Involvement of Ganglioside GM3 in G₂/M Cell Cycle Arrest of Human Monocytic Cells Induced by *Actinobacillus actinomycetemcomitans* Cytolethal Distending Toxin

Koji Mise,¹ Sumio Akifusa,^{1*} Shinobu Watarai,² Toshihiro Ansai,¹ Tatsuji Nishihara,³ and Tadamichi Takehara¹

Departments of Preventive Dentistry¹ and Oral Microbiology,³ Kyushu Dental College, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, and Laboratory of Veterinary Immunology, Division of Veterinary Science, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Gakuen-cho 1-1, Sakai, Osaka 599-8531,² Japan

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Actinobacillus actinomycetemcomitans produces a toxin called cytolethal distending toxin (CDT), which causes host cell DNA damage leading to the induction of DNA damage checkpoint pathways. CDT consists of three subunits, CdtA, CdtB, and CdtC. CdtB is the active subunit of CDT and exerts its effect as a nuclease that damages nuclear DNA, triggering cell cycle arrest. In the present study, we confirmed that the only combination of toxin proteins causing cell cycle arrest was that of all three recombinant CDT (rCDT) protein subunits. Furthermore, in order for rCDT to demonstrate toxicity, it was necessary for CdtA and CdtC to access the cell before CdtB. The coexistence of CdtA and CdtC was necessary for these subunits to bind to the cell. Cells treated with the glucosylceramide synthesis inhibitor 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol showed resistance to the cytotoxicity induced by rCDT. Furthermore, LY-B cells, which are deficient in the biosynthesis of sphingolipid, also showed resistance to the cytotoxicity induced by rCDT. To evaluate the binding of each subunit for glucosylceramides, we performed thin-layer chromatography immunostaining. The results indicated that each subunit reacted with the glycosphingolipids GM1, GM2, GM3, Gb3, and Gb4. The rCDT mixture incubated with liposomes containing GM3 displayed partially reduced toxicity. These results indicate that GM3 can act as a CDT receptor.

Actinobacillus actinomycetemcomitans, a gram-negative coccobacillus, is associated with several human diseases (38, 46, 48). These include endocarditis, meningitis, and osteomyelitis (35, 40), as well as various forms of periodontal disease: juvenile, early onset, refractory, and adult periodontitis (10, 12, 33, 48). Although the pathogenic mechanism by which this bacterial species acts to cause periodontal disease is not clear, this organism is able to produce a variety of virulence factors capable of facilitating the colonization, invasion, and destruction of the periodontal tissues (7, 24, 46). It also secretes a number of unusual proteins, including a leukocyte-specific leukotoxin with proapoptotic activity (17), a chaperonin 60 with osteolytic activity (15, 36), and various inhibitors of cell cycle progression (27, 43, 44). Several studies suggest that impaired host defense mechanisms may contribute to the infectious disease associated with *A. actinomycetemcomitans* (34, 38, 46).

One recently discovered class of bacterial toxins, called cytolethal distending toxin (CDT), has been isolated from a range of pathogenic bacteria, including *Escherichia coli*, *Campylobacter* species, *Shigella* species, *Haemophilus ducreyi*, *Helicobacter* species, and *A. actinomycetemcomitans* (3, 28, 30, 32, 37, 45, 47). CDT is a three-component toxin consisting of CdtA, CdtB, and CdtC (30, 31). This toxin is able to induce growth arrest at the G₂/M phase in epithelial cells and the

apoptosis of cultured B-cell lines in an ataxia telangiectasia-mutated kinase-dependent manner (5). Recently, it has been reported that CdtB exhibits limited amino acid sequence similarity with the DNase I family of proteins and that purified CdtB exhibits very low but measurable DNase activity (11, 19, 21). The cellular responses to DNA damage lead to the characteristic G₂/M cell cycle arrest, cellular distension, and nuclear enlargement observed in intoxicated cells (29). Although cytotoxicity caused by exogenously administered toxin requires all three CDT subunits, CdtB alone can reproduce all of the effects of CDT, provided that it is administered in very small quantities directly into the cytosol of target cells by either microinjection or transient expression (19). The mechanism by which CDT enters cells is not completely understood. However, the results of experiments using a number of pharmacological inhibitors have suggested that the toxin enters cells by means of receptor-mediated endocytosis, traveling deep into the endocytic pathway (4) before CdtB is delivered first into the cytosol and then into the nucleus of the target cell. While progress in understanding the function of CdtB has been made, there is still little known about the precise roles of CdtA and CdtC in CDT function. Recent evidence indicates that all three CDT subunits are present in the CDT holotoxin, probably in a 1:1:1 stoichiometry (20).

There are many protein toxins that bind to glycosphingolipids (GSLs) on the cell surface prior to invading host cells. The best known of these is the cholera toxin, an enterotoxin produced by *Vibrio cholerae* for which the specific cell surface receptor has been identified as ganglioside GM1. Cholera

* Corresponding author. Mailing address: Department of Preventive Dentistry, Kyushu Dental College, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan. Phone: 81 0935821131, ext. 2103. Fax: 81 0935917736. E-mail: akifusa@kyu-dent.ac.jp.

toxin consists of a pentameric B subunit that binds to GM1 and an A subunit with direct toxic activity. Other ganglioside-binding bacterial toxins include tetanus toxin (GD1b), botulinum toxin (GT1b and GQ1b), and delta toxin produced by *Clostridium perfringens* (GM2). Shiga toxin produced by *Shigella dysenteriae* and Vero toxin produced by enterohemorrhagic *Escherichia coli* bind to GSLs having an α -1,4 galabiose moiety in the sugar chain, such as Gb4 and Gb3 ceramide (2, 13, 16). Abundant evidence indicates that cholesterol plays a critical role in the endocytosis and transcytosis of these glycolipid-binding toxins via lipid rafts, which are highly enriched in cholesterol, GPI-linked proteins, and GSLs. CdtA shares some similarity in folding structure to the ricin B chain, which is involved in ricin binding and uptake (11, 20, 26). It is well known that the ricin B chain binds galactoside, but the cell surface component to which CdtA binds it is not yet known.

In the present study, we used purified recombinant *A. actinomycetemcomitans* CDT subunits to determine the activity of reconstituted holotoxin and investigated the binding of the holotoxin or individual subunits to U937 cells. In addition, we suggest that CDT binds to GSLs and identified GSLs that are bound by CDT.

MATERIALS AND METHODS

Cells and culture conditions. The human monocyte cell line U937 was grown in RPMI 1640 (GIBCO) containing L-glutamine, 10% fetal calf serum, streptomycin (100 μ g/ml), and penicillin (100 IU/ml) in an atmosphere containing 5% CO₂. For the depletion of sphingolipid, U937 cells were incubated for 10 days at 37°C in the presence of 8 μ M 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and then washed twice with phosphate-buffered saline (PBS) (18, 39). The serine-palmitoyl transferase-deficient cell line derived from CHO-K1, LY-B, and its complemented derivative, LY-B/cLCB1, were kind gifts from K. Hanada (9). Ham's F-12 medium supplemented with 10% newborn calf serum, streptomycin (100 μ g/ml), and penicillin (100 IU/ml) was used as the normal culture medium for LY-B and LY-B/cLCB1. Nutridoma medium (F-12 medium containing 1% Nutridoma-SP [Boehringer Mannheim] and gentamicin [10 μ g/ml]) and Nutridoma-BO medium (Nutridoma medium supplemented with 0.1% fetal calf serum and 10 μ M sodium oleate complexed with bovine serum albumin) were used as sphingolipid-deficient culture media. In this condition, the level of GM3 on the LY-B cells was approximately 30% of that on CHO-K1 and LY-B/cLCB1 (9).

Purification of recombinant proteins. *E. coli* transfected with the expression vector pET-28a containing the *cdtA*, *cdtB*, or *cdtC* gene was grown overnight in Luria-Bertani (LB) broth containing kanamycin (30 μ g/ml) and rifampin (200 μ g/ml), diluted 1:20 in fresh broth, and incubated for a further 3 h at 37°C. To stimulate gene expression, 1 mM isopropyl- β -D-thiogalactopyranoside was added and the culture was incubated for 6 h at 30°C. The cells were harvested by centrifugation at 5,000 $\times$ g for 30 min and then resuspended and lysed for 10 min in the protein extraction reagent B-PER (Pierce & Warriner Ltd., Cheshire, United Kingdom). As the expressed proteins were contained in inclusion bodies, purification was performed as described by the manufacturer of the B-PER reagent. Briefly, the lysates were centrifuged and the pellets were resuspended in the same volume of B-PER containing lysozyme (100 μ g/ml) and incubated for a further 5 min at room temperature. A 10-fold volume of B-PER diluted 1:10 was added to the lysates, and the inclusion bodies were collected by centrifugation at 15,000 $\times$ g for 20 min. After being washed twice in the same volume of diluted B-PER, the pellets were treated with 8 M urea in PBS, pH 7.5. The recombinant proteins were purified using Ni-nitrilotriacetic acid-agarose columns under denaturing conditions as specified by the manufacturer (QIAGEN Ltd.), except that after lysates were loaded onto the column, an additional column wash consisting of 2.5 mg of polymyxin B per ml of wash buffer was performed to remove lipopolysaccharide. The refolding of the denatured proteins was performed as described previously (1).

Analysis of cell cycle inhibition. To measure cell cycle arrest induced by the CDT proteins, U937 cells were seeded at a density of 1×10^5 cells/ml in a 24-well plate and incubated with 250 ng/ml of recombinant CDT (rCDT) protein/ml for 24 h. After incubation, the U937 cells were collected and stained in the dark at

4°C for 20 min with propidium iodide (10 μ g/ml) in PBS containing RNase (1 mg/ml). For each well, 1×10^4 cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter). Cell cycle analysis was performed using MultiCycle for Windows. All experiments examining cell cycle inhibition were repeated a minimum of three times and gave consistent results.

Extraction of nuclear material and Western blotting. The cells were collected after incubation for 24 h with CdtA, CdtB, and CdtC. After washing twice with PBS, the cells were resuspended in nucleous buffer (10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 7.4], 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 20 μ M cytochalasin B, protease inhibitors [Complete]). The cell membrane was fractured on ice using a glass Dounce homogenizer. Nuclear material was collected by centrifugation at 800 $\times$ g for 10 min and lysed in sodium dodecyl sulfate electrophoresis sample buffer. The lysate was boiled for 10 min, separated on a sodium dodecyl sulfate–12.5% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with antihistidine monoclonal antibody (COVANCE). Blots were developed with an ELC Western blotting analysis kit (Amersham Pharmacia) using a peroxidase-labeled secondary antibody.

Binding assay. The rCDT proteins were labeled with the Alexa Fluor 488 protein labeling kit (Molecular Probes) according to the manufacturer's protocol. Briefly, 1 M bicarbonate was added to each CDT protein; the protein was put into a lightproof tube containing Alexa Fluor and stirred for 1 h at room temperature. Unbound Alexa Fluor was removed by dialysis in PBS. After labeling, 5 μ g of each Alexa-labeled protein was added to cells seeded at a density of 1×10^5 cells/ml in a 24-well plate. The cells were then incubated for 3 h at 37°C in the dark and collected after being washed twice with PBS. The labeled protein bound to cells was detected using a flow cytometer.

TLC and TLC immunostaining test. Thin-layer chromatography (TLC) was carried out on high-performance TLC plates (silica gel 60 F254; Merck, Darmstadt, Germany) with a solvent system of chloroform-methanol-water (65:35:8, vol/vol/vol). Neutral GSLs were visualized by spraying the plate with orcinol reagent (0.2% orcinol in 2 N H₂SO₄). The TLC immunostaining of GSLs on TLC was performed according to the method of Watarai et al. (41) with slight modifications. Briefly, GSLs were spotted on a high-performance TLC plate and developed with chloroform-methanol-water (65:35:8, vol/vol/vol). The dried plate was soaked for 1 min in a 0.02% solution of polyisobutylmethacrylate (Tokyo Kasei Kogyo, Tokyo, Japan) dissolved in hexane, allowed to air dry, and then blocked by incubation in PBS containing 1% bovine serum albumin (Wako) and 0.02% Na₂S₂O₃ at 37°C for 30 min. The plate was then rinsed five times with PBS containing 0.1% Tween 20 (washing buffer; Wako) and incubated with His-tagged rCDT protein (His-CdtA, His-CdtB, or His-CdtC) diluted to 50 μ g/ml in PBS overnight at 4°C. After this, the plate was washed five times with washing buffer and incubated with a 1:1,000 dilution of anti-His mouse monoclonal antibody (COVANCE) at room temperature for 3 h. After washing with washing buffer, the plate was reincubated with a 1:2,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G antiserum (Amersham) at room temperature for 2 h. As a final step, the plate was washed five times with washing buffer and incubated with peroxidase substrate solution, which consisted of 2 ml of 0.3% 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) in methanol and five volumes of 100 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl and 0.01% H₂O₂ for 10 min at room temperature. The plate was washed with water to stop the reaction.

Preparation of liposomes. Liposomes for the adsorption experiment were prepared using the following method. Distearoylphosphatidylcholine (7 μ mol), cholesterol (2 μ mol), and GSLs (GM1, GM2, GM3, Gb3, or Gb4), each dissolved in an organic solvent, were mixed in a molar ratio of 1:1:0.1 in a conical flask. The lipids were dried on a rotary evaporator and then placed under high vacuum in a desiccator for 30 min. After the addition of 500 μ l of PBS and incubation at 50°C for 3 min, the lipid film was dispersed by vigorous vortexing and the resulting liposome suspension was centrifuged at 16,000 $\times$ g for 20 min at 4°C. After centrifugation, the liposome pellet was used for the adsorption experiment (42).

Adsorption of CDT by GSL. We tested the ability of GSLs (GM1, GM2, GM3, Gb3, and Gb4) to remove purified rCDT from a suspension. Briefly, 0.5 μ g of each rCDT subunit (CdtA, CdtB, and CdtC) was incubated with GSL-containing liposomes or liposomes without GSL at 37°C for 60 min. The mixture was centrifuged at 16,000 $\times$ g for 20 min at 4°C. After centrifugation, the supernatant was tested for cytotoxic activity against U937 cells. The cells were collected after incubation for 24 h and used for cell cycle analysis as described above (42).

Confirmation of GM3 expression on U937 cells. U937 cells were incubated with a 1:500 dilution of anti-GM3 monoclonal antibody for 1 h at 4°C. After being washed twice with PBS, the cells were incubated with a 1:400 dilution of

FIG. 1. The effects of the CDT complex and each individual subunit on cultured U937 cells. U937 cells were incubated for 24 h in the presence of 250 ng/ml of each protein (A). U937 cells were incubated for 3 h in the presence of 5.0 μ g/ml of each of the two proteins CdtB and CdtC (B), CdtA and CdtC (C), or CdtA and CdtB (D). Cells were then washed twice with PBS and incubated in the presence of 5.0 μ g of the third protein, CdtA (B), CdtB (C), or CdtC (D), for 24 h. The cells were analyzed for cell cycle distribution by the method described previously. At least 10,000 cells were analyzed per sample. The black lines represent CDT, and the grey lines are the control. The results are representative of three experiments.

Alexa-labeled secondary antibody for 30 min at 4°C. The cells were then washed twice with PBS and detected using a flow cytometer.

RESULTS

CdtA, CdtB, and CdtC are required for CDT toxicity. Several lines of evidence indicate that CDT causes sensitive eukaryotic cells to become blocked in the G_2 phase. To determine the contribution of each rCDT subunit in generating biological activity, the effects of independently expressed and purified rCDT proteins on cell cycle progression in U937 cells were analyzed by flow cytometry. Although no CDT activity was observed in cells with one or two subunits (data not shown), when all three subunits were incubated with U937 cells, the DNA content analysis clearly indicated that CDT caused cells to become blocked in either G_2 or early M phase for 24 h (Fig. 1A). In control cultures of U937 cells exposed to medium alone, only 12% of cells were in the G_2 phase, with a 2N DNA content. The treatment of U937 cells with all three subunits resulted in 87% of the cells being at the peak of propidium iodide fluorescence, indicating cells in the G_2 /M phase.

Premixing of CdtA and CdtC is required for the activity of CDT. In order to examine the hypothesis that CdtA and CdtC function as heteromeric subunits that mediate the delivery of the active subunit CdtB into host cells, we determined the sequence in which the subunits access the cell surface. Either one or a combination of two subunits was added to U937 cells, and the cells were incubated for 3 h. The cells were then washed twice with PBS, and the missing subunit(s) was added. After 24 h, the cell cycle distribution was analyzed by flow cytometry. When CdtB was added after premixing CdtA and CdtC, the U937 cells showed clear signs of CDT intoxication,

FIG. 2. Flow cytometric analysis of U937 cells treated with Alexa-labeled CDT protein. U937 cells were exposed to 5 μ g/ml of CdtA* (A), CdtB* (B), CdtC* (C), CdtA* and CdtC (D), CdtA and CdtC* (E), CdtA* and CdtB (F), or CdtB and CdtC* (G) for 3 h. Asterisks indicate Alexa-labeled proteins. The cells were washed twice with PBS and analyzed by flow cytometry. The grey lines represent medium only, and the black lines represent Alexa-labeled protein. The results are representative of three experiments.

indicated by G_2 /M cell cycle arrest (Fig. 1C). About 90% of the cells were in the G_2 /M phase, similar to the result when the three subunits were added simultaneously. No CDT toxicity was demonstrated, however, when CdtC was added after CdtA and CdtB or when CdtA was added after CdtB and CdtC (Fig. 1B and D).

The coexistence of CdtA and CdtC is required for binding of these subunits to the cell. Our hypothesis was that CdtA and CdtC would bind to the cell and introduce CdtB into the host. In order to confirm whether CdtA and CdtC actually bound to the cell, we examined the interaction using Alexa-labeled protein. CdtA and CdtC did not bind to the cell independently or in combination with CdtB (Fig. 2A, C, F, and G), and bound only to the cell, CdtA and CdtC were coexistent (Fig. 2D and E).

Depletion of sphingolipids from the cell causes resistance to rCDT cytotoxicity. U937 cells obtained after 10 days of culture in medium containing 8 μ M PPMP, a glucosylceramide synthesis

FIG. 3. The effect of treatment with PPMP on the cytotoxicity of CDT. U937 cells were cultured for 10 days either in medium containing 8 μ M PPMP (B and D) or without PPMP (A and C). The cells were washed twice with PBS and incubated for 24 h either in the presence of 250 ng/ml of each protein (B and C) or without CDT (A and D). All results are representative of at least three experiments.

inhibitor causing reversible GSL depletion, were cultured for 24 h with 250 ng per ml of each rCDT protein. After incubation, the cells were collected and the cell cycle distribution was analyzed by flow cytometry. After PPMP treatment, the cells showed greater resistance to cell cycle arrest than did control cells. Only 36% of the cells treated with PPMP concentrated in the G_2/M phase (Fig. 3D) compared with 61% of cells not treated with PPMP (Fig. 3C). To further investigate this observation, LY-B cells obtained after 2 days of culture in sphingolipid-deficient culture media were cultured with 1 μ g/ml of each rCDT protein for 24 h. The sphingolipid-depleted cells showed greater resistance to cell cycle arrest than did the

FIG. 4. The effects of CDT on cultured LY-B cells and LY-B/cLCB1 cells. LY-B cells (A and B) and LY-B/cLCB1 cells (C and D) were cultured for 48 h in Nutridoma-BO medium. LY-B cells and LY-B/cLCB1 cells were then cultured either in the presence of 1 μ g/ml of each CDT protein (B and C) or without CDT protein (A and C). All results are representative of at least three experiments.

FIG. 5. TLC of neutral glycosphingolipids and purified CDT subunits. Each GSL was spotted onto a TLC plate and developed with chloroform-methanol-water (65:35:8, vol/vol/vol). The TLC plates were incubated with His-tagged rCDT protein (His-CdtA, His-CdtB, or His-CdtC) at 4°C overnight. Each subunit was then detected by immunostaining blotting using anti-His mouse monoclonal antibody. Lane 1, Gb3; lane 2, Gb4; lane 3, GM3; lane 4, GM2; lane 5, GM1.

complemented derivative, LY-B/cLCB1. Only 51% of LY-B cells concentrated in the G_2/M phase (Fig. 4B), compared with 84% of LY-B/cLCB1 cells (Fig. 4D).

rCDT binds to GSL. The binding of CDT subunits to GSL was confirmed by a TLC immunostaining test. The binding of each toxin component was investigated using GM1, GM2, GM3, Gb3, Gb4, Lac-Cer, Gal-Cer, and Glc-Cer, which are well-known GSLs. The composition of each GSL and the binding of CDTs for it are shown in Fig. 5. CdtA reacted strongly with GM1, GM2, GM3, and Gb4. CdtB reacted strongly with GM1 and GM2 and weakly with Gb3 and Gb4. CdtC reacted strongly with GM1 and GM2. No toxin subunits reacted with Lac-Cer, Gal-Cer, or Glc-Cer. In order to corroborate the TLC results, we performed adsorption experiments using liposomes that included GSL. All CDT subunits and liposomes were incubated at 37°C for 2 h. After incubation, the mixture was centrifuged at $16,000 \times g$ for 20 min at 4°C. Each supernatant was tested for cytotoxic activity against U937 cells. Compared with liposomes without GSL, every supernatant obtained from liposomes containing GSLs reduced the toxicity of CTD and partially suppressed G_2/M arrest (Fig. 6A). Remarkably, the supernatant obtained from a mixture incubated with liposomes containing GM3 showed a greater reduction of toxicity and greater dose dependency than did the other supernatants (Fig. 6A and B). With this supernatant, only 19% of cells concentrated in the G_2/M phase, compared with 74% of control cells.

FIG. 6. Adsorption test (A to E). U937 cells were incubated with supernatant obtained from a mixture of CDT incubated for 24 h at 37°C with liposomes that included each GSL. After incubation, the cells were collected and analyzed for cell cycle distribution. At least 10,000 cells were analyzed per sample. The black lines represent supernatant obtained from a mixture incubated with liposomes that included the GSLs GM1 (A), GM2 (B), GM3 (C), Gb3 (D), and Gb4 (E). The grey lines represent supernatant obtained from a mixture incubated with liposomes without GSL as a control. The results are representative of at least three experiments. Panel F shows the percentage of U937 cells in the G₂/M phase for each volume of liposomes. ▲, liposome with GM1; ■, liposome with GM2; ●, liposome with GM3; ◆, GSL-free liposome; ×, medium alone.

DISCUSSION

We showed that a combination of all rCDT subunits of *A. actinomycetemcomitans* induced G₂/M arrest in U937 cells (Fig. 1A). One or two subunits alone could not cause G₂/M arrest (data not shown). Recently, Nesic et al. showed that the crystal structure of the CDT holotoxin from *H. ducreyi* reveals that CDT consists of an enzyme of the DNase I family bound

to two ricin-like lectin domains (26). They stated that CdtA, CdtB, and CdtC form a ternary complex with three interdependent molecular interfaces, characterized by globular as well as extensive nonglobular interactions. This supports our finding that all three subunits are required in order for CDT to demonstrate full toxicity.

While progress in understanding the function of CdtB has been made, there is still little known about the roles of CdtA and CdtC in CDT cytotoxicity. Therefore, to determine how CdtA and CdtC act on host cells, we performed a wash assay. G₂/M cell cycle arrest was caused only when CdtA and CdtC were incubated with U937 cells before the addition of CdtB (Fig. 1C). When two subunits were added after another subunit (for example, CdtB and CdtC were added after CdtA), CDT toxicity was not demonstrated (data not shown). In addition, we labeled each rCDT subunit using the Alexa fluorescence system to explore which subunits bind to cells. CdtA required CdtC to bind to cells and vice versa, but they did not bind to cells independently (Fig. 2D and E).

Mao and DiRienzo demonstrated by immunofluorescence that the recombinant product of the *CdtA* gene derived from *A. actinomycetemcomitans* binds to the surfaces of Chinese hamster ovary cells independently but that CdtB and CdtC do not (23). Lee et al. claimed that biotinylated CdtA and CdtC from *Campylobacter jejuni* bind specifically and independently to epithelial cells (HeLa cells) (22). These results differ from our findings. The differences may depend on the different cell species or the origin of the CDT. Deng and Hansen claimed that CdtA and CdtC from *Haemophilus ducreyi* together bind to HeLa cells (6). This is in agreement with our results. As another group has claimed (20), these results suggest that CDT is an AB₂ toxin composed of CdtB as the enzymatically active (A) subunit and CdtA and CdtC as the heterodimeric (B) subunit required for the delivery of CdtB into the target cell. We also confirmed by Western blotting that CdtB enters the nucleus (data not shown).

It is known that many bacterial toxins use neutral GSLs, present on the cell membrane, as receptors. We used PPMP to prevent neutral GSL synthesis in order to explore the possibility of a similar receptor being used for the internalization of CDT. Cells treated with PPMP showed tolerance to CDT toxicity, and G₂/M arrest was partially released (Fig. 3D). It was possible to prevent CDT from binding to the cell and to reduce the toxicity of CDT by preventing GSL synthesis. Similar results were observed in the sphingolipid-deficient cells (Fig. 4). To examine the possibility that rCDT uses a protein receptor on the cell surface, we examined the effect on CDT intoxication of treatment of the cell surface with proteinase K. CDT toxicity was not reduced by this treatment (data not shown). This finding suggests that the intoxication by rCDT requires cell surface GSLs but not proteins. To identify the GSLs that act as CDT receptors, we analyzed the specificity of binding of each subunit for each GSL by TLC. All three subunits reacted strongly with GM1 and GM2. CdtA also reacted strongly with GM3 and Gb4 (Fig. 5). Next, we performed an adsorption experiment using liposomes, including each GSL, in a manner similar to the cell membrane to elucidate the biological role of GSL in the induction of CDT toxicity. In the actual cell membrane, GSLs are incorporated in the lipid bilayer and are a part of the structure. The supernatant of a

reaction mixture of rCDT and liposomes including each GSL (GM1, GM2, GM3, Gb3, and Gb4) or GSL-free liposomes was incubated with U937 cells. Remarkably, the incubation with liposomes including GM3 reduced CDT toxicity (Fig. 6C). Additionally, G₂/M arrest was released in a manner dependent on the dose of GM3 (Fig. 6F). Recently, it has been reported that the HA1, HA3, and HA3b subcomponents of hemagglutinin, which is a component of the *Clostridium botulinum* progenitor toxin, use GM3 as a receptor (8, 14). Misasi et al. suggested that GM3-protein complexes are structural components of the prosaposin receptor involved in cell differentiation (25). We used flow cytometry with a monoclonal antibody to confirm that GM3 was expressed in U937 cells (data not shown). These results show that rCDT may use GM3 as a cell surface receptor. As CdtA reacts with GM1, GM2, GM3, and Gb4, CdtA would recognize GalNAc β 1-3 (or -4) Gal or NA α 2-3Gal β 1-4Glc. CdtB and CdtC react with GM1 and GM2 and would thus recognize GalNAc β 1-4(NeuAc α 2-3)Gal. If GM3 acts as a CDT receptor, the NA α 2-3Gal β 1-4Glc moiety of GM3 may be required for the binding of CDT onto the cell surface. Furthermore, GM3 interacted with only CdtA, which is considered the most essential subunit for CDT binding to the cell. It is known that CdtA shares some similarity in folding structure to the ricin B chain which binds galactoside (11, 20). This evidence strongly suggests that this structure is involved with CDT binding. Liposomes containing GM3 caused a critical reduction of rCDT cytotoxicity, but the reduction was partial. This finding indicates that other glycolipids or biological materials may function as alternative CDT receptors.

In summary, the cells treated with PPMP showed resistance to cytotoxicity. Each rCDT subunit has a binding ability for GSLs. In addition, the rCDT mixture incubated with liposomes containing GM3 showed significantly reduced toxicity. These results indicate that GM3 may play an important role as the receptor of CDT. Although there have been few reports about the internalization and transportation of CDT, Cortes-Bratti et al. have reported that CDT enters cells by endocytosis via clathrin-coated pits and is transported via the Golgi complex (4). Efforts continue in our laboratory to examine the precise mechanism of endocytosis after *A. actinomycetemcomitans* CDT binds to mammalian cells.

REFERENCES

- Akifusa, S., S. Poole, J. Lewthwaite, B. Henderson, and S. P. Nair. 2001. Recombinant *Actinobacillus actinomycetemcomitans* cytolethal distending toxin proteins are required to interact to inhibit human cell cycle progression and to stimulate human leukocyte cytokine synthesis. *Infect. Immun.* **69**: 5925–5930.
- Bock, K., M. E. Breimer, A. Brignole, G. C. Hansson, K. A. Karlsson, G. Larson, H. Leffler, B. E. Samuelsson, N. Stromberg, C. S. Eden, et al. 1985. Specificity of binding of a strain of uropathogenic *Escherichia coli* to Gal alpha 1-4Gal-containing glycosphingolipids. *J. Biol. Chem.* **260**:8545–8551.
- Cope, L. D., S. Lumbley, J. L. Latimer, J. Klesney-Tait, M. K. Stevens, L. S. Johnson, M. Purven, R. S. Munson, Jr., T. Lagergard, J. D. Radolf, and E. J. Hansen. 1997. A diffusible cytotoxin of *Haemophilus ducreyi*. *Proc. Natl. Acad. Sci. USA* **94**:4056–4061.
- Cortes-Bratti, X., E. Chaves-Olarte, T. Lagergard, and M. Thelestam. 2000. Cellular internalization of cytolethal distending toxin from *Haemophilus ducreyi*. *Infect. Immun.* **68**:6903–6911.
- Cortes-Bratti, X., C. Karlsson, T. Lagergard, M. Thelestam, and T. Frisan. 2001. The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways. *J. Biol. Chem.* **276**:5296–5302.
- Deng, K., and E. J. Hansen. 2003. A CdtA-CdtC complex can block killing of HeLa cells by *Haemophilus ducreyi* cytolethal distending toxin. *Infect. Immun.* **71**:6633–6640.
- Fives-Taylor, P. M., D. H. Meyer, K. P. Mintz, and C. Brissette. 1999. Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontol.* **20**:136–167.
- Fujinaga, Y., K. Inoue, S. Watarai, Y. Sakaguchi, H. Arimitsu, J. C. Lee, Y. Jin, T. Matsumura, Y. Kabumoto, T. Watanabe, T. Ohya, A. Nishikawa, and K. Oguma. 2004. Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes. *Microbiology* **150**:1529–1538.
- Hanada, K., T. Hara, M. Fukasawa, A. Yamaji, M. Umeda, and M. Nishijima. 1998. Mammalian cell mutants resistant to a sphingomyelin-directed cytolysis. Genetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. *J. Biol. Chem.* **273**:33787–33794.
- Haraszthy, V. L., G. Hariharan, E. M. Tinoco, J. R. Cortelli, E. T. Lally, E. Davis, and J. J. Zambon. 2000. Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. *J. Periodontol.* **71**:912–922.
- Hassane, D. C., R. B. Lee, M. D. Mendenhall, and C. L. Pickett. 2001. Cytolethal distending toxin demonstrates genotoxic activity in a yeast model. *Infect. Immun.* **69**:5752–5759.
- Haubek, D., K. Poulsen, J. Westergaard, G. Dahlen, and M. Kilian. 1996. Highly toxic clone of *Actinobacillus actinomycetemcomitans* in geographically widespread cases of juvenile periodontitis in adolescents of African origin. *J. Clin. Microbiol.* **34**:1576–1578.
- Holmgren, J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. *Nature* **292**:413–417.
- Inoue, K., Y. Fujinaga, K. Honke, K. Yokota, T. Ikeda, T. Ohya, K. Takeshi, T. Watanabe, and K. Oguma. 1999. Characterization of haemagglutinin activity of *Clostridium botulinum* type C and D 16S toxins, and one subcomponent of haemagglutinin (HA1). *Microbiology* **145**:2533–2542.
- Kamin, S., W. Harvey, M. Wilson, and A. Scutt. 1986. Inhibition of fibroblast proliferation and collagen synthesis by capsular material from *Actinobacillus actinomycetemcomitans*. *J. Med. Microbiol.* **22**:245–249.
- Karlsson, K. A. 1989. Animal glycosphingolipids as membrane attachment sites for bacteria. *Annu. Rev. Biochem.* **58**:309–350.
- Kirby, A. C., S. Meghji, S. P. Nair, P. White, K. Reddi, T. Nishihara, K. Nakashima, A. C. Willis, R. Sim, M. Wilson, et al. 1995. The potent bone-resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. *J. Clin. Invest.* **96**:1185–1194.
- Kovbasnjuk, O., M. Edidin, and M. Donowitz. 2001. Role of lipid rafts in Shiga toxin 1 interaction with the apical surface of Caco-2 cells. *J. Cell Sci.* **114**:4025–4031.
- Lara-Tejedo, M., and J. E. Galan. 2000. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science* **290**:354–357.
- Lara-Tejedo, M., and J. E. Galan. 2001. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. *Infect. Immun.* **69**:4358–4365.
- Lara-Tejedo, M., and J. E. Galan. 2002. Cytolethal distending toxin: limited damage as a strategy to modulate cellular functions. *Trends Microbiol.* **10**:147–152.
- Lee, R. B., D. C. Hassane, D. L. Cottle, and C. L. Pickett. 2003. Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. *Infect. Immun.* **71**:4883–4890.
- Mao, X., and J. M. DiRienzo. 2002. Functional studies of the recombinant subunits of a cytolethal distending holotoxin. *Cell. Microbiol.* **4**:245–255.
- Meyer, D. H., and P. M. Fives-Taylor. 1997. The role of *Actinobacillus actinomycetemcomitans* in the pathogenesis of periodontal disease. *Trends Microbiol.* **5**:224–228.
- Misasi, R., M. Soric, T. Garofalo, T. Griggi, W. M. Campana, M. Giammatteo, A. Pavan, M. Hiraiwa, G. M. Pontieri, and J. S. O'Brien. 1998. Colocalization and complex formation between prosaposin and monosialoganglioside GM3 in neural cells. *J. Neurochem.* **71**:2313–2321.
- Nesic, D., Y. Hsu, and C. E. Stebbins. 2004. Assembly and function of a bacterial genotoxin. *Nature* **429**:429–433.
- Ohguchi, M., A. Ishisaki, N. Okahashi, M. Koide, T. Koseki, K. Yamato, T. Noguchi, and T. Nishihara. 1998. *Actinobacillus actinomycetemcomitans* toxin induces both cell cycle arrest in the G₂/M phase and apoptosis. *Infect. Immun.* **66**:5980–5987.
- Okuda, J., M. Fukumoto, Y. Takeda, and M. Nishibuchi. 1997. Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the *cdtABC* genes of *Shigella dysenteriae*. *Infect. Immun.* **65**:428–433.
- Peres, S. Y., O. Marches, F. Daigle, J. P. Nougayrede, F. Herauld, C. Tasca, J. De Rycke, and E. Oswald. 1997. A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G₂/M phase. *Mol. Microbiol.* **24**:1095–1107.
- Pickett, C. L., D. L. Cottle, E. C. Pesci, and G. Bikah. 1994. Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. *Infect. Immun.* **62**:1046–1051.
- Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin. 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. *Infect. Immun.* **64**:2070–2078.

32. Pickett, C. L., and C. A. Whitehouse. 1999. The cytolethal distending toxin family. *Trends Microbiol.* **7**:292–297.
33. Rodenburg, J. P., A. J. van Winkelhoff, E. G. Winkel, R. J. Goene, F. Abbas, and J. de Graff. 1990. Occurrence of *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. *J. Clin. Periodontol.* **17**:392–399.
34. Shenker, B. J. 1987. Immunologic dysfunction in the pathogenesis of periodontal diseases. *J. Clin. Periodontol.* **14**:489–498.
35. Slots, J., and M. Ting. 1999. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontol.* **20**:82–121.
36. Suda, T., I. Nakamura, E. Jimi, and N. Takahashi. 1997. Regulation of osteoclast function. *J. Bone Miner. Res.* **12**:869–879.
37. Sugai, M., T. Kawamoto, S. Y. Peres, Y. Ueno, H. Komatsuzawa, T. Fujiwara, H. Kurihara, H. Suganaka, and E. Oswald. 1998. The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. *Infect. Immun.* **66**:5008–5019.
38. Sullivan, P., W. L. Clark, and P. K. Kaiser. 2002. Bilateral endogenous endophthalmitis caused by HACEK microorganism. *Am. J. Ophthalmol.* **133**:144–145.
39. Tetaud, C., T. Falguieres, K. Carlier, Y. Lecluse, J. Garibal, D. Coulaud, P. Busson, R. Steffensen, H. Clausen, L. Johannes, and J. Wiels. 2003. Two distinct Gb3/CD77 signaling pathways leading to apoptosis are triggered by anti-Gb3/CD77 mAb and verotoxin-1. *J. Biol. Chem.* **278**:45200–45208.
40. van Winkelhoff, A. J., and J. Slots. 1999. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in nonoral infections. *Periodontol.* **20**:122–135.
41. Watarai, S., S. Handa, T. Tadakuma, and T. Yasuda. 1987. Application of liposomes to generation of monoclonal antibody to glycosphingolipid: production of monoclonal antibody to GgOse4Cer. *J. Biochem. (Tokyo)* **102**:59–67.
42. Watarai, S., Tana, K. Inoue, Y. Kushi, E. Isogai, K. Yokota, K. Naka, K. Oguma, and H. Kodama. 2001. Inhibition of Vero cell cytotoxic activity in *Escherichia coli* O157:H7 lysates by globotriaosylceramide, Gb3, from bovine milk. *Biosci. Biotechnol. Biochem.* **65**:414–419.
43. White, P. A., M. Patel, S. Nair, J. Ashmore, P. Galgut, M. Wilson, B. Henderson, and I. Olsen. 1998. Control of the human cell cycle by a bacterial protein, gapstatin. *Eur. J. Cell Biol.* **77**:228–238.
44. White, P. A., M. Wilson, S. P. Nair, A. C. Kirby, K. Reddi, and B. Henderson. 1995. Characterization of an antiproliferative surface-associated protein from *Actinobacillus actinomycetemcomitans* which can be neutralized by sera from a proportion of patients with localized juvenile periodontitis. *Infect. Immun.* **63**:2612–2618.
45. Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett. 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G₂-phase cell cycle block. *Infect. Immun.* **66**:1934–1940.
46. Wilson, M., and B. Henderson. 1995. Virulence factors of *Actinobacillus actinomycetemcomitans* relevant to the pathogenesis of inflammatory periodontal diseases. *FEMS Microbiol. Rev.* **17**:365–379.
47. Young, V. B., K. A. Knox, and D. B. Schauer. 2000. Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus*. *Infect. Immun.* **68**:184–191.
48. Zambon, J. J. 1985. *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J. Clin. Periodontol.* **12**:1–20.

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