INFECTION AND IMMUNITY, Sept. 1986, p. 573–581
0019-9567/86/090573-09$02.00/0

Vol. 53, No. 3

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

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Received 14 April 1986/Accepted 12 May 1986

This study was undertaken to determine whether a binding site for *Clostridium 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*. 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 bound 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 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 hamster BBMs 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. 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, bound 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.

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 toxins A and 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 of 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, 24, 31). However, a receptor for either toxin A or toxin B has not been identified. The present report describes studies designed to explore the interaction of toxin A with brush border membranes (BBMs) from the hamster, 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 increased at lower temperatures. A similar binding phenomenon was 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.

(A preliminary report of this work was presented at The CIBA Foundation Symposium 112 on 9–12 July 1984 in London, England, on microbial toxins and diarrheal disease, and at the American Society for Microbiology annual meeting held on 23–28 March 1986 in Washington, D.C.)

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 (30). 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* β-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-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-α-L-fucoside were purchased from Sigma.

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(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, melibiose, 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-*O*-methyl-α-D-galactose, 1-*O*-methyl-β-D-galactose, stachyose, calf thyroglobulin, asialofetuin, fetuin, orosomucoid, human glycophorin, ovomucoid, ovalbumin, α-lactalbumin, β-lactoglobulin, bovine brain gangliosides (type III), cerebroside types 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 α1-3 galactose was purchased from BioCarb Chemicals AB, Lund, Sweden.

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

Preparation of toxin A. A highly toxigenic strain of *C. difficile*, VPI 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 37°C for 48 h in brain heart infusion 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-Freez. Defibrinated, sterile rabbit blood was purchased from Brown Laboratories (Topeka, Kans.) and Hazelton Dutchland, Inc. (Denver, Pa.). Fresh rabbit blood (drawn in EDTA) was obtained from animals maintained in the laboratory. Blood from other animal species was obtained from Pel-Freez and the Department of Veterinary Medicine, Virginia Polytechnic Institute and State University. Erythrocytes were sedimented by centrifugation at 1,500 × *g* for 5 min and washed four times in isotonic TBS (0.1 M Tris buffer containing 50 mM NaCl, pH 7.2) at 4°C before use.

Preparation of membranes. (i) Isolation of BBMs. BBMs were isolated by 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. The purity of the BBMs was monitored by phase-contrast microscopy.

(ii) Preparation of ghost membranes. Hemoglobin-free rabbit erythrocyte membranes (ghosts) were prepared by using a hemolysis procedure similar to the method of Dodge et al. (10) with 5 mM sodium phosphate buffer (pH 8.0, 4°C). Sedimented membranes were creamy white and were resuspended to the original blood volume in TBS at 4°C before use.

Detergent solubilization. BBMs and ghosts 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). BBMs and ghosts were incubated for 60 min at 37 and 4°C, respectively, and centrifuged at 20,000 × *g* for 60 min. The clear supernatant, representing the solubilized extract, was aspirated from the gelatinous pellets, filter-sterilized (0.22-μm-pore filter) and stored at 4°C before 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 of bovine serum albumin (BSA) per ml before use. Rabbit erythrocytes were washed four 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 of 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 37°C, the cells were sedimented by centrifugation at 12,000 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 h. 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 anti-goat immunoglobulin conjugated to alkaline phosphatase, and alkaline phosphatase substrate. A_{405} readings 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 (2.5×10^8 cells per ml) and hamster BBMs (2 mg of membrane protein per ml) in 0.05 M sodium citrate–0.1 M NaCl containing 0.04% sodium azide (citrate-buffered saline) were incubated at room temperature or at 37°C with either 1.5 U of coffee bean α-galactosidase (pH 6.0) or 2.5 U of either jack bean β-galactosidase (pH 4.0) or *E. coli* β-galactosidase (pH 4.0). At various times, 0.15-ml portions were removed and washed two 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 1 N HCl to pH 6.0 or 4.0 before incubation (37°C, 1.5 h) with 1 U 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 of membrane protein per ml) in citrate-buffered saline (pH 5.5) were incubated at 37°C for 12 h with 5 U of α-L-fucosidase. The membranes were removed and washed three times in excess ice-cold TBS at 4°C before use. The efficacy of α-L-fucosidase was examined by its ability to hydrolyze *p*-nitrophenyl-α-L-fucoside.

(iii) Proteolytic digestion. *S. griseus* pronase (2 mg) was added to Triton X-100-solubilized extracts in 2 ml and incubated at 37°C for 3 h. The incubation was terminated by placing the reaction tube in a boiling water bath for 5 min

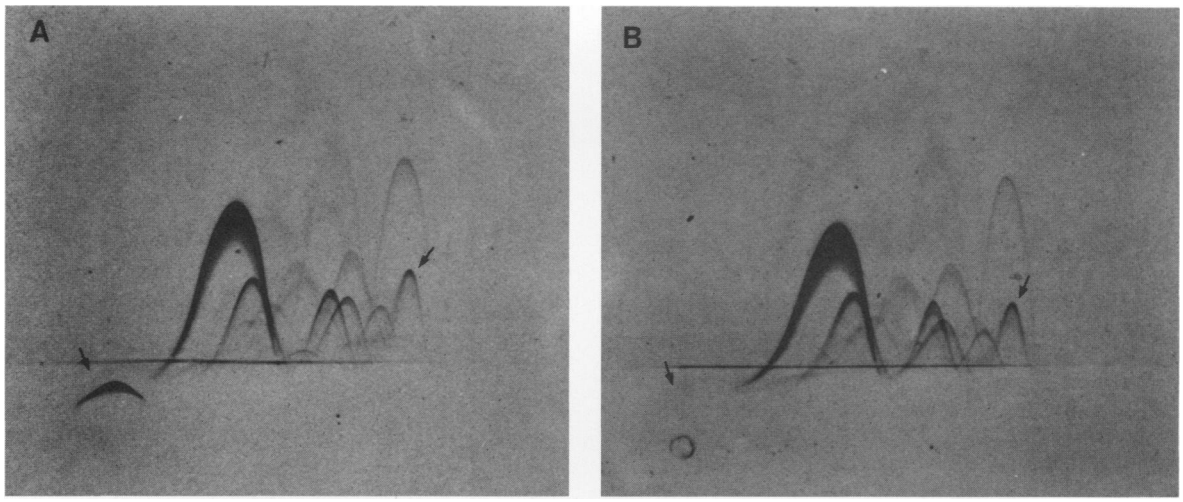

FIG. 1. Analysis by crossed IEP of *C. difficile* 10463 culture filtrate adsorbed with hamster BBMs at 4°C. The upper portion of the gel in each plate contained 0.1 ml of goat antiserum against *C. difficile* VPI 10463 culture filtrate (11). (A) The well contained 50 μg of 10463 culture filtrate in 25 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 BBMs from hamsters. Note the absence of toxin A (left arrow) in plate B, demonstrating that hamster BBMs bind only toxin A. The toxin B arc in the adsorbed culture filtrate (right arrow) is still present.

before assaying for binding activity. Mild proteolytic treatment of washed erythrocytes (2.5×10^8 cells per 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 were washed three times in TBS. No apparent hemolysis was encountered under these conditions.

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

Hemagglutination and hemagglutination inhibition assay. Erythrocytes were washed four times in 10 volumes of TBS (pH 7.2) and diluted to a 2.5% suspension. Twofold 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 erythrocytes were allowed to settle at either 4, 22, or 37°C. 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 twofold series) in small (12 by 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 samples of erythrocyte suspension were added to V-bottom microtiter plates which contained either TBS or *C. difficile* 10463 antitoxin (1:2,000 dilution for room temperature in TBS; 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 for 30 min at 4 or 22°C. 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 of membrane protein per ml) were incubated in TBS at room temperature in a final volume of 1 ml with each of the two 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 (with or without *N*-acetyl-D-galactosamine). After treatment, lectins were removed by centrifugation, and membranes were assayed for toxin binding at 22°C by the toxin A ELISA. All lectins were purchased from Sigma.

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

RESULTS

Toxin specificity. We examined the ability of hamster BBMs 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 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 (100°C, 10 min) did not reduce the binding activity.

Erythrocyte specificity. Because erythrocyte membrane surfaces contain a variety of carbohydrate sequences, erythrocytes from the following animal species were studied for their ability to bind the toxins: human (types A, B, and O), rhesus monkey, hamster, 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 (43; H. C. Krivan and D. M. Lyerly, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C245, p. 340). Toxin B did not agglutinate rabbit erythrocytes.

The hemagglutination assay was most sensitive for detecting toxin A at 4°C, and hemagglutination was not observed at 37°C. Toxin A agglutinated rabbit erythrocytes at concentrations as low as 0.8 μg/ml at 4°C. 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 of toxin A per ml. Agglutination of the erythrocytes by toxin A at 4°C disappeared when the cells were warmed to 37°C. The possibility that toxin A was enzymatically destroying its binding site at 37°C was ruled out because erythrocytes agglutinated after being incubated with toxin at 37°C for 60 min and then cooled to 4°C. 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 BBMs and erythrocyte ghosts. (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 4°C 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 37°C 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 4°C, and residual toxin was removed by three cycles of centrifugation and washing at 4°C. Bound toxin dissociated from both hamster BBMs and rabbit erythrocytes when warmed to 37°C, although not 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 37°C, and the sensitivity of the hemagglutination assay is greatest at 4°C.

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).

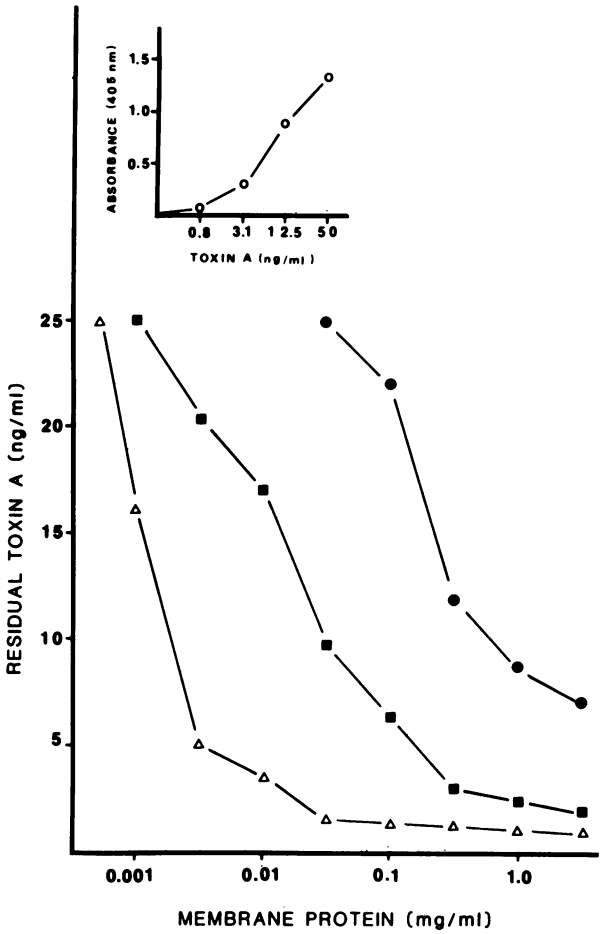

FIG. 2. Effect of membrane concentration on the binding of *C. difficile* toxin A. The indicated concentration of membranes (membrane protein, milligrams per milliliter) was incubated 30 min at 4°C in 0.3 ml of TBS containing 0.2% BSA and 25 ng of toxin A per ml. Concentrations of toxin A were determined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

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 similarly to extracts from rabbit erythrocytes; however, only rabbit erythrocytes were agglutinated by the toxin in the hemagglutination assay. This suggests that binding structures exist on calf erythrocytes which could be detected only 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 calf and rabbit erythrocyte membranes 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 either with pronase or neuraminidase or by boiling did not abolish binding.

(ii) Enzyme degradation of the membrane-bound binding

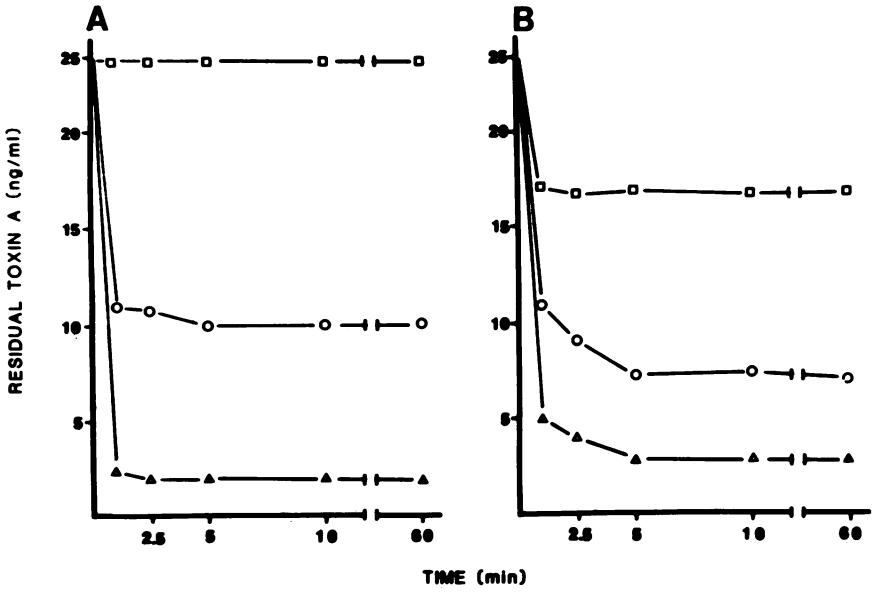

FIG. 3. Time course of binding of *C. difficile* toxin A to rabbit erythrocytes (A) and hamster BBMs (B) at 4°C (△), 22°C (○), and 37°C (□). Reaction mixtures contained 10^8 rabbit erythrocytes or BBMs (1 mg of membrane protein per ml) and 25 ng of toxin A per ml in 0.3 ml of TBS–0.2% BSA. Concentrations of residual toxin were determined by ELISA as described in Materials and Methods.

activity. Pretreatment of rabbit erythrocytes and hamster BBMs 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 4 and 37°C.

Hemagglutination inhibition. We tested a large number of saccharides, α- and β-substituted galactosides, neutral serum, 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 erythrocyte 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. BS-1 blocked binding of the toxin to both hamster BBMs and rabbit erythrocytes (Table 3). BS-1 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. *D. biflorus* agglutinin did not block binding of the toxin to either membrane type.

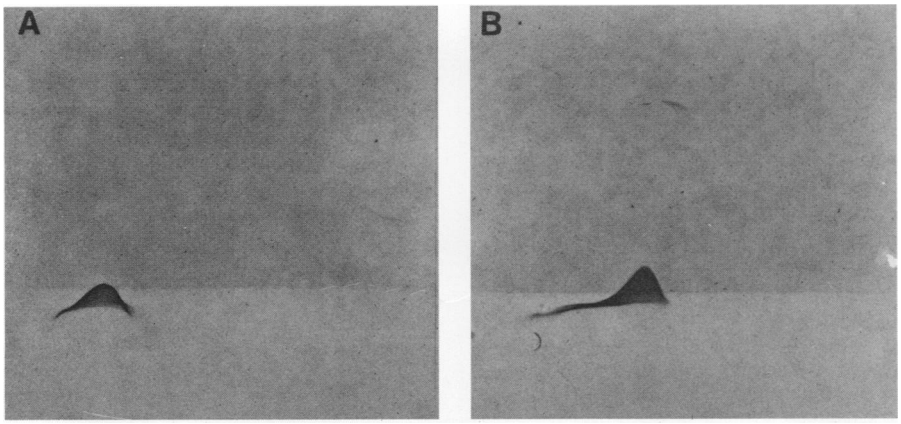

FIG. 4. Analysis by crossed IEP of *C. difficile* VPI 10463 purified toxin A combined with Triton X-100 solubilized extract from rabbit ghost membranes. The upper portion of the gel in each plate contained 0.1 ml of goat antiserum against *C. difficile* 10463 culture filtrate (11). (A) The well contained 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 panel B was also observed when extract from hamster BBMs or calf thyroglobulin was combined with toxin A and analyzed.

TABLE 1. Effect of enzymatic treatment of rabbit erythrocytes and hamster BBMs on binding of *C. difficile* toxin A[a]

Enzyme	Activity (U)	Rabbit erythrocyte hemagglutinin titer	% Inhibition of hamster BBMs
None		512	0
α-Galactosidase	1.5	8	42
β-Galactosidase	2.5	512	0
Neuraminidase	1.0	512	0

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

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 (Galα1-3[Fucα1-2]Galβ1-4GlcNAc) contains an α-1-2 fucosyl unit linked to the penultimate galactose, experiments were designed to test whether 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 of toxin A per mg of membrane protein). Toxin A did not bind to membranes from human type A or O erythrocytes which had been pretreated with fucosidase.

TABLE 3. Effect of lectin blockade on binding of *C. difficile* toxin A to hamster BBMs and rabbit erythrocytes[a]

Lectin	Specificity	% Binding of *C. difficile* to[b]:	
		Hamster BBMs	Rabbit ghosts
BS 1	α-D-Gal	31.5 (25)	51.0 (0.5)
DBA	α-D-GalNAc	0 (25)	0 (25)

[a] Hamster 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 BS-1 and *Dolichos biflorus* agglutinin incubated with BBMs and rabbit ghosts are indicated in parentheses.

DISCUSSION

This study was undertaken to determine whether receptors for *C. difficile* enterotoxin (toxin A) exist in the BBMs of the hamster, 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 hamster BBMs was specific. 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 labeling procedures (Bolton-Hunter, lactoperoxi-

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

Carbohydrate compounds (concn)	
Sera (undiluted)	Other saccharides (10 mg/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
Asialofetuin	D-Glucuronic acid
Fetuin	D-Glucosamine
Orosomucoid	D-Galactosamine
Ovomucoid	*N*-Acetyl-D-glucosamine
α-Macroglobulin	*N*-Acetyl-D-galactosamine
α-Lactalbumin	*N*-Acetylneuraminic acid
β-Lactoglobulin	Lactose
Fibronectin	Sucrose
Human thyroglobulin	Maltose
	Cellobiose
	N-Acetylneuramin-lactose
Glycolipids (1 mg/ml)	*N*,*N*′-Diacetylchitobiose
Gangliosides	
Cerebroside type I	
Cerebroside 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 22°C 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 22°C. Gal, Galactose; Glc, glucose; Fru, fructose.

TABLE 4. Glycoconjugates containing terminal α-galactoside linkages

Structure[a]	Source	Reference
Galα1-3Galβ1-4GlcNAcβ1-3Gal... | α1-2 | Fuc	Human erythrocyte	42
Galα1-3Galβ1-4GlcNAcβ1 ↘ 3 Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide 6 ↗ Galα1-3Galβ1-4GlcNAcβ1	Rabbit erythrocyte	20
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide	Calf and rabbit erythrocytes	9, 12
(Galα1-3Galβ1-4GlcNAc...)$_n$	Calf thyroglobulin	36

[a] Gal, Galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Fuc, fucose; Man, mannose.

dase, Chloramine T, and Iodogen) rendered the toxin biologically inactive and destroyed the hemagglutinating activity of the toxin (unpublished results). An ELISA procedure has been used to quantitate binding of *E. coli* heat-labile enterotoxin 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 has 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 showed that hamster BBMs 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 the 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 recently been 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 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 thyroglobulin, and there was a complete absence in human thyroglobulin. 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, 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 α1-3-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 BBMs 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 5.6 (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 caused by the presence of α-linked anhydrogalactose which is known to be a chemical constituent of agarose (34). The possibility that toxin A interacts with agarose has also been investigated by others. Lonnroth and Lange (27) reported that toxin A bound to Bio-Rad agarose A columns and could be eluted with galactose (like cholera and *E. coli* heat-labile enterotoxins). We have not been able to reproduce these results (unpublished data).

We have purified large amounts of toxin A based on affinity chromatography with rabbit erythrocyte membranes or immobilized thryoglobulin (H. C. Krivan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B60, p. 34; H. C. Krivan and T. D. 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 37°C. 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 hamster BBMs and not to rabbit erythrocytes at 37°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; there 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 hamster 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 are 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 cells but not on 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.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI 15749 and AM 30331 from the National Institutes of Health, grant PCM-8408920 from the National Science Foundation, and State Support grant 2124520 from the Commonwealth of Virginia. G.F.C. is the recipient of National Research Service Award HD-06639 from NICHHD.

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