Induction of Cell Cycle Arrest in Lymphocytes by *Actinobacillus actinomycetemcomitans* Cytolethal Distending Toxin Requires Three Subunits for Maximum Activity

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Induction of Cell Cycle Arrest in Lymphocytes by
Actinobacillus actinomycetemcomitans Cytolethal Distending
Toxin Requires Three Subunits for Maximum Activity

Bruce J. Shenker,* Dave Besack,* Terry McKay,* Lisa Pankoski,* Ali Zekavat,* and
Donald R. Demuth†

We have previously shown that Actinobacillus actinomycetemcomitans produces an immunosuppressive factor encoded by the cytolethal distending toxin (cdt)B gene. In this study, we used rCdt peptides to study the contribution of each subunit to toxin activity. As previously reported, CdtB is the only Cdt subunit that is capable of inducing cell cycle arrest by itself. Although CdtA and CdtC do not exhibit activity alone, each subunit is able to significantly enhance the ability of CdtB to induce G₂ arrest in Jurkat cells; these effects were dependent upon protein concentration. Moreover, the combined addition of both CdtA and CdtC increased the ED₅₀ for CdtB >7000-fold. In another series of experiments, we demonstrate that the three Cdt peptides are able to form a functional toxin unit on the cell surface. However, these interactions first require that a complex forms between the CdtA and CdtC subunits, indicating that these peptides are required for interaction between the cell and the holotoxin. This conclusion is further supported by experiments in which both Jurkat cells and normal human lymphocytes were protected from Cdt holotoxin-induced G₂ arrest by pre-exposure to CdtA and CdtC. Finally, we have used optical biosensor technology to show that CdtA and CdtC have a strong affinity for one another (10⁻¹⁰ M). Furthermore, although CdtB is unable to bind to either CdtA or CdtC alone, it is capable of forming a stable complex with CdtA/CdtC. The implications of our results with respect to the function and structure of the Cdt holotoxin are discussed. The Journal of Immunology, 2005, 174: 2228–2234.

The cytolethal distending toxins (Cdts)³ are a family of heat-labile protein cytopathic toxins produced by several different bacterial species including diarrheal disease-causing enteropathogens such as some Escherichia coli isolates, Campylobacter jejuni, Shigella species, Haemophilus ducreyi, Salmonella typhi, and Actinobacillus actinomycetemcomitans (1–9). There is now clear evidence that Cdt is encoded by three genes, designated cdtA, cdtB, and cdtC, which are cotranscribed (1, 5, 6, 8). The cdt genes encode polypeptides with apparent molecular masses of ~24–35 kDa. Cdts were first characterized by their ability to cause progressive cellular distension and finally death in some cell lines; it should be noted that the gross cellular changes associated with Cdt activity are clearly different from those caused by other known toxins that induce rapid morphological alterations culminating in cell death (10).

We have recently shown that A. actinomycetemcomitans produces an immunosuppressive toxin capable of inhibiting both B and T cell function by interfering with the normal cell cycle progression of lymphocytes resulting in G₂ arrest (11–13). The active toxin was shown to be the product of the cdtB gene (8). There is now general agreement that the Cdt holotoxin consists of all three Cdt subunits: CdtA, CdtB, and CdtC, and maximum expression of Cdt activity requires the presence of all three subunits (1, 14–18). However, limited information is available on the role of the individual subunits, in particular, CdtA and CdtC, in toxin activity. In this regard, we have previously shown that, following exposure to the holotoxin, all three subunits are detected associated with lymphocytes. Several investigators have suggested that CdtA and CdtC are responsible for association of the toxin complex to the cell membrane, which in turn leads to the internalization of CdtB (9, 19, 20). However, to date, it has been difficult to ascribe a specific function to these peptides with any confidence. To overcome these difficulties and more accurately define a role for the Cdt peptides in toxin activity, we have used an in vitro transcription/translation protein expression system that enables us to produce sufficient amounts of protein for characterization and functional assessment. The expressed proteins contain a C-terminal His tag for easy isolation and identification. Using these recombinant peptides, we now report that only CdtB is capable of inducing cell cycle arrest by itself. However, both CdtA and CdtC influence the activity of CdtB and are required to achieve maximum toxin activity. Furthermore, it appears that CdtA and CdtC, but not CdtB, are required for interaction of the toxin with the cell surface. Finally, our results suggest that assembly of the holotoxin into a stable complex requires both CdtA and CdtC, and that these two peptides must form a complex before CdtB is able to become associated with the holotoxin.

Materials and Methods

Cell culture and analysis of cell cycle

The T cell leukemia cell line Jurkat (E6–1; American Type Tissue Culture Collection) was maintained as previously described (15). Briefly, cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were harvested in mid-log growth phase and plated at 5 × 10⁵.
cells/ml in 24-well tissue culture plates. The cells were exposed to medium or Cdt peptide(s) as indicated and incubated for 18 h. To measure Cdt-induced cell cycle arrest, Jurkat cells were washed and fixed for 60 min with cold 80% ethanol. After washing, the cells were stained with 10 μg/ml propidium iodide containing 1 mg/ml RNase (Sigma-Aldrich) for 30 min. Samples were analyzed on a BD Biosciences FACStar®PLUS flow cytometer. Propidium iodide fluorescence was excited by an argon laser operating at 488 nm, and fluorescence was measured with a 630/22-nm bandpass filter using linear amplification. A minimum of 15,000 events were collected on each sample; cell cycle analysis was performed using ModFit (Verity Software House).

Human PBMC (HPBMC) were prepared as described previously (8). Briefly, HPBMC were isolated by buoyant density centrifugation on Ficoll-Hypaque (Amersham Biosciences). HPBMC were washed twice with RPMI 1640, and viable-cell counts were performed by trypan blue dye exclusion. Lymphocytes (1 × 10⁶ cells/ml) were activated with PHA (1 μg/ml; Abbott Laboratories) following pretreatment for 45 min with Cdt. Cell cycle was determined as described above.

Construction of plasmid containing Cdt genes

Individual expression plasmids were constructed for each Cdt subunit in pGEM: full-length CdtA (pGEMCdtAF), truncated CdtA (pGEMCdtA), truncated CdtB (pGEMCdtB), and truncated CdtC (pGEMCdtC). Each construct contains a RBS site and a T7 promoter upstream of the cdt gene and incorporates a hexa-His C-terminal tag. Construction of these plasmids involved two sequential PCR. The first reaction used the primers indicated in Table I and is used as a template (15); the primers consisted of 15–20 bp of genomic Cdt sequence and additional sequence required to perform the second round of PCR. The products of the first PCR were then used as templates for the second PCR according to the manufacturer’s directions (Roche Linear Template Generation Kit; Roche Applied Science). The resulting PCR products were then cloned into pGEM-T Easy, and the plasmids were transformed into E. coli JM109. Cultures of transformed JM109 were grown in 500 ml of LB broth and used to purify the recombinant plasmids for use in the in vitro expression system.

To express CdtB as a GFP fusion protein, pAaCdtB-GFP was constructed using two sequential PCR. First, the cd tB gene was amplified as described above using primer pair P9/P10. The amplified product was used as a template in a second PCR (primer pair P11/P12) to incorporate a T7 promoter and an N-terminal penta-His sequence. The product from this reaction was ligated into pcDNA3.1/CT-GFP-TOPO (Invitrogen Life Technologies) and used to transform TOP10 competent cells.

Expression and purification of Cdt peptides

In vitro expression of Cdt peptides was performed using the Rapid Translation System (RTS 500 ProteoMaster; Roche Applied Science), and reactions were run according to the manufacturer’s specification (Roche Applied Science) using 10–15 μg of template DNA. After 20 h at 30°C, the reaction mix was removed, and the expressed Cdt peptides were purified by nickel affinity chromatography as previously described (15). Flow cytometric analysis of Jurkat cells for the presence of CdtB-GFP

Jurkat cells were treated with CdtA, CdtC, and CdtB-GFP as described in Results. The cells were then incubated for 2 h at 37°C, washed, and fixed in 2% paraformaldehyde. Flow cytometry was performed using an argon laser (488 nm) to excite the fluorochrome; emission fluorescence was analyzed using a 530/15-nm bandpass filter.

Measurement of Cdt subunit binding with an optical biosensor

All surface plasmon resonance (SPR) experiments were conducted on a Biacore X (Biacore) with active temperature control at 25°C. The running buffer for the experiments was PBS containing 0.01 M HEPES (pH 7.4), 3 mM EDTA, and 0.005% Surfactant P20 (Biacore). Approximately 2000 response units (RU) of either purified CdtA, CdtB, or CdtC was coupled to flow cell 2 (Fc2) of a CM5 sensor chip via primary amines according to the manufacturer’s specifications. Fc1 was activated and blocked without the addition of protein. To characterize the binding of individual Cdt peptides to one another, the flow path was set to include both flow cells, the flow rate was 50 μl/min, and the data collection rate was set to high. Protein samples were serially diluted in the running buffer. Binding of each Cdt sample was allowed to occur for 2 min, with the wash delay set for an additional 2 min to allow for a smooth dissociation curve. The chip surface was regenerated by injecting brief pulses of 0.2 M sodium carbonate (pH 10) until the response signal returned to baseline. SPR data were analyzed with BLAevaluation, version 3.0, software, which employs global fitting. Sensorgrams were corrected for nonspecific binding by subtracting the control sensogram (Fc1) from the Cdt surface sensogram (Fc2). Model curve fitting of individual peptide binding was done with a 1:1 Langmuir binding model with drifting baseline. This is the simplest model for the interaction between receptor and ligand; it follows the equation A + B ⇄ AB. The rate of association (kₐ) is measured from the forward reaction, whereas kₐ is measured from the reverse reaction (21). Model curve fitting of CdtB to CdtA/CdtC was done with the Bivalent analyte model. This model describes the binding of a bivalent analyte to immobilized ligand, where one analyte molecule can bind to one or two ligand molecules. Binding to the second ligand molecule is described by a single set of rate constants, so that the two sites on the analyte are equivalent in the first binding step (A + B ⇄ AB). Binding to the second ligand molecule is described by a second set of rate constants, allowing the model to take cooperative effects into account (AB + B ⇄ AB2).

Results

We have previously generated several plasmids that express various combinations of the A. actinomycetemcomitans cdt genes to determine the requirement of individual genes for the expression of toxin activities. These experiments indicated that, although the

<table>
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* Bold text represents primer sequence based upon cdt gene sequence.

** 5’ promoter supplied with commercial kit (Roche Linear Template Generation Kit; Roche Applied Science).
CdtB peptide alone is sufficient to induce G2 arrest in human lymphocytes, the presence of all three Cdt peptides was necessary to induce maximum toxin activity. In this study, we investigated the contribution of the Cdt subunits to toxin activity using individual Cdt peptides expressed using an in vitro-coupled translation/transcription system. The templates for each of the Cdt peptides included a C-terminal hexa-histidine tag. It should be noted that in our previous study, we observed that the Cdt holotoxin was composed of truncated peptides (15); this was not a surprising observation for the CdtB and CdtC peptides that contain a well-recognized signal sequence. In contrast, CdtA does not contain a common signal sequence; however, the CdtA peptide contained in the Cdt holotoxin lacked the first 59 aa (15). Therefore, the DNA templates were constructed to encode truncated Cdt peptides; to determine the requirement for CdtA truncation, we expressed not only the truncated CdtA but also the full-length peptide (CdtA^F). As shown in Fig. 1, A and B, each peptide exhibited the expected molecular mass: 28 kDa (CdtA^F), 18 kDa (CdtA), 32 kDa (CdtB), and 20 kDa (CdtC), and could also be detected with anti-His mAb. Identity of each peptide was confirmed by Western blot using mAbs specific for each peptide (Fig. 1, C–E).

In the first series of experiments, we assessed the capacity of each of the Cdt peptides to influence toxin activity expressed by CdtB. As shown in Fig. 2, CdtB alone was able to induce G2 arrest in Jurkat cells; the arrest was dose dependent when the cells were treated with 0.6–10 μg/ml CdtAB. The CdtB peptide exhibited an ED_{50} of 12.6 μg/ml, which was comparable to that previously reported for rCdtB expressed in E. coli transformed with a plasmid containing the cdhB gene (15). Jurkat cell proliferation was not altered when treated with either CdtA (either full-length or truncated) or CdtC alone (data not shown). Exposure of Jurkat cells to CdtB in the presence of CdtA resulted in increased toxin activity as evident by the percentage of cells arrested in the G2 phase of the cell cycle (Fig. 3A); enhancement of CdtB activity by CdtA was dose dependent. In the presence of 50 and 250 ng of CdtA, the ED_{50} for CdtB was reduced to 1.3 and 0.26 μg/ml, respectively, representing a 10- to 40-fold increase in toxin activity. CdtA^F had no effect on CdtB activity. CdtC similarly affected the ability of CdtB to induce Jurkat cells to undergo G2 arrest (Fig. 3B); the addition of 50 and 250 ng of CdtC resulted in a decrease in the CdtB ED_{50} to 2.4 and 0.72 μg/ml, respectively, representing a 5- to 17-fold increase in activity. In contrast, there was a substantial increase in toxin activity when Jurkat cells were treated with CdtB in the presence of both CdtA and CdtC (Fig. 3C). In the presence of 50 and 250 ng of CdtA and CdtC, the ED_{50} for CdtB was reduced to 0.7 and 0.04 ng/ml, respectively. This represents an increase in activity of >1800-fold (50 ng of CdtA/CdtC) and >7000-fold (250 ng of CdtA/CdtC). It should be noted that 10 ng of CdtA and CdtC significantly increased CdtB activity (ED_{50} = 3.8 ng/ml); the addition of 10 ng of CdtA or CdtC alone had no effect on CdtB activity (data not shown). CdtA^F did not alter CdtB activity in the presence of CdtC (data not shown).

We have previously shown that the Cdt holotoxin is a heterotrimer consisting of truncated CdtA, CdtB, and CdtC. Although CdtB alone is capable of inducing lymphocyte cell cycle arrest, our current studies clearly demonstrate that both CdtA and CdtC have a profound effect on activity. It has previously been suggested that CdtA and CdtC serve to deliver CdtB to the cell (14, 20). To investigate this possibility, Jurkat cells were exposed to three different Cdt peptide pairs: CdtA/CdtB, CdtA/CdtC, and CdtB/CdtC. After 30 min, the cells were washed and then exposed to the third Cdt peptide, respectively. As shown in Fig. 4, Jurkat cells were only induced to undergo G2 arrest when they were first exposed to CdtA and CdtC followed by CdtB. These results suggest that CdtA and CdtC are indeed the subunits required for holotoxin interaction with the cell. It should also be noted that sequential exposure of cells to individual Cdt peptides did not lead to cell cycle arrest regardless of the order of exposure (data not shown). In a similar experiment, Jurkat cells were exposed to CdtB-GFP alone (Fig. 5B) or following treatment with CdtA and CdtC (C). As shown, CdtB-GFP cell-associated fluorescence was marginally detected when cells were exposed to the GFP fusion protein alone; there was a significant increase in fluorescence in the presence of CdtA and CdtC. The mean channel fluorescence was 5.8 (control cells), 9.1 (CdtB-GFP only), and 15.3 (CdtA/CdtC/CdtB-GFP treated). Exposure of cells to either CdtA or CdtC alone did not result in a detectable increase in cell-associated CdtB-GFP fluorescence (data not shown).

**FIGURE 1.** Isolation and analysis of Cdt peptides expressed in vitro. Cdt peptides were expressed using an in vitro translation/transcription system, purified by nickel affinity chromatography, and analyzed by PAGE and Western blot: lane 1, CdtA^F; lane 2, CdtA; lane 3, CdtB; and lane 4, CdtC. A, Coomassie-stained PAGE gel; B, Western blot stained with anti-His mAb; C, Western blot stained with anti-CdtB mAb; D, Western blot stained with anti-CdtB mAb; and E, Western blot stained with anti-CdtA mAb.

**FIGURE 2.** Induction of G2 arrest by CdtB. Jurkat cells were treated with varying concentrations of CdtB for 18 h. The cells were then analyzed for cell cycle distribution by flow cytometry based upon propidium iodide fluorescence. The percentage of G2 cells (mean ± SD) is plotted vs CdtB concentration; the ED_{50} value is presented. Control cells exposed to medium only averaged 15.8%; results represent the mean of three experiments.
Our results are consistent with the notion that CdtA and CdtC together are required for Cdt holotoxin interaction with the cell surface. To further confirm this requirement, we next tested the individual Cdt peptides for their ability to block the induction of G2 arrest by the holotoxin. As shown in Fig. 6, Jurkat cells exposed to the Cdt holotoxin (CdtABC) exhibited 57.9% G2 cells; control cells contained 16.9% G2 cells. Pre-exposure of cells to either CdtA or CdtC had no affect on the ability of the holotoxin to induce cell cycle arrest. In contrast, pretreatment of Jurkat cells with both CdtA and CdtC blocked cell cycle arrest by the holotoxin; under these conditions, the percentage of G2 cells was reduced to 20.4%. Similar experiments were conducted on normal human lymphocytes (Fig. 7). HPBMC activated by PHA exhibit 7.1% cells in the G2 phase (Fig. 7B); in contrast, a 3-fold increase in G2 cells (22.1%) was observed following pretreatment with Cdt holotoxin (Fig. 7C). Exposure to CdtA and CdtC before treatment with holotoxin protected the cells from toxin-induced G2 arrest (7.6% G2; Fig. 7D). These results provide further evidence that indeed CdtA and CdtC interact with the cell surface on not only lymphoid cell lines, but also normal lymphocytes as well. Furthermore, our collective results suggest that CdtA and CdtC are most likely required to form a complex before their interaction with the cell.

FIGURE 3. Effect of CdtA and CdtC on CdtB-induced G2 arrest. Jurkat cells were exposed to varying concentrations of CdtB in the presence of CdtA (A), CdtC (B), or both CdtA and CdtC (C). , Represents results from cells exposed to CdtB in the presence of 10 ng/ml Cdt peptide; , represents 50 ng/ml Cdt peptide; , represents 250 ng/ml Cdt peptide; and , represent the effect of 250 ng/ml CdtA'. The cells were analyzed for cell cycle distribution by flow cytometry based upon propidium iodide fluorescence. The percentage of G2 cells (mean ± SD) is plotted vs CdtB concentration. ED50 value is presented. Control cells exposed to medium only averaged 14.1%; results represent the mean of three experiments.

FIGURE 4. Sequential exposure of Jurkat cells to Cdt peptides. Jurkat cells were exposed to 250 ng/ml each of either CdtA/CdtB, CdtA/CdtC, or CdtB/CdtC for 1 h, washed and then treated with the third Cdt peptide as indicated. The cells were then analyzed for cell cycle distribution 18 h later by flow cytometry based upon propidium iodide fluorescence. The percentage of G2 cells (mean ± SD) is plotted; results represent the mean of three experiments.

FIGURE 5. Flow cytometric analysis of Jurkat cells treated with CdtB-GFP. Jurkat cells were exposed to medium only (A), 25 µg/ml CdtB-GFP (B), and CdtB-GFP in the presence of 250 ng/ml each of CdtA and CdtC (C) for 2 h. The cells were washed and analyzed for GFP fluorescence. Results are representative of three experiments; log GFP fluorescence is plotted vs relative cell number.
In a final series of experiments, we assessed the kinetics and thermodynamic binding parameters of Cdt subunit interaction using optical biosensor technology; this method provides quantitative affinities (\(K_D\) (equilibrium dissociation constant)) as well as on (\(k_{on}\)) and off (\(k_{off}\)) rates for the formation of each Cdt complex. To calculate kinetic and thermodynamic binding parameters for Cdt peptide interactions, 2-fold serial dilutions of each Cdt peptide were injected onto a chip with one of the peptides covalently bound to it. Initially, CdtC was coupled to a sensor chip surface (Fig. 8A); CdtB or CdtA was allowed to flow over the chip surface.

Fc2 contained the immobilized CdtC, whereas Fc1 was activated and blocked without the addition of protein. In the initial 50 s of each sensorgram, a buffer baseline was established. Sample was injected and the association of CdtA (or CdtB) and CdtC followed for 2 min. The sample was then replaced with buffer, and the dissociation of complex followed for another 2 min. The response on the y-axis is measured in response units. In preliminary experiments we determined that there was very little nonspecific binding of protein to the activated and blocked surface on Fc1; nonetheless, data from Fc1 were subtracted from the data from Fc2 to correct for changes in bulk refractive index and nonspecific binding to the sensor chip surface. Fig. 8A shows a group of sensorgram overlays for the binding of CdtA to immobilized CdtC. The data were analyzed using the best global fit to a simple 1:1 Langmuir binding model with drifting baseline, assuming that CdtA is a monomer in solution (21). Using this model, the P2 values (a standard statistical measure of the closeness of fit) were all >10, and residuals, which correspond to the difference between the actual and fitted data, are within ±0.6 RU, indicating a good fit (not shown). Assessment of the fit is important, because the on and off rates are calculated from the fitted data. Calculation of CdtA affinity for the immobilized CdtC was 10^{-7} M (Table II), indicating the formation of a stable complex. It should be noted that CdtA\(^{F}\) was able to form a complex with CdtC, but with lower affinity (10^{-6} M; data not shown). In contrast, CdtB was unable to bind to CdtC (Fig. 8A; dotted line).

To determine whether the kinetics of binding between CdtC and CdtA was dependent on the orientation of the complex, the reverse experiment was performed. CdtA was immobilized to the sensor chip, and CdtC was allowed to flow over the chip (Fig. 8B). Analysis of the data indicates that the binding kinetics for the formation of the complex was approximately the same as when the molecules were in the reverse orientation (Table II). CdtB was not able to

FIGURE 6. Inhibition of Cdt holotoxin induced G2 arrest. Jurkat cells were pre-exposed to 5 \(\mu\)g/ml CdtA (C), 5 \(\mu\)g/ml CdtC (D), or 5 \(\mu\)g/ml CdtA and CdtC (E) for 2 h. The cells were washed, treated with 50 pg/ml Cdt holotoxin for 60 min, washed, and incubated for 18 h. The cells were analyzed for cell cycle distribution by flow cytometry based upon propidium iodide fluorescence. The percentages of cells in G0/G1, S, and G2/M are shown. A shows the cell cycle distribution for control cells exposed to medium, and B for Jurkat cells exposed to holotoxin without prior exposure to any Cdt subunits. Results are representative of three experiments.
CdtB was allowed to first form a complex. Under these conditions, CdtB was able to bind to the CdtA/CdtC complex to form a highly stable tripartite complex as evinced by the affinity constant and low off rate. Indeed, SPR analysis clearly demonstrates that CdtA and CdtC subunits and their role in the holotoxin, we used several approaches. First, we attempted to assemble a functional holotoxin on the lymphocyte surface by sequentially exposing cells to the individual subunits; this approach initially failed to result in an active toxin unit. However, we were able to generate a functional toxin by using a modified approach in which we first used pairs of Cdt peptides followed by the addition of the missing subunit. Under these conditions, a functional toxin unit could only be assembled when the cells were simultaneously exposed to CdtA and CdtC followed by the addition of CdtB. Similarly, pre-exposure of lymphocytes to CdtA and CdtC resulted in a significant increase in cell-associated CdtB. Therefore, we concluded that the interaction between the holotoxin and cells was dependent upon a complex formed by CdtA and CdtC; specifically, it appears that these units directly bind to the cell surface. This interpretation was further confirmed by our observation that prior exposure of lymphocytes to the combination of CdtA and CdtC prevented the holotoxin from interacting with the cell and inducing cell cycle arrest. Once again, exposure to either subunit alone failed to have any effect on the ability of the holotoxin to interact with the cell.

Cdt subunit interactions were further assessed by SPR, which provides sensitive detection of molecular interactions in real time. Assembly of a functional holotoxin in the presence of cells demonstrated that not only are all three subunits required for maximal activity, but these studies also suggest that CdtA and CdtC must be assembled when the cells were simultaneously exposed to CdtA and CdtC subunits and their role in the holotoxin, we used several approaches. First, we attempted to assemble a functional holotoxin on the lymphocyte surface by sequentially exposing cells to the individual subunits; this approach initially failed to result in an active toxin unit. However, we were able to generate a functional toxin by using a modified approach in which we first used pairs of Cdt peptides followed by the addition of the missing subunit. Under these conditions, a functional toxin unit could only be assembled when the cells were simultaneously exposed to CdtA and CdtC followed by the addition of CdtB. Similarly, pre-exposure of lymphocytes to CdtA and CdtC resulted in a significant increase in cell-associated CdtB. Therefore, we concluded that the interaction between the holotoxin and cells was dependent upon a complex formed by CdtA and CdtC; specifically, it appears that these units directly bind to the cell surface. This interpretation was further confirmed by our observation that prior exposure of lymphocytes to the combination of CdtA and CdtC prevented the holotoxin from interacting with the cell and inducing cell cycle arrest. Once again, exposure to either subunit alone failed to have any effect on the ability of the holotoxin to interact with the cell.

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According to the study, Cdt is a complex of three subunits: CdtA, CdtB, and CdtC. The crystal structure supports this notion (24). Also, demonstration of DNase activity has been most often shown on plasmid DNA; this raises the interesting possibility that this may facilitate processing of the cdtABC genes of Shigella dysenteriae. Infect. Immun. 65:428.

Several investigators have also suggested that CdtB functions as a DNase-like moiety whereby it cleaves DNA and in turn activates cell cycle checkpoints as a result of DNA damage (16, 26, 28). It should be noted, however, that the link between DNase and CdtB is based upon weak sequence similarity, although analysis of the crystal structure supports this notion (24). Also, demonstration of DNase activity has been most often shown on plasmid DNA; this often requires enormous amounts of protein (micrograms) compared with the small amounts of toxin (picograms) required to induce cell cycle arrest. Thus, it remains unclear as to whether DNA degradation represents the only functional activity for CdtB as it relates to the induction of G2 arrest.

The role for CdtA and CdtC also remains unclear; however, there is growing evidence that these subunits are involved in forming the stabilized holotoxin complex and for recognition of cell-associated receptors. Indeed, our results support the notion that not only are CdtA and CdtC required for maximum toxin activity but also that they are involved in toxin-cell interaction. Furthermore, the SPR data suggest that not only do CdtA and CdtC interact with one another in a 1:1 manner, but that CdtB interacts with a complex of these two units. Furthermore, the best of these data suggests that the CdtA/CdtC complex also functions as a single unit in its interaction with CdtB. These conclusions are also supported by analysis of the crystal structure of the Cdt holotoxin from H. ducreyi. In these analyses, Nesic et al. (24) showed direct contact between CdtA-CdtB, CdtA-CdtC, and CdtB-CdtC, and further that the trimeric complex shows no signs of oligomerization. Further analysis suggests the CdtA and CdtC exhibit ricin-like domains. Based upon the crystal structure and our observations as well as those of others, Cdt holotoxin appears to be an AB2 toxin where CdtB is the active (A) unit and the complex of CdtA and CdtC comprise the binding (B) unit (14, 24). Although we and others have demonstrated that CdtA and CdtC are required for formation of the holotoxin and for the interaction of the toxin with the cell, it should not be assumed that the role of these two subunits is limited to these functions.

In conclusion, our results demonstrate that, whereas CdtB alone is a potent immunoinhibitory factor capable of inducing G2 arrest in lymphocytes, it is considerably more potent in the presence of CdtA and CdtC. Moreover, the A. actinomycetemcomitans holotoxin is composed of a stable trimeric complex composed of CdtA, CdtB, and CdtC. Clearly, further investigation is required to identify the cellular target(s) and complete molecular pathway by which each of the Cdt subunits acts to induce cell cycle arrest. A thorough understanding of the mechanism of action by which Cdt functions will provide important insights into the pathogenesis of infections caused by Cdt-producing microbial species.

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References