INFECTION AND IMMUNITY, Jan. 1991, p. 73–78
0019-9567/91/010073-06$02.00/0

Vol. 59, No. 1

Toxin A of *Clostridium difficile* Binds to the Human Carbohydrate Antigens I, X, and Y

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Received 21 May 1990/Accepted 8 October 1990

***Clostridium difficile* causes pseudomembranous colitis in humans. The enterotoxin (i.e., toxin A) from this organism is believed to be responsible for the initial intestinal pathology associated with this disease. Previous work shows that this toxin binds to carbohydrates that contain Galα1-3Galβ1-4GlcNAc. However, this carbohydrate is not present on normal human cells. Therefore, this study was undertaken to identify potential receptors for toxin A that do exist on human intestinal epithelium. Using enzyme-linked immunosorbent assay, affinity chromatography, and altered migration in an electric field, we assayed the binding of toxin A to purified carbohydrates and glycoproteins. We found that toxin A bound to the carbohydrate antigens designated I, X, and Y. Each of these carbohydrates exist on the intestinal epithelium of humans.**

Clostridium difficile is one of the most common causes of bacterial diarrhea and colitis among hospitalized patients. The organism cannot compete successfully with the normal microflora of the adult colon but can grow to high numbers when the colonic flora has been disturbed by antibiotic treatments (35, 45). *C. difficile* then keeps the colonic flora from reestablishing itself by producing an enterotoxin, toxin A, which causes destruction of the colonic epithelium, resulting in fluid secretion into the intestine (25, 27). A second toxin produced by *C. difficile*, toxin B, has no effect on undisturbed intestinal epithelium but is an extremely active cytotoxin on nonepithelial cells (1, 25, 27, 29, 40, 41). This toxin may gain access to the underlying cells through the destructive action of toxin A on the epithelial cells (27). Hamsters are the most susceptible laboratory animal to this disease, and in these animals the two toxins seem to act synergistically. Toxin B alone has no effect when given orally to hamsters but kills them when it is combined with sublethal amounts of toxin A (27).

An enterotoxin must bind to the intestinal epithelial cells, and toxins often accomplish this by binding to specific carbohydrate structures on the membrane of the epithelial cells. Previous work in this laboratory has shown that toxin A binds to a trisaccharide (Galα1-3Galβ1-4GlcNAc) which occurs in large amounts on the intestinal cells of hamsters (6, 22). Hamsters can be protected from this disease by immunizing them with a recombinant peptide which contains the carbohydrate-binding portion of toxin A (8). This recombinant protein contains repeating amino acid domains which bind multiple copies of the trisaccharide receptor for the toxin (8, 33). Recently, we have shown that cell lines which express large amounts of this carbohydrate on their cell membranes are more sensitive to the cytotoxic action of toxin A than are other cell lines (42, 44). This evidence led us to speculate that Galα1-3Galβ1-4GlcNAc was the biological receptor for toxin A in humans as well as hamsters.

A search of the literature revealed that there was a problem with this postulate. Normal human cells do not express this trisaccharide (11, 13). Furthermore, most people produce antibodies against this carbohydrate (12). Preliminary experiments showed that toxin A did bind to human intestinal epithelium; therefore, we started to test for the binding of toxin A to carbohydrate structures that have been reported to exist on human intestinal mucosa. The first structure we tested was a carbohydrate (Galβ1-4[Fucα1-3]GlcNAc) which appeared to be conformationally similar to the reported receptor for toxin A (Galα1-3Galβ1-4GlcNAc). We found that toxin A bound to this carbohydrate and two other related oligosaccharides, all of which exist on human intestinal epithelial cells.

MATERIALS AND METHODS

Biochemicals. Carbohydrates isolated from human milk and chemically conjugated to human serum albumin (HSA) were kindly provided by Howard Krivan of BioCarb Chemicals or were purchased from BioCarb Chemicals (Lund, Sweden). See Table 1 for a description of the oligosaccharides and their names. In order to determine if toxin A from *C. difficile* bound to these oligosaccharide-HSA conjugates, we performed two assays: crossed immunoelectrophoresis (IEP) and enzyme-linked immunosorbent assay (ELISA) (as described below). We also assayed the binding of toxin A to bovine thyroglobulin (Sigma Chemical Co., St. Louis, Mo.), which contains Galα1-3Galβ1-4GlcNAc (37), and to the following human proteins, both of which contain the X antigen (4, 30): secretory component (Chemicon, Temecula, Calif.) and carcinoembryonic antigen (CEA) (Calbiochem, San Diego, Calif.).

Toxin purification. *C. difficile* VPI 10463 was grown in dialysis cultures (39). A culture filtrate was prepared by filtering the contents of the dialysis sac with a 0.45-μm-pore-size filter. Toxin A was purified from the culture filtrate by affinity chromatography, using immobilized bovine thyroglobulin (23). The toxin was determined to be homogeneous by procedures previously described (39). The concentration of toxin A was estimated by the method of Bradford (3) with bovine gamma globulin as the standard.

Antiserum. Antiserum to culture filtrate of *C. difficile* was prepared in goats (9). Affinity-purified antibody to toxin A was prepared as previously described (28). The affinity-purified antibody to the toxin was biotinylated with biotina-

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midocaproate *N*-hydroxysuccinimide ester (Sigma) (16). The biotin-antibody conjugate was used at a 1:1,000 dilution.

IEP. The binding of toxin A to glycoproteins was assayed by IEP (22) by mixing 100 μg of toxin A with 500 μg of glycoprotein per ml and incubating on ice for 10 min before loading on the gel. The migration of toxin A with the glycoprotein was compared with the migration of toxin A alone. Interpretation of the results is based on the fact that toxin A normally migrates slowly in IEP. If toxin A binds to a glycoprotein that migrates rapidly in IEP, then the bound toxin will be pulled by the more rapidly migrating glycoprotein, thus causing an increase in the migration of toxin A in IEP. If toxin A does not bind to the glycoprotein, then the mixture does not affect the migration of the toxin.

ELISA. ELISAs were performed in Immulon II 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). Each well was coated with 150 μl of 1:2 serially diluted glycoprotein, starting at 20 μg of glycoprotein per ml of 50 mM carbonate buffer (pH 9.6). Plates were incubated for 16 h at 4°C and then washed twice with PBS-T (0.8% NaCl, 0.02% KH_2PO_4, 0.22% Na_2HPO_4, 0.02% KCl, 0.05% Tween 20, 0.02% NaN_3 [pH 7.4]). The plates were blocked with 0.5% casein in 100 mM Tris–150 mM NaCl (pH 7.0) for 1 h at 22°C and then were washed twice with PBS-T. To each well was added 100 μl of toxin A at 10 μg/ml in PBS-T. The plates were incubated at either 4 or at 37°C for 3 h. After this step, all buffers were at 4°C and all treatments were performed in a 4°C cold room, unless otherwise stated. The plates were washed twice with PBS-T, and 100 μl of a 1:1,000 dilution of biotinylated antibody to toxin A in PBS-T was added to each well and incubated for 1 h. The plates were washed twice with PBS-T, and 100 μl of 0.1-U/ml alkaline phosphatase conjugated to avidin (Sigma) was added to each well. The plates were incubated for 1 h and washed twice with PBS-T. Then the plates were moved to room temperature and 100 μl of *para*-nitrophenylphosphate (1 mg/ml) in 100 mM diethanolamine buffer (pH 9.8) was added to each well. All assays were developed for 90 min at room temperature before the A_{405} was read. Control wells included wells without toxin A and/or without glycoprotein. The highest A_{405} obtained with wells that either did not contain toxin A or did not contain glycoprotein was used as the value for background, which was subtracted from the values presented in the figures.

The possibility that mono- or disaccharides could inhibit the binding of toxin A to the Y antigen was investigated at 37°C by the ELISA procedure described above, except that the Immulon II plates were coated with 2 μg of Y antigen-HSA per ml. One microgram of toxin A was added to each well either alone or with 1 mg of carbohydrate. See results for the sugars used in this experiment.

Latex agglutination. In order to confirm the reaction of toxin A with carbohydrates that contain the X antigen, we assayed the binding of toxin A to chemically synthesized carbohydrates linked to latex beads (Chembiomed Ltd., Edmonton, Alberta, Canada). The oligosaccharide-latex reagent was blocked with 0.5% casein for 4 h. Five microliters of latex beads was added to either 10 μl of toxin A (100 μg/ml) or 10 μl of TBS (100 mM Tris, 150 mM NaCl [pH 7.0]) on a glass plate. This was incubated on ice for 2 min, and then 10 μl of a monoclonal antibody to toxin A (i.e., PCG-4 at 1 mg/ml [26]) was added to the mixtures. The plate was rocked for 3 min and then observed for agglutination.

Toxin A isolation. Isolation of toxin A from all other *C. difficile* antigens was attempted using the basic protocol developed for the affinity purification of toxin A on immobilized Galα1-3Galβ1-4GlcNAc (23); however, the X antigen was used in place of Galα1-3Galβ1-4GlcNAc. Culture filtrate was concentrated fivefold on a MiniCon concentrator (Amicon, Danvers, Mass.), and then 25 μl of this was mixed with a 20-μl pellet of latex beads containing the X antigen. This was incubated on ice for 10 min, and then the latex beads were pelleted by centrifugation. The supernatant was saved, and the pellet was washed three times with ice-cold TBS. The latex beads were then warmed to 37°C in 25 μl of TBS and then pelleted by centrifugation. Each step of the isolation was monitored using IEP with 5 μl of sample in the first dimension and 100 μl of antiserum to culture filtrate of *C. difficile* in the second dimension.

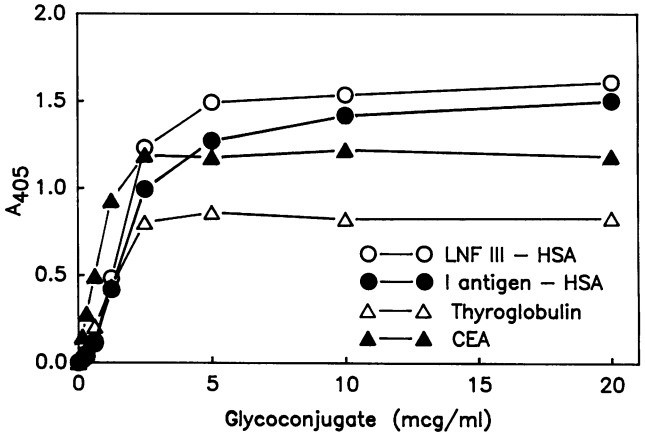

FIG. 1. ELISA of toxin A binding to glycoconjugates at 4°C. CEA and LNF III-HSA contain the X antigen, and thyroglobulin contains Galα1-3Galβ1-4GlcNAc. The assay was developed for 90 min at room temperature before the A_{405} was recorded. The A_{405} for toxin incubated with the glycoproteins at 37°C was insignificant. See Table 1 for carbohydrates that did not bind toxin A, as determined by IEP and ELISA.

RESULTS

Examination of molecular models suggested to us that the X antigen (Galβ1-4[Fucα1-3]GlcNAc) was conformationally similar to the previously identified receptor Galα1-3Galβ1-4GlcNAc. Polysaccharides that contain the X antigen are present on the naturally occurring glycoproteins CEA and the secretory component of human antibodies (4, 30). Mixing these proteins with toxin A caused an alteration in the migration of toxin A in an electric field; this was most easily observed in IEP. We then confirmed by ELISA that toxin A was binding to these two glycoproteins. This assay showed that toxin A was binding at 4°C but not at 37°C (Fig. 1). Similar results were obtained with bovine thyroglobulin, which contains the previously described trisaccharide receptor (Galα1-3Galβ1-4GlcNAc).

Although the binding of toxin A to these proteins suggested that the toxin was binding to the X antigen, there was a possibility that toxin A was binding to other carbohydrate structures shared by these two glycoproteins (e.g., BiAO, see Table 1 for structure; 4, 30). The conformation of the trisaccharide that defines the X antigen is part of the pentasaccharide LNF III. Therefore, we tested the binding of toxin A to the purified pentasaccharide which had been conjugated to HSA. The LNF III-HSA conjugate also altered the migration of toxin A in IEP (data not shown) and bound toxin A in an ELISA (Fig. 1); the BiAO conjugate did

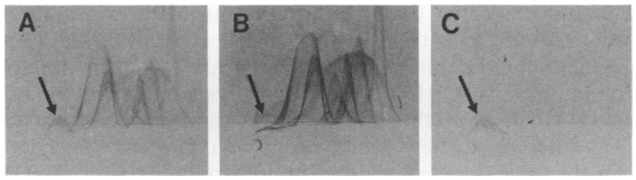

FIG. 2. IEP showing the stages of isolation of toxin A, using immobilized X antigen. Arrows mark the position of toxin A. The second dimension of each plate contains antisera to culture filtrate of *C. difficile*. (A) Culture filtrate of *C. difficile* before exposure to the immobilized X antigen. (B) Culture filtrate of *C. difficile* after exposure to the immobilized X antigen at 4°C; only toxin A was removed from the culture filtrate. (C) Eluant obtained by warming the immobilized X antigen that had bound toxin A; only toxin A was detected.

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

LNF III contains two carbohydrates more than the X antigen, which is defined by a trisaccharide. To determine if toxin A would bind to the X antigen alone, we tested for agglutination of latex beads to which this trisaccharide had been attached. Toxin A agglutinated these coated beads, and the beads specifically removed the toxin from culture filtrate at 4°C. Warming the beads to 37°C eluted the purified toxin (Fig. 2).

To further define the minimal carbohydrate structure that bound toxin A, we assayed the binding of toxin A to the i antigen conjugated to HSA. This tetrasaccharide is similar to LNF III but lacks the α1-3-linked fucose. As has been previously reported (6), we found that the i antigen did not bind toxin A (Table 1). Unexpectedly, we found that toxin A did bind to the branched form of this antigen, which is called the I antigen (Fig. 1). The I antigen contains two type 2 core structures (Galβ1-4GlcNAc) per oligosaccharide (Table 1). The toxin did not bind to any of the oligosaccharides we tested that contain the similar type 1 core structure (Galβ1-3GlcNAc [Table 1]).

We then tested the Y antigen, which is similar to the X antigen except that it contains an additional α1-2-linked fucose. Y antigen conjugated to HSA altered the migration of toxin A in IEP (Fig. 3), and in an ELISA toxin A bound to this antigen in a temperature-dependent manner (Fig. 4). Over 10 times more toxin was bound to the Y antigen than to LNF III at 4°C, and significant amounts of toxin also were bound at 37°C, whereas we could not detect binding of toxin to LNF III or I antigen at 37°C.

Binding of proteins to complex oligosaccharides sometimes can be inhibited by simple sugars or by disaccharides; therefore, we tested a number of these for the ability to inhibit the binding of toxin A to the Y antigen at 37°C. Of the sugars tested (glucose, galactose, *N*-acetylglucosamine, *N*-

TABLE 1. Oligosaccharide-HSA glycoconjugates tested for binding of toxin A

Name[a]	Trivial name(s)[b]	Structure[c]	Binding of toxin A[d]
LNF I	Le^d	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc) 2 Fucα1′	No
LNF II	Le^a	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc) 4 Fucα1′	No
LND I	Le^b	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc) 2 4 Fucα1′ Fucα1′	No
LNF III	X Le^x	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc) 3 Fucα1′	Yes
Le^y	Y	Galβ1-4GlcNAcβ1 2 3 Fucα1′ Fucα1′	Yes
LNnT	i	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)	No
LNnH	I	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc) 6 Galβ1-4GlcNAcβ1′	Yes
BiAO		Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4(GlcNAc) 6 Galβ1-4GlcNAcβ1-2Manα1′	No

[a] The names used in this table are the conventional designations used for these carbohydrates.

[b] The X and Y antigens are also referred to as the Lewis X (Le^x) and Lewis Y (Le^y) antigens, respectively. We use the designation X and Y because theses antigens are not actually members of the Lewis blood group (43). The X antigen is also designated by several other names, including My-1 (19), VEP8- and VEP9-antigen (15), and stage-specific embryonic antigen 1 (14).

[c] Parentheses around a carbohydrate indicate that the carbohydrate was chemically modified during the linking of the oligosaccharide to HSA.

[d] Binding of toxin A to oligosaccharides was determined by IEP and by ELISA.

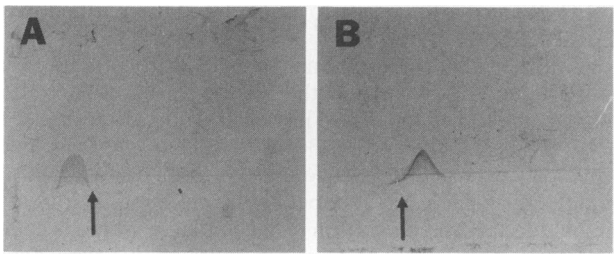

FIG. 3. IEP of toxin A (A) or toxin A mixed with Y antigen-HSA (B). Arrows indicate the position of the leading edge of toxin A without the Y antigen. The Y antigen-HSA glycoconjugate caused an increased migration of toxin A. This was also observed with either I or X antigen glycoconjugates, as well as when CEA or secretory component was mixed with toxin A. Other carbohydrates conjugated to HSA (Table 1) did not alter the migration of toxin A.

acetylgalactosamine, mannose, fucose, sucrose, fructose, xylose, mannitol, lactose, *N*-acetyllactosamine, melibiose, arabinose, stachyose, and 2'-fucosyllactose), only *N*-acetyllactosamine (Galβ1-4GlcNAc) had any effect on binding. This compound, which is similar to the type 2 core, inhibited binding by over 20%.

DISCUSSION

All of the carbohydrates that bound toxin A (I, X, and Y antigens and Galα1-3Galβ1-4GlcNAc) contain the type 2 core (Galβ1-4GlcNAc). The β1-4-linkage of the type 2 core produces oligosaccharides with a conformation that is very different from that of oligosaccharides with the β1-3-linkage present in the type 1 core (Galβ1-3GlcNAc; 24). This suggests that the binding of toxin A to the carbohydrates was dependent on the conformation of the type 2 core. Supporting this belief is our observation that *N*-acetyllactosamine (Galβ1-4GlcNAc) inhibited the binding of toxin A to the Y antigen, whereas the monosaccharides present in the type 2 core did not affect the binding. The specificity of toxin A for the type 2 core is further indicated by the observation that lactose did not inhibit the binding of toxin A. Lactose has a conformation that is identical to that of *N*-acetyllactosamine, except that lactose does not contain an *N*-acetyl amine (24).

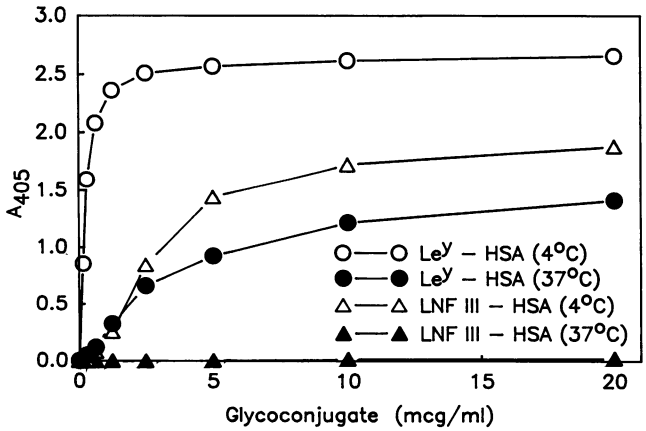

FIG. 4. ELISA of toxin A binding to either LNF III, which contains the X antigen, or Y antigen conjugated to HSA. The assays were performed in parallel at 4 and 37°C and were developed for 90 min at room temperature before the A_{405} was recorded.

This indicates that toxin A requires the *N*-acetyl amine in order to bind to carbohydrates. Thus, Galβ1-4GlcNAc appears to be the minimum carbohydrate structure that is bound by toxin A. However, the i antigen and BiAO did not bind toxin A, even though they also contain the type 2 core.

The difference between the oligosaccharides which contain the type 2 core and that bound toxin A (I, X, and Y antigen) and the ones that did not bind toxin A (i antigen and BiAO) is that the former contain a branch either on or immediately adjacent to the type 2 core. These branches sterically hinder changes in the conformation of the core; this may hold the carbohydrate in a conformation that favors the binding of toxin A. Conversely, the conformations of the type 2 core in nonbranched oligosaccharides may be too dynamic to bind toxin A. If this postulate is correct, it raises the question of how the disaccharide *N*-acetyllactosamine could inhibit the binding of toxin A. The answer may be that the molar concentration of *N*-acetyllactosamine used to inhibit the binding of toxin was at least 10,000-fold more than the concentration of either i antigen or BiAO used to assay the binding of the toxin. With such a high concentration of *N*-acetyllactosamine, it is possible that a small percentage of the molecules were in the correct conformation to bind to toxin A, thereby inhibiting the binding of toxin A to the Y antigen. Therefore, we believe that for toxin A to bind to a carbohydrate, the carbohydrate must contain the type 2 core.

Toxin A contains multiple binding sites for the carbohydrate ligands (8, 33). Therefore, a molecule of toxin A can bind more ligands as the number of ligands increases. Therefore, increasing the density of the ligands will increase the avidity of toxin A for the ligand because the avidity is determined by the summation of the affinities of the individual binding sites that are actually bound to the ligand. One way to increase the density of a carbohydrate ligand is to increase the number of branches on an oligosaccharide that contain the carbohydrate structure. Initially this appeared to explain why the branched I antigen, which contains two copies of the type 2 core, bound toxin A, whereas the linear i antigen, which contains only one copy of the type 2 core, did not bind the toxin. However, BiAO did not bind toxin A, and this carbohydrate also contains two copies of the type 2 core. Therefore, a high density of the type 2 core is not enough to bind toxin A. Instead, a receptor for toxin A apparently also must be branched immediately on or adjacent to the type 2 core.

The I, X, and Y antigens are present on human intestinal epithelium (10, 18, 29, 36). The binding of toxin A to the I, X, and Y antigens suggests to us that these carbohydrates may be receptors for toxin A in humans. Supporting this postulate is our observation that toxin A binds to the human glycoproteins CEA and the secretory component of antibodies; both of these glycoproteins contain the X antigen (4, 30) and are present on human intestinal epithelial cells. Toxin A has the greatest affinity for the Y antigen, but the I and X antigens also may function as receptors even though we could not detect binding to the I and X antigens at 37°C. After all, toxin A binds to the I antigen, X antigen, and Galα1-3Galβ1-4GlcNAc equally and toxin A appears to use Galα1-3Galβ1-4GlcNAc as a receptor on hamster intestinal mucosa (22).

Human granulocytes express large amounts of the X antigen (15, 19, 38), which indicates that toxin A may be targeted to these cells. This may be of significance, because large numbers of granulocytes occur in the pseudomembranes that are characteristic of pseudomembranous colitis.

Furthermore, toxin A causes granulocytes to release factors that can cause intestinal secretion and enteritis (31, 32). This had led some researchers to speculate that toxin A is not directly active on intestinal epithelium but that the factors released from the granulocytes cause the tissue damage and diarrhea associated with toxin A in vivo (41). We believe toxin A does act directly on intestinal epithelium, because toxin A is toxic to cell lines in vitro that are physiologically similar to intestinal epithelium (17). Even so, we agree that some of the symptoms associated with pseudomembranous colitis may be due to the factors released by the granulocytes. These factors may add to the tissue damage associated with toxin A, thus increasing the severity of the disease.

Most human infants apparently are insensitive to toxin A, since they can be colonized by toxigenic *C. difficile* with no ill effects (2, 7, 34). Previously, this laboratory proposed that the insensitivity of infants to toxin A may be due to either the lack of a receptor for toxin A or a low density of the receptor (5, 22). However, human infants express the Y antigen on their intestinal epithelium (20, 21) and the Y antigen binds toxin A at 37°C. Therefore, our data conflict with the postulate that infants are insensitive to toxin A because they lack a receptor for the toxin.

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

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 15749 from the National Institute of Allergy and Infectious Diseases and by state support grant 2124520 from the Commonwealth of Virginia.

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