed these results. Tsai and coworkers (33) showed that treatment of both human and dog erythrocytes with neuraminidase abolished the binding activity of the cold agglutinin; however, when the cells were allowed to adsorb large amounts of exogenous ganglioside receptor, both types of cells could agglutinate at both 0°C and 37°C. These investigators have calculated that 7×10^4 to 10^6 molecules of receptor per cell are required for agglutintion to occur at 0°C, but that greater than 10^6 per cell are needed for agglutination to occur at 37°C.

The cooperative binding inherent in a multivalent ligand/receptor (7) may be the mechanism by which toxin A interacts with its binding site. Multivalent binding between toxin A and it carbohydrate binding site has been demonstrated by the ability of toxin A to agglutinate rabbit erythrocytes

and bind to agarose beads containing covalently attached calf thyroglobulin. There is also evidence to support that toxin A may require a high density of receptors to bind at 37°C. The finding that the percentage recovery of thermal eluted toxin A was inversely related to the amount of thyroglobulin coupled to the gel was unexpected (Section III), but it may be related to the density of the toxin binding sites in the gel. At high concentrations of calf thyroglobulin there may be an irreversible interaction of the toxin with its binding site at 37°C, and hence less toxin was eluted off the column at this temperature. Like the Waldenstrom's macroglobulin receptors on human erythrocytes, the density of the toxin binding sites on rabbit erythrocytes may not be high enough to allow multivalent attachment and agglutination of cells at 37°C. The brush border membranes, on the other hand, may contain areas that are densely packed with receptors (like the calf thyroglobulin affinity column) accounting for the binding of the toxin at 37°C.

Multivalent binding has also been shown to exist with other bacterial toxins. Using fluorescein-labeled cholera toxin, Craig and Cuatrecasas (6) demonstrated that a temperature-dependent redistribution of cell surface receptors occurred in rat lymph node cells. They showed that once lymphocytes incubated at 0°C with labeled cholera toxin were warmed to 37°C, the surface fluorescence, which was initially diffuse and patchy, redistributed and became concentrated

preferentially at one pole of the cell. These investigators pointed out that although receptor mobility in the plane of the membrane is critical for "patch and cap formation", it is equally important that the toxin is multivalent so as to "crosslink" the concentrated receptors. These investigators concluded that multivalent binding of cholera toxin to cells with few receptors requires lateral movement of the receptors and is time and temperature dependent. Multivalent binding to cells with many receptors is rapid and occurs at low temperatures where lateral diffusion is minimal. Thus, multivalent binding appears to be essential for cholera toxin action but lateral redistribution may only be required to achieve multivalent binding of the toxin to cells with few receptors.

Future research areas

As is often the case, a research problem is really never completed but instead leads to other questions and areas that require investigation. For example, it will be important to determine if the toxin A binding site which has been identified in brush border membranes is the physiological receptor to which the toxin binds during disease. Does the binding of toxin A to this receptor result in the tissue damage and fluid seen during the disease? A receptor for toxin B has not been identified and it remains to be determined whether toxins A and B act on organs outside the intestine. Once the

toxins bind to their respective target tissues, what is their mechanism of action? The structure of toxin A is not known but evidence which has already been discussed suggests it is an aggregate. Does the entire toxin aggregate enter the cell and shut off some vital function or are small fragments or subunits of each toxin responsible for the toxicity?

Another area that requires investigation is to test the receptor density hypothesis with toxin A. If it is assumed that toxin A binds to its receptor in a multivalent fashion, it would be of interest to determine whether lateral redistribution of the toxin-receptor complex occurs with brush border membranes or intestinal epithelial cells of the hamster. One way of doing this would be to take the approach of Craig and Cuatrecasas (9) and use fluorescein-labeled toxin A and look for the same patching and capping phenomena. It is important to note that the exudative plaques on the colonic mucosa (pseudomembranes) of patients with C. difficile disease are localized in patches.

Another experiment that would support the receptor density hypothesis would involve treatment of rabbit erythrocytes with alpha-galactosidase, which was shown in Section II to abolish toxin binding, and then to subsequently restore binding activity by treatment of the cells with exogenous decasaccharide ceramide, which contains the toxin A binding sequence Galα1-3Galβ1-4GlcNAc. Following the same approach as Tsai et al. (33), when large amounts of

decasaccharide are adsorbed the cells are tested for agglutination in the cold and at 37°C. Agglutination of rabbit erythrocytes by toxin A at 37°C would demonstrate that the density of receptors on the rabbit erythrocyte could influence the thermal amplitude of toxin A.

At present there is an ongoing collaborative effort between Dr. David F. Smith's laboratory in the Department of Biochemistry and Nutrition and with Dr. Tracy D. Wilkin's laboratory where the research contained in this dissertation was conducted. Research in Dr. Smith's laboratory concerns the elucidation of the different structures on glycolipids that contain the toxin A binding site Galα1-3Galβ1-4GlcNAc. Future research areas between the two laboratories hopefully will lead to an understanding of the nature of the physiological receptor for toxin A in both hamsters and humans.

The results of the research presented in this dissertation provide the framework for further studies in the molecular mechanism of action of toxin A and C. difficile disease. The carbohydrate binding site and thermal dependency of toxin A have other potential uses in Microbiology, particularly in the field of diagnostics. Toxin A can also be regarded as a highly specific probe for terminal alpha-linked galactose residues, which may be important in other areas of membrane biology and carbohydrate biochemistry. Hopefully the work included in this dissertation will be helpful to other investigators interested in these areas.

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IDENTIFICATION AND CHARACTERIZATION OF A RECEPTOR FOR *CLOSTRIDIUM DIFFICILE* ENTEROTOXIN

by

Howard C. Krivan

(ABSTRACT)

Clostridium difficile and its toxins are implicated as the cause of pseudomembranous colitis in patients undergoing antibiotic therapy. Very little information is known about how these toxins bind to cells and cause disease. In an attempt to understand how these toxins work this dissertation research was undertaken to determine whether a receptor for *C. difficile* enterotoxin (toxin A) exists in the brush border membranes (BBMs) of the hamster, an animal known to be extremely sensitive to the action of the toxin.

Toxin A was the only antigen adsorbed by the BBMs from the culture filtrate of *C. difficile*. Erythrocytes from various animal species were also examined for binding activity. Only rabbit erythrocytes could bind the toxin, and the cells agglutinated. A binding assay based on an enzyme-linked immunosorbent assay method for quantifying *C. difficile* toxin A was used to compare binding of the toxin to hamster BBMs, rabbit erythrocytes, and to BBMs from rats, which are less susceptible to the action of *C. difficile* toxin A than hamsters. Results of this comparison indicated the following order of toxin binding frequency: rabbit erythrocytes >

hamster BBMs > rat BBMs. Binding of toxin A to BBMs from hamsters at 37°C was comparable to what has been observed with cholera toxin, but binding was enhanced at 4°C. A similar binding phenomenon was observed with rabbit erythrocytes.

Examination of the cell surfaces of hamster BBMs and rabbit erythrocytes with lectins and specific glycosidases revealed a high concentration of terminal α-linked galactose. Treatment of both membrane types with α-galactosidase destroyed the binding activity. The glycoprotein, calf thyroglobulin, also bound the toxin and inhibited toxin binding to cells. An efficient, single-step method for isolation of highly purified toxin A from C. difficile culture filtrate has been developed based on toxin association with calf thyroglobulin.

Toxin A did not bind to human erythrocytes from blood group A, B, or O donors. However, after fucosidase treatment of human erythrocytes, only blood group B erythrocytes could bind the toxin. This indicated that toxin A was likely binding to Galα1-3Galβ1-4GlcNAc, a carbohydrate sequence also found on calf thyroglobulin and rabbit erythrocytes.

All of the results indicate that hamster BBMs contain a carbohydrate binding site for toxin A that has at least a Galα1-3Galβ1-4GlcNAc nonreducing terminal sequence.