Carbohydrate-Binding Specificity of the *Escherichia coli* Cytolethal Distending Toxin CdtA-II and CdtC-II Subunits

Leslie A. McSweeney and Lawrence A. Dreyfus*

Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri—Kansas City, Kansas City, Missouri

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Intoxication by cytolethal distending toxin depends on assembly of CdtB, the active A component of this AB toxin, with the cell surface-binding (B) component, composed of the CdtA-CdtC heterodimer, to form the active holotoxin. Here we examine the cell surface binding properties of *Escherichia coli*-derived CdtA-II (CdtA-II_{Ec}) and CdtC-II_{Ec}, and their capacity to provide a binding platform for CdtB-II_{Ec}. Using a flow cytometry-based binding assay, we demonstrate that CdtB-II_{Ec} binds to the HeLa cell surface in a CdtA-II_{Ec} and CdtC-II_{Ec}-dependent manner and that CdtA-IIEc and CdtC-IIEc compete for the same structure on the HeLa cell surface. Preincubation of cells with glycoproteins (thryoglobulin and fetuin), but not simple sugars, blocks surface binding of CdtA-IIEc and CdtC-IIEc. Moreover, CdtA-IIEc and CdtC-IIEc bind immobilized fetuin and thyroglobulin as well as fucose and to a lesser degree N-acetylgalactosamine and N-acetylglucosamine. Removal of N- but not O-linked carbohydrates from fetuin and thyroglobulin prevents binding of CdtA-IIEc and CdtC-IIEc to these glycoproteins. In addition, removal of N- but not O-linked surface sugar attachments prevents CDT-II_{Ec} intoxication. To characterize the cell surface ligand recognized by CdtA-IIEc and CdtC-IIEc, lectins having various carbohydrate specificities were used to block CDT activity and the cell surface binding of CdtA-II_{Ec} and CdtC-II_{Ec}. Pretreatment of cells with AAA, SNA-I, STA, UEA-I, GNA, and NPA partially or completely blocked CDT activity. AAA, EEA, and UEA-I lectins, all having specificity for fucose, blocked surface binding of CdtA-II_{Ec} and CdtC-II_{Ec}. Together, our data indicate that CdtA-IIEc and CdtC-IIEc bind an N-linked fucose-containing structure on HeLa cells.

Cytolethal distending toxin (CDT) is a heterotrimeric protein toxin produced by several pathogenic bacteria. CDT activity is dependent on the expression of three closely linked genes, *cdtA*, *cdtB*, and *cdtC*, encoding proteins with molecular masses of approximately 27, 29, and 20 kDa, respectively (1, 9, 19, 23, 25, 28). Several reports show that the CDT holotoxin is composed of all three proteins bound together to form a tripartite complex (9, 13, 15, 22, 24). Considerable evidence supports the notion that CdtB is the biologically active subunit. The direct introduction or expression of CdtB in mammalian cells results in all the cytotoxic effects associated with the CDT holotoxin (7, 10, 12). The CdtB subunit is a homolog of ricin B chain-like lectin molecules that associate to form a trimeric toxin in which CdtB is the active A component and CdtA and CdtC comprise the B (binding) component. Recent X-ray diffraction and three-dimensional structure modeling of the *H. ducreyi* CDT holotoxin suggests that CdtA_{Ha} and CdtC_{Ha} are ricin B chain-like lectin molecules that associate to form a scaffold for CdtB{\textsubscript{{Ha}}} association and a binding domain for the cell surface (17).

This study was originated to examine the role that CdtA-IIEc and CdtC-IIEc play in CDT binding and intoxication and to characterize the interaction of these subunits with the cell surface. We report here that cell surface carbohydrates play a key role in CDT subunit binding and subsequent intoxication.

* Corresponding author. Mailing address: Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri—Kansas City, Kansas City, MO 64110. Phone: (816) 235-2590. Fax: (816) 235-5158. E-mail: dreyfusl@umkc.edu.
Our findings indicate CdtA-II_{Ec} and CdtC_{II} are carbohydrate-binding proteins that bind N-linked carbohydrate moieties on the cell surface and provide a scaffold for CdtB-II_{Ec} binding. The characteristics of the putative CDT receptor are discussed.

MATERIALS AND METHODS

CDT nomenclature. General reference to CDT or the CDT subunits CdtA, CdtB, and CdtC will be as written. Specific reference to a particular CDT will include a subscript designating the bacterial species of origin as follows: CDT_{Aa}, CDT_{Bb}, A. actinomycetemcomitans; CDT_{Cf}, Campylobacter jejuni; CDT_{Tg}, E. coli, with sub-varieties CdtA_{IEc}, CdtB_{IEc}, and CdtC_{IEc}; and CDT_{Hd}, H. ducreyi.

Bacterial strains, plasmids, and culture conditions. The previously described plasmid pG3 containing the CDT_{Hd} operon (7) was used in the expression of CDT_{Hd}. Holotoxin as described below. E. coli XL1 Blue (Stratagen, La Jolla, Calif.) was used for general cloning experiments and plasmid isolation. E. coli BL21 (DE3) (Invitrogen, Carlsbad, Calif.) was used for expression of CdtA-II_{Ec}.

E. coli TOP10 (Invitrogen) was used to express CdtB-II_{Ec}-His6 and CdtC-II_{Ec}. The arabinose-inducible expression vector pBAD/HisB containing the E. coli cdtB-II gene (pBAD/EcCd-t-II) was used as the source of His-tagged CdtB-II_{Ec} (His6-CdtB-II_{Ec}) expression vector pET16b containing the E. coli cdtB-II gene was used as the source for CdtB-II_{Ec}. Bacterial strains were grown on L agar plates or in L broth at 37°C containing the following antibiotics and chemicals when appropriate: carbenicillin (100 μg/ml), arabinose (0.02%), and IPTG (isopropyl-β-D-thiogalactopyranoside; 0.5 mM). HeLa cells (American Type Culture Collection, Manassas, Va.) were maintained in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal calf serum, 1 mg of sodium citrate per ml, 0.3% NP-40, and 20 μg/ml propidium iodide per ml, 100 mM of sodium dodecyl sulfate (SDS) loading buffer, and 25% glycerol.

Purification of CDT subunits. The expression and purification of CdtB-II_{Ec} and CdtC-II_{Ec} were performed as described by Elwell et al. (6). The expression and purification of CdtA-II_{Ec} and CdtC-II_{Ec} were performed as described previously by McSweeney and Dreyfus (16).

CDT activity and cell cycle distribution analysis. Unless specified, the CDT holotoxin used in all experiments was a polysynthetic B extract of the periplasmic contents of E. coli XL1 Blue (pG3) (7). Holotoxin activity was assessed by DNA content-based cell cycle distribution analysis of CDT-treated HeLa cells as determined by flow cytometry. An aliquot of CDT was added to 5 × 10^5 HeLa cells in 100-mm-diameter culture dishes containing 5 ml of complete medium 24 h before assay. The amount of CDT required to cause a 50% block in the cell cycle after 24 h of incubation was designated 1 effective dose (ED). In most experiments, cells were treated with 3 EDs of CDT. This amount of toxin consistently blocked >95% of the cell population at the G2/M transition point after 24 h. After CDT treatment, HeLa cells were washed in phosphate-buffered saline (PBS) and removed from the culture dishes by treatment with trypsin. Cells were washed in PBS, fixed in 70% ethanol for 1 h on ice, and following removal of ethanol by washing with PBS, the cells were stained with propidium iodide (50 μg of propidium iodide per ml, 1 mg of sodium citrate per ml, 0.3% NP-40, and 20 μg/ml RNase per ml) for 1 h at room temperature. Cellular fluorescence was analyzed by flow cytometry with a FACSCalibur cytometer (Becton Dickinson). Background was set with a cell suspension of approximately 10^6 cells per ml that were incubated with forward- and side-scatter emission, was analyzed for fluorescence intensity on the fluorescein isothiocyanate emission channel of a FACSCalibur flow cytometer (BD Biosciences). Background was set with a cell suspension of approximately 10^6 cells per ml that were incubated with primary and secondary antibody but without the addition of CDT subunits. Gain and voltage settings were adjusted to position the background (control) population within in the first decade of fluorescence emission. For each experiment, more than 10^5 cells were recorded.

Carbohydrate- and lectin-mediated inhibition of CDT activity. Fucose, lactose, ovalbumin, mannos, N-acetylglactosamine (GalNAc), N-acetylgalactosamine (GalNAc), fetuin, asialofetuin, bovine submaxillary mucin (BSM), α1-3-glycoprotein, thyroglobulin, and transferrin receptor were purchased from Sigma Aldridge. The following lectins were purchased from EY Laboratories (San Mateo, Calif.): EEA, ECA, AAA, SNA-I, STA, UEA-I, GNA, NPA, ConA, PNA, MPA, and SBA. The lectins were used at 1:100 in PBS (Table 2). For carbohydrate inhibition experiments, 3 EDs of native CDT holotoxin was combined with various concentrations of sugars or glycoproteins (dissolved in PBS) in a total volume of 1 ml. After 1 h of incubation at room temperature, the holotoxin-sugar or holotoxin-glycoprotein mixtures were added to 2 × 10^5 HeLa cells in six-well plates and incubated at 37°C for 20 min. The samples were washed twice with 5 ml of PBS to remove unbound toxin. Fresh DMEM was added to the cells that were then incubated for 24 h at 37°C in atmosphere containing 5% CO2. Cell cycle distribution analysis was performed as described above.

For lectin inhibition experiments, individual lectins (100 μg) were added to 2 × 10^5 HeLa cells in six-well plates and incubated for 1 h at room temperature. Unbound lectins were washed away with two 5-ml PBS washes. Following removal of unbound lectins, 3 EDs of CDT holotoxin were added to the lectin-treated cells, which were further incubated for 20 min at 37°C in atmosphere containing 5% CO2. The treated cells were washed twice with PBS to remove unbound toxin and suspended in 5 ml of complete DMEM followed by plating in 100-mm-diameter culture dishes. After 24 h of incubation at 37°C in atmosphere containing 5% CO2, cell cycle distribution analysis was performed as described above.

Binding of CdtA-II_{Ec} and CdtC-II_{Ec} to immobilized carbohydrates and glycoproteins. Agarose beads matrices coupled with lactose, mannos, fucose, GalNAc, GlcNAc, fetuin, thyroglobulin, and BSM were obtained from EY Laboratories. Binding assays were performed in PBS with 25 μg of either CdtA-II_{Ec} or CdtC-II_{Ec} and 100 μg of the carbohydrate gel in a final volume of 300 μl. Samples were mixed on a tube rotator at 4°C for 1 h. Following incubation, the tubes were centrifuged at 4,000 × g for 5 min at 4°C. The supernatant fraction was removed, and the pellets were washed twice with 1 ml of ice-cold PBS. The pellets were suspended in 300 μl of sodium dodecyl sulfate (SDS) loading buffer and boiled, and aliquots were loaded on a 12% polyacrylamide gel and separated by SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting, blocked with 5% skim milk, and incubated for 2 h with anti-CdtA-II_{Ec} antibody, anti-CdtB-II_{Ec} antibody, and anti-CdtC-II_{Ec} antibody. Following incubation and subse-
RESULTS

Binding of CDT subunits to HeLa cells. Several lines of evidence implicate CdtB as the biologically active CDT subunit. CdtB alone, however, is not cytotoxic unless introduced into the cell by microinjection, electroporation, or expression from a transgene construct. The normal route for triggering the cytolethal activity of CDT requires the presence of all three CDT subunits. Recent reports indicate that CdtA and CdtC are the binding subunits of the CDT holotoxin (5, 14, 16). These data are supported by structural analysis of the CDT-Hd holotoxin, indicating that CdtA-Hd and CdtC-Hd possess structural folds that resemble those of the ricin B chain (17). To investigate the association of CDT subunits to HeLa cells, we developed a flow cytometry-based binding assay modeled after a previous study by Warner et al. (26). In this assay, live, unfixed HeLa cells are incubated with the CDT subunits, either alone or in combination. Cells are then incubated with the appropriate primary antibody followed by Alexa 488-conjugated secondary antibody and examined by flow cytometry. A subsequent increase in the surface fluorescence intensity above background is indicative of subunit binding. Cells incubated with CdtA-II Ec or CdtC-II Ec alone displayed an approximate 10-fold increase in fluorescence above the background (Fig. 1A and C). The background cell population was represented by control cells incubated in the presence of primary and secondary antibody but without CDT subunits (Fig. 1A and C). The levels of binding, or fluorescence intensity, observed with either CdtA-II Ec or CdtC-II Ec alone were assigned arbitrary scores of 3+, as shown to the right of Fig. 1A and C, respectively. In the case of the CdtA-II Ec binding, fluorescence was decreased when an equimolar amount of CdtC-II Ec was included in the reaction mixture (Fig. 1A). The same pattern was observed with the binding of CdtC-II Ec in the presence of CdtA-II Ec (Fig. 1C). When the binding of CdtA-II Ec was examined in the presence of CdtB-II Ec, the results were the same as those observed with CdtA-II Ec alone (binding pattern shown to the right of Fig. 1A). When the binding of CdtA-II Ec was examined in the presence of CdtB-II Ec and CdtC-II Ec, the pattern was identical to that of CdtA-II Ec in the presence of CdtC-II Ec (binding pattern to the right of Fig. 1A): that is to say, the fluorescence intensity signal for CdtA-II Ec was reduced to the 2+ level and CdtB-II Ec had no influence on the binding of CdtA-II Ec. These results were identical to those observed for the binding of CdtC-II Ec in the presence of CdtA-II Ec and/or CdtB-II Ec (binding pattern to the right of Fig. 1C). These observations suggested that CdtA-II Ec and CdtC-II Ec compete for the same binding site on HeLa cells. By comparison to the cell surface binding capacity of CdtA-II Ec and CdtC-II Ec, CdtB-II Ec bound poorly, if at all, to HeLa cells in the absence of CdtA-II Ec and CdtC-II Ec (Fig. 1B). The presence of either CdtA-II Ec or CdtC-II Ec alone had no influence on CdtB-II Ec binding; however, in the presence of both CdtA-II Ec and CdtC-II Ec subunits, the cell surface binding of CdtB-II Ec was equivalent to that of CdtA-II Ec and CdtC-II Ec (Fig. 1B).

Competitive binding of CdtA-II Ec and CdtC-II Ec subunits. To further explore the apparent competitive nature of CdtA-II Ec and CdtC-II Ec binding, we tested the binding of each subunit in the presence of increasing concentrations of the other subunit. The standard binding assay with either CdtA-II Ec or CdtC-II Ec added alone (1 nmol of subunit per 5 × 10^5 cells) was performed, and results identical to those shown in Fig. 1 were obtained (Fig. 2). The bold black histograms represent the binding of CdtA-II Ec and CdtC-II Ec individually, whereas, as before, the gray areas represent the background fluorescence of cells incubated with primary and secondary antibodies in the absence of CDT subunits (Fig. 2). In the case of CdtA-II Ec binding, the presence of one-, two-, and fourfold molar excess of CdtC-II Ec in the binding reaction (denoted by dashed gray histograms) steadily decreased the fluorescence intensity to the level of the background (Fig. 2). Identical results were obtained for the binding of CdtC-II Ec that is, increasing amounts of CdtA-II Ec in the reaction mixture re-
assess the specific function of CdtA-II Ec and CdtC-IIEc, PSI-molar excess of CdtC-IIEc, respectively. The bottom panel represents the binding of CdtC-II Ec to HeLa cells as detected with homologous primary and Alexa 488-conjugated secondary antibody, but no CdtA-IIEc. The 1X, 2X, and 4X peaks represent the binding of CdtA-IIEc in the presence of an equimolar concentration, twofold molar excess, and fourfold molar excess of CdtC-IIEc, respectively. The CdtA-IIEc panel represents the binding of CdtA-II Ec (1 nmol) to 5 × 10^5 HeLa cells (solid black tracing) compared to the background level consisting of HeLa cells incubated with homologous primary and Alexa 488-conjugated secondary antibody, but no CdtA-IIEc. The 1X, 2X, and 4X peaks represent the binding of CdtC-II Ec to HeLa cells as detected with homologous primary and Alexa 488-conjugated secondary antibody in the absence and presence of an equimolar concentration, twofold molar excess, and fourfold molar excess of CdtA-II Ec, respectively.

duced the binding of CdtC-II Ec. These data support the notion that CdtA-II Ec and CdtC-II Ec subunits compete for the same binding site or binding ligand on the HeLa cell surface.

Relationship of CdtA-II Ec and CdtC-II Ec to other known proteins. The binding patterns observed for CDT subunits suggested that CdtA-II Ec and CdtC-II Ec independently bind to the HeLa cell surface and that the binding of CdtB-II Ec is dependent upon both CdtA-II Ec and CdtC-II Ec. To further assess the specific function of CdtA-II Ec and CdtC-II Ec, PSI-BLAST analysis was performed on these protein sequences in an attempt to infer functionality based on sequence similarity. Results of a PSI-BLAST search suggested that CdtA-II Ec is related to a number of carbohydrate-binding proteins with either galactose or mannose specificity, including ricin B chain, abrin B chain, misteltoe lectin I B chain, and nigirin B chain. In addition, CdtA-II Ec displayed apparent similarity to carbohydrate-specific enzymes, including N-acetylgalactosaminyltransferase, β-mannase, xylanase, and α-galactosidase. All of the lectins and enzymes having similarity to CdtA-II Ec had final E-values of less than 1e^-24. These data inferred that CdtA-II Ec may function as a carbohydrate-binding protein, the target for which may be a cell surface structure. The result of a PSI-BLAST analysis of CdtC-II Ec was less conclusive than that with CdtA-II Ec. After the final PSI-BLAST iteration, proteins identified with significant similarity to CdtC-II Ec were the CdtC and CdtA proteins from other bacterial species. Early iterations of the CdtC-II Ec PSI-BLAST analysis resulted in the same list of lectins and carbohydrate-modifying enzymes present in the final search analysis for CdtA-II Ec. These observations suggest that CdtA-II Ec and CdtC-II Ec may have a similar structural fold and/or the same function.

Glycoproteins but not simple sugars inhibit CDT activity. Based on the PSI-BLAST results for CdtA-II Ec and CdtC-II Ec and X-ray diffraction analysis of CDT holotoxin (17), it is reasonable to speculate that CdtA-II Ec and CdtC-II Ec bind to carbohydrate-containing receptors on the target cell surface. In this set of experiments, we examined the effect of various simple sugars and glycoproteins on the toxicity of CDT. CDT was preincubated with individual simple sugars or glycoproteins before the addition to HeLa cells for a brief exposure. The ligands tested were lactose, mannose, fucose, GalNAc, GlcNAc, fetuin, asialofetuin, thyroglobulin, ovalbumin, α1-glycoprotein, and transferrin receptor. Following preincubation of CDT with blocking sugars and glycoproteins, the mixture was added to HeLa cells and toxicity was assessed by an examination of the cell cycle distribution of treated and untreated cells following 24 h of incubation (Materials and Methods). None of the simple sugars tested inhibited CDT action at doses as high as 1 mg/ml (Table 1). In contrast, thyroglobulin, BSM, and α1-glycoprotein inhibited the effects of CDT, with various half-maximal inhibitory concentrations ranging from 75 to 550 µg/ml (Table 1). Thyroglobulin, the strongest inhibitor of CDT activity, resulted in a 50% decrease in toxin activity at a dose of 75 µg/ml. BSM and α1-glycoprotein blocked CDT activity by 50% at concentrations of 350 and 550 µg/ml, respectively (Table 1). These data suggested that complex carbohydrates, such as those on glycoproteins, may inhibit CDT

### Table 1. Inhibition of CDT activity by simple sugars and glycoproteins

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<th>Inhibitor</th>
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<tr>
<td>Glycoproteins (µg/ml)</td>
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<tr>
<td>Thyroglobulin</td>
<td>75</td>
</tr>
<tr>
<td>Bovine submaxillary mucin</td>
<td>350</td>
</tr>
<tr>
<td>α1-Glycoprotein</td>
<td>550</td>
</tr>
<tr>
<td>Fetuin</td>
<td>&gt;1,000</td>
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<tr>
<td>Asialofetuin</td>
<td>&gt;1,000</td>
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<tr>
<td>Transferrin receptor</td>
<td>&gt;1,000</td>
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<tr>
<td>Sweets (µM)</td>
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</tr>
<tr>
<td>Fucose</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Mannose</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>GalNAc</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>&gt;1,000</td>
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<tr>
<td>Lactose</td>
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a Minimum concentration required to inhibit CDT activity by 50%.
CdtA-IIec and CdtC-IIec binding to carbohydrates. Based on the above results, we examined the binding of CdtA-IIec and CdtC-IIec to immobilized carbohydrates and glycoproteins. The binding of CdtA-IIec and CdtC-IIec to immobilized sugar and sugar-containing ligands was assessed with the following sugar- or glycoprotein-conjugated agarose beads: lactose, mannose, fucose, GalNAc, GlcNAc, fetuin, thyroglobulin, and BSM. CdtA-IIec and CdtC-IIec were incubated with the carbohydrate- or glycoprotein-conjugated matrices, which were subsequently washed free of unbound CDT subunits. The gel matrices were then prepared for SDS-PAGE analysis, and CDT subunit binding was assessed by Western blot analysis. CdtA-IIec and CdtC-IIec bound the same set of ligands, including thyroglobulin, fetuin, and fucose (Fig. 3A). To a lesser extent, CdtA-IIec and CdtC-IIec also bound to GalNAc- and GlcNAc-agarose (Fig. 3A). Binding of CdtA-IIec and CdtC-IIec to the unmodified agarose matrix was negligible, as was the binding of these subunits to mannose and lactose (Fig. 3A).

Fetuin and thyroglobulin are glycoproteins containing both N- and O-linked complex carbohydrates (21, 27). In addition, the carbohydrate moieties on these glycoproteins may or may not contain terminal sialic acid residues (21, 27). In an attempt to identify the linkage specificity of the carbohydrate moiety bound by CdtA-IIec and CdtC-IIec, fetuin- and thyroglobulin-coupled agarose matrices were subjected to various enzyme pretreatments prior to incubation with the CDT subunits. The binding of CdtA-IIec and CdtC-IIec to fetuin- and thyroglobulin-coupled matrices was completely abolished by pretreatment with PNGase F to remove N-linked sugars (Fig. 3B). Pretreatment of the glycoprotein-conjugated matrices with O-glycosidase or neuraminidase to remove O-linked sugars and sialic acid residues, respectively, had no effect on CdtA-IIec and CdtC-IIec binding (not shown).

N-linked sugars on the HeLa cell surface mediate CDT-dependent intoxication. A hallmark of CDT action is disruption of cell cycle progression. Histograms depicting DNA content of HeLa cells following various treatments are shown in Fig. 4. Cells in G0 or G1 occupy the left-most population of cells in each histogram representing a DNA content of 2N (Fig. 4). Cells at the G2/M transition of the cell cycle possess 4N DNA content and thus occupy the right-most peak of each histogram (Fig. 4). Compared to untreated HeLa cells (Fig. 4), cells treated with CDT accumulated at the G2/M transition of the cell cycle (Fig. 4). To determine whether an interaction of CDT with cell surface carbohydrates was necessary for cellular intoxication, we first removed or prevented the formation of N- and O-linked surface glycan structures on HeLa cells prior to treatment with CDT. Treatment of HeLa cells with benzyl-GalNAc, an inhibitor of O-linked oligosaccharide glycoprotein attachments, had no effect on subsequent intoxication (Fig. 4). Likewise, pretreatment of cells with O-glycosidase to remove O-linked carbohydrate structures had no effect on subsequent CDT intoxication (Fig. 4). In contrast to these observations, cells pretreated with tunicamycin to block the surface expression of N-glycosylation completely blocked the action of CDT (Fig. 4). Likewise, treatment of HeLa cells with PNGase F to remove surface-N-linked oligosaccharides also blocked subsequent CDT intoxication (Fig. 4). Together, these data suggest that HeLa cells bear N-linked surface carbohydrate moieties involved in CDT intoxication. Presumably, the N-linked sugar residues are responsible for CDT binding to the HeLa cell surface.

In preliminary experiments, we noted that pretreatment of HeLa cells with neuraminidase to remove exposed sialic acid residues did not appear to inhibit CDT action. Instead, the data were suggested that neuraminidase pretreatment may enhance CDT activity. To verify this, HeLa cells were either pretreated, or not, with neuraminidase to remove terminal sialic acid residues from surface glycoproteins and glycolipids and then treated with 1 ED of CDT. By definition, 1 ED of CDT is that amount which induces a 50% G2/M cell cycle block in 95% of the cells blocked at the G2/M transition (Fig. 4). Neuraminidase pretreatment followed by 1 ED of CDT, however, consistently resulted in >95% of the cells blocked at the G2/M interface (Fig. 4). Treatment with neuraminidase alone had no effect on the cell cycle distribution of HeLa cells (not shown).

Several carbohydrate-binding proteins identified by PSI-BLAST analysis as having similarity to CdtA-IIec and CdtC-IIec had binding specificities of galactose and/or mannose oligosaccharides. We examined the effect of CDT on HeLa cells following pretreatment with endo-β-N-acetylgalactosaminidase H (endoH) and α- or β-galactosidase for removal of mannose...
FIG. 4. Effect of the removal of N- and O-linked carbohydrates from the HeLa cell surface on subsequent intoxication by CDT. The ability of CDT (3 ED) to induce cell cycle arrest in HeLa cells was examined following various treatments. Cells were pretreated with various chemicals or enzymes as described in Materials and Methods and then intoxicated with CDT. Following 24 h of incubation, the cells were examined for cell cycle distribution analysis by DNA content-based flow cytometry. The data were analyzed for DNA content with ModFit based flow cytometry. The data were analyzed for DNA content with ModFit, and the histograms were then overlaid on each other for comparison. Each experiment was performed at least three times. The data shown are typical of each replicate experiment.

Effect of lectins on cell surface binding of CdtA-II-Ec and CdtC-II-Ec. If CdtA-II-Ec and/or CdtC-II-Ec is responsible for holotoxin binding to the cell surface, the inhibitory effect of certain lectins on CDT toxicity is likely the result of inhibition of CdtA-II-Ec and/or CdtC-II-Ec binding to the cell surface. Therefore, we investigated the capacity of various lectins to block the cell surface binding of CdtA-II-Ec and CdtC-II-Ec. HeLa cells were preincubated with the lectins that reduced or diminished CDT activity (AAA, SNA-I, STA, UEA-I, GNA, and NPA). Following preincubation with lectins, HeLa cells were incubated with CdtA-II-Ec or CdtA-II-Ec alone or with all three CDT subunits. The binding of individual CDT subunits was then assessed by flow cytometry. As with the data from previous binding experiments, the shaded histograms in each panel of Fig. 5 represent the background level of fluorescence obtained following incubation of HeLa cells with primary and secondary antibody without the addition of CDT subunits. A second control consisting of HeLa cells incubated with the various lectins followed by primary and secondary antibody was also performed. The results of these controls to assess the potential cross-reactivity between CDT subunit-specific antibody and the various lectins to be tested were identical to those with the standard background fluorescence controls (not shown). The bold black-traced histograms shifted to the right of background in each panel of Fig. 5 represent the binding of either CdtA-II-Ec (left panels) or CdtC-II-Ec (right panels) in the absence of lectin preincubation to HeLa cells and thus are identical to the results shown in Fig. 1. The light gray-traced histograms in each panel represent the fluorescence intensity yielded by subunit interaction with HeLa cells following preincubation with the various lectins shown to the left of each panel. Superimposition (or near superimposition) of the gray trace with the bold black trace, such as observed following preincubation with GNA, NPA, SNA, and STA lectins, indicated that lectin preincubation did not interfere with the capacity of HeLa cells to bind CdtA-II-Ec or CdtC-II-Ec. Lectin interference as observed with AAA, EEA, and UEA resulted in a leftward shift or downward shift in fluorescence intensity, indicating that the binding activity of CdtA-II-Ec or CdtC-II-Ec was blocked or inhibited by lectin preincubation. The consequences of lectin preincubation with HeLa cells were
TABLE 2. Inhibition of CDT activity by lectins

<table>
<thead>
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<th>Lectin (source)</th>
<th>Specificity</th>
<th>Inhibition of:</th>
<th>CdtA/C binding</th>
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<tbody>
<tr>
<td>EEA (spindle)</td>
<td>α-Gal[1,3](α-fuc(1,2)]Gal</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>ECA (coral tree)</td>
<td>β-Gal(1,4)GlcNAc</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AAA (freshwater cell)</td>
<td>α-1-Fucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SNA-I (elderberry)</td>
<td>α-NeuNac(2,6)GlcNAc; lactose</td>
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<td>+</td>
</tr>
<tr>
<td>STA (potato)</td>
<td>β-GlcNAc(1,4)GlcNAc</td>
<td>+</td>
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<td>UEA-I (gorse)</td>
<td>α-1-Fucose</td>
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<td>GNA (snowdrop bulb)</td>
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<td>NPA (daffodil)</td>
<td>α-d-Mannose; α-glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ConA (jack bean)</td>
<td>α-Mannose; α-galactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CSA (Scotch broom)</td>
<td>GalNAc; galactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WGA (wheat germ)</td>
<td>GlcNAc; NeuNAc</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MPA (Osage orange)</td>
<td>GlcNAc; galactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SBA (soybean)</td>
<td>GalNAc</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*In each case, unless otherwise noted, the end letter “A” represents agglutinin obtained from the source abbreviated according to its Latin designation (except for SBA): EEA, Euonymus europaeus; ECA, Erythrina cristagalli; AAA, Anguilla anguilla; SNA, Sambucus nigra; STA, Solanum tuberosum; UEA, Ulex europaeus; GNA, Galanthus nivalis; NPA, Narcissus pseudonarcissus; ConA, concanavalin A from Canavalia enulisima; CSA, Cytisus scoparius; WGA, Triticum vulgare; MPA, Maclura pomifera; SBA, Glycine max.

a CDT activity was assessed as described in Materials and Methods. A score of + + indicated that activity was completely inhibited to the level of the control, which received no lectin pretreatment. A score of + indicated that activity was reduced consistently from >95% of cells blocked in G2/M to <50% of cells blocked in G2/M. Identical results were obtained in each of three experiments with each lectin tested.

b Inhibition of binding was as described in the legend to Fig. 5.

d The requirement for both CdtA-II_Ec and CdtC-II_Ec in CDT intoxication coupled with the lack of binding of CdtB-II_Ec to target cells in the absence of CdtA-II_Ec and CdtC-II_Ec is supported by the proposed model for the three-dimensional structure of the H. ducreyi holotoxin (17). The proposed structural model for the CdtT_Hd holotoxin suggests that the CdtA_Hd and CdtC_Hd subunits form a dimer with two functional faces (17). One CdtAC face binds the cell surface, while the other CdtA_Hd face binds CdtB_Hd. Of particular interest to this report is the proposed cell surface-binding domain of the CdtA_Hd-CdtC_Hd dimer. The CdtA_Hd domain of the proposed cell surface binding face formed by the CdtA_Hd mutant region contains a patch of aromatic residues highly conserved among CdtAs of all species of origin. Mutagenesis of four conserved residues (W91G, W98G, W100G, and Y102A) eliminated toxicity but preserved the ability of the CdtA_Hd deletion mutant to form a holotoxin structure with CdtB_Hd and CdtC_Hd (17).

The authors propose that this region defines the cell surface-binding of CdtT_Hd. It is interesting to note that these conserved residues are all contained within an in-frame 43-amino-acid C. jejuni CdtA deletion mutant prepared by Lee et al. (14). The CdtA_Cj deletion mutant still bound HeLa cells but was unable to participate in the formation of CDT holotoxin and thus was inactive. In addition, the CdtA_Cj deletion mutant competed with CDT holotoxin for cell surface binding. These authors suggested that residues contained within the 43-amino-acid deletion were involved in the association of CdtA_Cj and CdtC_Cj (14). The aromatic patch identified on the surface of CdtT_Hd by structural analysis fits a projected model for a carbohydrate-binding domain on a globular protein (20). The amino acids defining the binding activity of CdtA and CdtC, as well as the CdtA-Cdimer, although presently unknown, will undoubtedly lie within the surface area of the CdtA contact region as defined for the H. ducreyi CDT by Nesic and Stebbins (17).

In this report, we examined the binding of CdtA-II_Ec and CdtC-II_Ec to HeLa cells, using a flow cytometry-based binding
FIG. 5. Lectin pretreatment of HeLa cells blocks cell surface binding by CDT subunits. The capacity of lectin pretreatment of HeLa cells to block subsequent binding by CDT subunits was examined by flow cytometry as described in the legends to Fig. 1 and 2. Lectin pretreatments,
assay as a method to better define the binding activities of the CDT subunits. Our findings are consistent with the X-ray diffraction model for the CDT holotoxin that suggests carbohydrate-binding roles for CdtA-IIEc and CdtC-IIEc (17). Here we demonstrate for the first time functional lectin-like activity for both CdtA-IIEc and CdtC-IIEc. Both CDT subunits bound various immobilized carbohydrates, including fucose, GalNAc, and GlcNAc, to various degrees. We also examined the binding of CdtA-IIEc and CdtC-IIEc to fetuin and thyroglobulin, two model glycoproteins containing well-characterized carbohydrate linkages. Thyroglobulin contains 16 confirmed N-linked glycosylation sites, of which are linked to complex oligosaccharide units containing fucose, galactose, mannose, and glucosamine (27). Other confirmed N-linkage sites are coupled to a mixture of high mannose, galactose, and glucosamine. N-linked sugars on fetuin are highly fucosylated and carry the fucose- and galactose-containing Lewis X or asialo-Lewis X epitopes (21). CdtA-IIEc and CdtC-IIEc both bound efficiently to each of these glycoproteins. The increase in CDT activity for HeLa cells observed following treatment of cells with neuraminidase suggests that removal of terminal sialic acid residues exposes additional binding sites for CdtA-IIEc and CdtC-IIEc. Release of the N-linked sugars from thyroglobulin and fetuin completely abolished their binding capacity for CdtA-IIEc and CdtC-IIEc. However, O-linked sugars were apparently not involved in CDT subunit binding, since removal of these linkages from the model glycoproteins had no effect on CdtA-IIEc or CdtC-IIEc binding. In addition to these data, treatment of HeLa cells with PNGase F to remove N-linked glycan structures or tunicamycin to prevent N glycosylation completely inhibited subsequent CDT intoxication. Treatments to block, or remove, O-linked sugars from HeLa cells had no effect on subsequent toxicity, suggesting again that O-linked sugars are not involved in the association of CDT with target cells.

Lectins with a number of different specificities including fucose, galactose, mannosone GalNAc, and GlcNAc reduced or diminished subsequent CDT intoxication. These results were in contrast to the subunit blocking experiments, in which only three lectins, AAA, EEA, and UEA, blocked the binding of CdtA-IIEc and CdtC-IIEc to HeLa cells. This apparent discrepancy may indicate that lectins that block CDT activity, but do not block binding of individual CdtA-IIEc and CdtC-IIEc subunits, sterically interfere with holotoxin binding. The common ligand specificity of the three lectins that block individual subunits binding is fucose.

These data, coupled with the results of our lectin blocking experiments suggest that CdtA-IIEc and CdtC-IIEc bind N-linked fucose containing complex carbohydrates on the HeLa cell surface. A determination of the specific limits of the carbohydrate-binding specificity of CdtA-IIEc and CdtC-IIEc awaits verification by additional carbohydrate binding analyses. We are presently attempting to refine the carbohydrate specificity of the cell surface ligands bound by CdtA-IIEc and CdtC-IIEc and define the subunit residues responsible for the carbohydrate-binding activity described in this report.

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REFERENCES


