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Actinobacillus actinomycetemcomitans Cytolethal Distending Toxin (Cdt): Evidence That the Holotoxin Is Composed of Three Subunits: CdtA, CdtB, and CdtC

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

We have shown the Actinobacillus actinomycetemcomitans produces an immunosuppressive factor encoded by the cytolethal distending toxin (cdt)B gene, which is homologous to a family of Cdts expressed by several Gram-negative bacteria. We now report that the capacity for CdtB to induce G2 arrest in Jurkat cells is greater in the presence of the other Cdt peptides: CdtA and CdtC. Plasmids containing the cdt operon were constructed and expressed in Escherichia coli; each plasmid contained a modified cdt gene that expressed a Cdt peptide containing a C-terminal His tag. All three Cdt peptides copurified with the His-tagged Cdt peptide. Each of the peptides associated with the complex was truncated; N-terminal amino acid analysis of CdtB and CdtC indicated that the truncation corresponds to cleavage of a previously described signal sequence. CdtA was present in two forms in crude extracts, one of which still expressed a Cdt peptide containing a C-terminal His tag. Expression of maximum toxic activity (27–29) of the Cdt holotoxin. Although we have shown that CdtB alone is capable of inducing all the biological effects typically associated with Cdt, our previous studies did not rule out a role for CdtA and/or CdtC (13). Although several investigators agree with our conclusion that CdtB is indeed the functional subunit, there is currently a controversy as to whether CdtC is also capable of inducing G2 arrest in the cell cycle of mitogen-activated human T cells (12, 13). However, it should be emphasized that Cdt-treated lymphocytes do not exhibit the morphological alterations that are commonly observed with cell lines such as HeLa cells, which are often used as a target cell to define the action of the Cdt.

To date, limited information is available that defines the nature of the Cdt holotoxin. Although we have shown that CdtB alone is capable of inducing all the biological effects typically associated with Cdt, our previous studies did not rule out a role for CdtA and/or CdtC (13). Although several investigators agree with our conclusion that CdtB is indeed the functional subunit, there is currently a controversy as to whether CdtC is also able to fulfill this role or whether perhaps all three peptides are required to form the holotoxin and for the expression of maximum toxic activity (27–29). We now report that, although CdtB alone is indeed sufficient to induce G2 arrest in human lymphocytes, both CdtA and CdtC are required to achieve maximum cell cycle arrest. Moreover, we
demonstrate that the holotoxin consists of a heterotrimeric complex of CdtA, CdtB, and CdtC. It should be noted that this complex is composed of mature peptides in which a portion of its N-terminal sequence has been cleaved. Finally, we provide evidence that all three peptides can be found associated with the lymphocytes within 2 h of exposure to 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, Manassas, VA) was maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were harvested in mid-log growth phase and plated at 5 × 10⁶ cells/ml in 24-well tissue culture plates. The cells were exposed to medium or the toxin preparation 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, St. Louis, MO) for 30 min. Samples were analyzed on a FACStar Plus flow cytometer (BD Biosciences, San Jose, CA). 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 was collected on each sample; cell cycle analysis was performed using Modfit (Verity Software House, Topsham, ME).

Construction of plasmids expressing cdt genes

Several of the cdt gene constructs used in this study were derived from the pUCAcdb2 plasmid as previously described (13); this plasmid contains cdtA, cdtB, cdtC, a small upstream open reading frame (orf2), and an additional 2.5 kb of sequence downstream of the cdtC gene. A series of plasmids were constructed that lacked one or more of the open reading frames present in pUCAcdb2. These plasmids were prepared by first digesting pUCAcdb2 with HphI, which cleaves within the cdtA gene, and EcoRI, which cleaves in the pUC multiple cloning region (13). The resulting 3.2-kb DNA fragment contains the plasmid vector, orf2, and the first 250 residues of the cdtA gene. PCR products were then generated from several primer pairs (see Table I) to generate a product that possesses an upstream HphI site and a downstream EcoRI site. The resulting fragments were subsequently ligated to the 3.2-kbp product from the restriction digestion above. Ligation with the 1946-bp P1/P1 product yielded pUCAcdbABC, which contains orf2, cdtA, cdtB, and cdtC, but lacks the 2.5-kb sequence downstream of the cdt operon. Plasmid pUCAcdbAB, which contains orf2, cdtA, and cdtB, was produced by ligation with the 1350-bp P3/P4 product, and pUCAcdbA, containing only orf2 and cdtA, resulted from ligation with the 430-bp P3/P19 product.

Plasmid pUCAcdbAC, containing the cdtA and cdtC genes, was prepared by first using P5/P6 and P7/P8 to amplify cdtA (including orf2) and

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<td><strong>Plasmid</strong></td>
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CdTC, respectively. The PCR products were ligated to pGEM-T (Promega, Madison, WI), and the plasmids were transformed into E. coli DH5α (Invitrogen, Carlsbad, CA). The inserts were isolated by digestion with XmaI/SacI (cdtA) and SacI/EcoRI (cdtB); cdtC was then ligated to pUC19 following digestion of the plasmid with SacI/EcoRI; the resulting plasmid was then digested with XmaI/SacI and ligated to cdtA.

Plasmid pUCAcadtBC, in which cdtA was inactivated, was prepared by digesting pUCAcadtABC with NheI, and then blunting with Klenow polymerase, followed by religation. The resulting plasmid thus contains a frameshift mutation in the cdtA gene.

PUCAcadtC, which contains only cdtC, was constructed from the P20/P21 PCR product, which was first ligated to pGEM-T, digested with PstI and BamHI, and then ligated to pUC19.

Three additional plasmids were constructed that contain orf2 and the three cdt genes; each plasmid contains a penta-His sequence that encodes a C-terminal His tag on either cdtA (pUCAcadtABC his), cdtB (pUCAcadtABC(C) his), or cdtC (pUCAcadtABC(G) his). To construct pUCAcadtABC(C), two PCR products were generated using the P9/P10 (Smal-cdtABC(A)/AflII) and P11/P12 (Smal-AflII-cdtB-RsrII) primer pairs; the latter PCR product and pUCAcadtABC were digested with Smal/RsrII and ligated. The intermediate plasmid and the Smal-cdtA(C)-AflII PCR product were digested with Smal/AflII and ligated. PUCAcadtABC(C) was constructed from two PCR products P13/P14 (RsrII-cdtB(C)(A)/AflII) and P15/P16 (RsrII-AflII-cdtC-EcoRI), the latter PCR product and pUCAcadtABC were digested with EcoRI/RsrII and ligated. The intermediate plasmid along with the other PCR product were digested with RsrII/AflII and ligated. PUCAcadtABC(C) was generated from the PCR product produced with P17/P18 (RsrII-cdtB(C)-cdtC/EcoRI). The PCR product and pUCAcadtABC were digested with EcoRI and RsrII and then ligated.

Analysis and isolation of expressed peptides

The plasmids were constructed so that the cdt genes were under control of the lac promoter; all ligation mixtures were transformed into E. coli DH5α. Cultures of transformed E. coli were grown in 500 ml of LB broth and induced with 0.1 mM isopropyl-β-D-thiogalactoside for 2 h; bacterial cells were harvested, washed, and resuspended in 50 mM Tris (pH 8.0). The cells were frozen overnight, thawed, and sonicated. Extracts were analyzed for the presence of Cdt peptides by Western blot (described below) and for immunosuppressive activity, which was defined based upon the induction of G2 arrest in Jurkat cells (see above; Ref. 12).

Histidine-tagged peptides were isolated by nickel affinity chromatography as previously described (13). Briefly, the sonicated bacterial extracts were applied to a histidine-binding column (HiTrap Chelating HP; Amersham Biosciences, Uppsala, Sweden). The column was washed, and Histagged proteins were eluted with 500 mM imidazole.

The CdtABC(C) peptides were subjected to N-terminal amino acid analysis using Edmund degradation. Sequencing was performed on an Applied Biosystems (Foster City, CA) Procise sequencer using manufacturer’s software by the Wistar Protein and Molecular Biology Core facility (Wistar Institute, Philadelphia, PA). The derived N-terminal peptide sequence was compared with known protein sequences using the National Center for Biotechnology Information Blast program.

Expression of cdtB(C) and cdtA-gst gene and isolation of recombinant protein

PUCAcadtB(C) encodes cdtB with a C-terminal histidine tag, but lacking the signal sequence. The plasmid was prepared as previously described (13). The resulting plasmid was digested with PstI and BamHI to remove the insert, which was subsequently ligated into pUC19 under control of the lac operator and used to transform E. coli DH5α.

Cultures of transformed E. coli pUCAcadtB(C) were grown as described above. The cells were frozen overnight, thawed, and sonicated. The expressed protein was contained in inclusion bodies, which were isolated, solubilized, and refolded using a modification of the procedure that we previously described (13). Briefly, the inclusion bodies were isolated by centrifugation (10,000 × g) and washed in 50 mM Tris (pH 8.0) containing 2 M urea. The inclusion bodies were solubilized in 50 mM Tris (pH 8.0) containing 8 M urea and 100 mM 2-ME; solubilization was allowed to proceed for 2 h at 37°C. Following centrifugation, the solubilized protein was isolated on a histidine-binding column. The isolated protein was then refolded by sequential dialysis in 4, 2, 1, and 0.5 M urea in PBS (pH 7.4); the final dialysis was with PBS (pH 7.4) containing 200 μM glutathione and 0.4 M 1-arginine.

A plasmid that directs the expression of the CdtC protein was constructed in pGEX-6p-2 to generate a GST fusion protein as previously described (13). PUCAcdtA-GST was similarly prepared by ligation of the p20 PCR product, which was then ligated to pUC19 under control of the lac operator and used to transform E. coli DH5α.

The GST-fusion proteins were purified as previously described (13) and used to generate antiserum and mAb.

FIGURE 1. Effect of single-gene deletions on A. actinomycetemcomitans Cdt-induced G2 arrest. A, Jurkat cells were exposed to varying concentrations (micrograms per milliliter) of cell extract derived from E. coli transformed with pUCAcadtABC (●), pUCAcadtAB (△), pUCAcadtBC (□), pUCAcadtAC (○), or pUC19 (○) and subjected to cell cycle distribution based upon propidium iodide fluorescence using flow cytometry. Results are plotted as percentage of G2 cells (mean ± SD) of three experiments vs protein concentration; SD is indicated by bars. ED50 values, which represent the concentration required to induce 50% G2 cells, are shown in the inset. Cell cycle distribution for control cells (exposed to medium only) was 48.9 ± 2.0% (G2/G1), 35.7 ± 2.3% (S), and 15.4 ± 1.4% (G2/M). B, Shown is a Western blot analysis of E. coli extracts derived from cdt gene-containing plasmids. Cell extracts were fractionated by SDS-PAGE and analyzed by Western blot using anti-CdtB mAb, anti-CdtC mAb, and anti-CdtA polyclonal sera. The blots were analyzed by digitized scanning densitometry; the numbers indicate the relative density in comparison to pUCAcadtABC. Results are representative of three experiments.
Western blot analysis of Cdt plasmid constructs and Cdt-treated Jurkat cells

Relative expression of Cdt peptides by E. coli transformed by the various plasmid constructs was assessed by Western blot analysis. Briefly, 20 μg of each extract was separated by 10% SDS-PAGE and then transferred to nitrocellulose. The membrane was blocked with BLOTTO and then incubated with primary Abs for 18 h at 4°C (12). CdtA was detected with a polyclonal rabbit antiserum; CdtB and CdtC were detected with mAb: CdtB19D6 and Cdtc6C11, respectively. His-tagged proteins were detected with anti-His mAb (Novagen, Madison, WI). Membranes were washed, incubated with either goat anti-mouse Ig (1/1000 dilution; Southern Biotechnology Associates, Birmingham, AL) or donkey anti-rabbit (1/1000; Jackson ImmunoResearch, West Grove, PA) and finally incubated with either goat anti-mouse Ig (1/1000; Southern Biotechnology Associates, Birmingham, AL) or donkey anti-rabbit (1/1000; Amersham Biosciences) conjugated to HRP; the blots were developed using chemiluminescence and analyzed as described above.

Flow-cytometric and Western blot analysis of Jurkat cells for Cdt peptides

Jurkat cells (2 × 10⁶) were incubated in the presence of medium alone or 2 μg/ml A<sup>B</sup>B<sub>C</sub>, AB<sup>B</sup>C<sub>C</sub>, or ABC<sup>B</sup>C<sub>C</sub> for 2 h. The cells were washed, exposed to normal mouse IgG (10 μg/ml; Zymed Laboratories, San Francisco, CA), and then stained (30 min) for cell surface Cdt peptides with anti-His mAb (2.5 μg; Novagen, Madison, WI). Membranes were washed, incubated with either goat anti-mouse Ig (1/1000 dilution; Southern Biotechnology Associates, Birmingham, AL) or donkey anti-rabbit (1/1000; Amersham Biosciences) conjugated to Alexa Fluor 488 (Zenon, San Francisco, CA), and then stained (30 min) for cell surface Cdt peptides with anti-His mAb (2.5 μg; Novagen) conjugated to Alexa Fluor 488 (Zenon One Alexa Fluor; Molecular Probes, Eugene, OR) according to the manufacturer’s directions; normal mouse IgG similarly conjugated was used as a control. After washing, the cells were fixed in 2% paraformaldehyde and analyzed by flow cytometry as previously described (12).

The association of Cdt peptides with Jurkat cells was also analyzed by Western blot. Jurkat cell cultures were incubated as described for the FACS experiments. Replicate cultures were pooled, washed, and resuspended in PBS containing 0.1 mM PMSF (12); SDS sample buffer and reducing agent (Invitrogen) were added, and the samples were fractionated by 10% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with BLOTTO and then incubated with anti-His mAb (100 ng/ml; Novagen) for 18 h at 4°C. Membranes were washed and incubated with goat anti-mouse Ig (1/1000 dilution; Southern Biotechnology Associates) conjugated to HRP; the blots were developed using chemiluminescence and analyzed as described above.

Immunoprecipitation of Cdt peptides from A. actinomycetemcomitans cell extract

Anti-CdtC mAb (Cdtc6C11) was immobilized using protein G (Seize X protein G immunoprecipitation kit; Pierce, Rockford, IL) according to the manufacturer’s specifications. The immobilized Ab (500 μg) was incubated overnight with 500 μg of crude soluble sonic extract prepared from A. actinomycetemcomitans as previously described (11). After extensive washing of the Ab-gel matrix, Cdt peptides were eluted at pH 2.8; the elution was neutralized by the addition of 1 M Tris (pH 9.5). Samples were then fractionated by SDS-PAGE and analyzed by Western blot as described above.

Production of polyclonal antisera and mAbs to Cdt peptides

CdtA and CdtC were expressed as GST fusion proteins and purified as described above; rCdtB containing a C-terminal histidine tag was purified as described. Anti-CdtB and -CdtC mAb were generated as previously described (24). Briefly, BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA), 10–12 wk old, were immunized by i.p. injection with 10–20 μg of Cdt peptide on days 0, 10, 20, and 30, and allowed to rest for 30 days. Three days before fusion, the animals received 10 μg of peptide i.v. Splenocytes were fused to Sp2/0-Ag14 myeloma cells in the presence of 50% polyethylene glycol (Kodak 1450). The cells were then dispersed in Kennett’s HY medium containing 20% FBS, glutamine, oxaloacetate, pyruvate, hypoxanthine, and asparagine. The cells were fed 7
While the nature of the Cdt holotoxin is not known, our results suggest that, to produce maximum toxicity, the toxin is most likely composed of all three Cdt peptides. To explore this possibility, we constructed three plasmids, pUCAcadtA, pUCAcadtB, and pUCAcadtC, all of which contain the cdtA, cdtB, and cdtC genes. The next series of experiments were conducted to further demonstrate the requirement for all three Cdt peptides in expression of maximum toxic activity. Previously, we reported that rCdtB alone is able to induce G2 arrest in lymphocytes; similarly, as shown in Fig. 2, rCdtB is capable of inducing G2 arrest in Jurkat cells. Furthermore, the addition of 1 μg/ml of CdtB was determined from pUCAcadtA and pUCAcadtC significantly increased CdtB toxicity; the ED50 for CdtB alone was reduced from 2.5 to 0.02 μg/ml in the presence of extracts containing CdtA and CdtC. Similar results were observed for extracts derived from pUCAcadtAC (results not shown). It should be noted that the effect of CdtA and CdtC was concentration dependent; further increases in extract concentration lowered the ED50 value for CdtB, and likewise, decreases in extract levels resulted in higher ED50 values (results not shown). The addition of extract from either pUCAcadtA and pUCAcadtC caused a small, but reproducible, decrease in the ED50 for CdtB. At concentrations tested (0.1–20 μg/ml), pUCAcadtA and pUCAcadtC alone were not capable of inducing G2 arrest; furthermore, the addition of control extracts derived from pUC19 had no effect on CdtB toxicity.

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mass corresponding to ~18 kDa, suggesting that the complexes contain the truncated form of CdtA, which was also present in other E. coli extracts (Fig. 1B). Identity of each peptide was also confirmed using mAbs that recognize CdtB or CdtC, or a polyclonal sera that recognizes CdtA (Fig. 3A). Because CdtB and CdtC have been shown to contain a signal sequence, we wanted to determine whether the toxin complex was composed of full-length peptides or the cleaved product. N-terminal amino acid analysis was performed on each of the three peptides isolated from the extract derived from the pUCAacdtABChis plasmid (Fig. 3B). The N terminus for CdtB and CdtC corresponds with residues 23 and 21, respectively, of the deduced amino acid sequence for these proteins. In both instances, this is consistent with cleavage of the signal sequence of these proteins (12). As noted in Fig. 1B, two immunoreactive CdtA bands were observed in the E. coli extracts. Interestingly, only the smaller fragment was found to be associated with the complexes isolated on the His-binding columns. Analysis of this peptide indicates that the N terminus of the truncated CdtA peptide corresponds to residue 59 of the deduced amino acid sequence of this protein (Fig. 4). It is noteworthy that, under the conditions that we ran the histidine-binding columns, the full-length CdtAhis eluted at lower concentrations of imidazole; no other Cdt peptides were associated with this form of CdtA. In addition, immunoprecipitation of the holotoxin from A. actinomycetemcomitans extracts with anti-CdtC mAb coprecipitated CdtA and CdtB (Fig. 5A). It should be noted that, although both the full-length and truncated CdtA peptides were present in the A. actinomycetemcomitans extract, only the truncated form of CdtA was immunoprecipitated with the holotoxin by anti-CdtC mAb. Thus, CdtA may undergo unique processing during assembly of the toxin.

Each of the His-tagged toxin complexes were also assessed for their ability to interact with Jurkat cells. First, we tested the
complexes for toxin activity; Jurkat cells were exposed to 40 pg of each of the purified complexes and then subjected to cell cycle analysis 18 h later. As shown in Fig. 6, untreated cells exhibited a typical cell cycle profile for Jurkat cells: 49.4% G0/G1 phase, 35.1% S phase, and 15.5% G2/M phase. In contrast, cells exposed to either CdtABBC, CdtABCC, or CdtABCBC exhibited a significant increase in the percentage of cells in the G2/M phase: 69.4, 61.6, and 69.1%, respectively. The A. actinomycetemcomitans Cdt holotoxin was biologically active; exposure of Jurkat cells to 10 ng of holotoxin resulted in G2 arrest of 73% of the cells (Fig. 5, B and C). We also analyzed Jurkat cells for the presence of Cdt peptides by using immunofluorescence in conjunction with flow cytometry (Fig. 7). Following exposure to the toxin complexes for 2 h, both CdtB and CdtC were detected on the surface of Jurkat cells; mean channel fluorescence increased from 7.8 in control cells to 12.7 (CdtB) and 29.9 (CdtC). The presence of CdtA was not detected in these experiments. However, all three Cdt peptides were detected in the Western blots of Jurkat cell lysates with the anti-His mAb (Fig. 8). This suggests that the level of cell-associated CdtA may be lower than CdtB and CdtC.

**Discussion**

All known Cdt operons contain three genes, cdtA, cdtB, and cdtC, encoding proteins with similar molecular masses (20–35 kDa). However, there are conflicting reports regarding whether a single gene or multiple cdt genes encode the holotoxin responsible for the induction of cell cycle arrest in target cells. Our previous studies demonstrated that CdtB alone was sufficient to induce lymphocytes to undergo G2 arrest; however, these studies did not rule out the possibility of a role for either CdtA or CdtC. Likewise, Frisk et al. (28) and Wising et al. (30) showed that CdtB is the active component of the H. ducreyi Cdt; however, they also proposed that toxicity is dependent upon the other two Cdt components. In another study, Lara-Tejero and Galan (31) used recombinant C. jejuni peptides to demonstrate that the holotoxin was likely composed of the three Cdt proteins. In contrast, Stevens et al. (29) reported that cdtC encodes the structural toxin of H. ducreyi. In the present study, we quantitatively analyzed a series of plasmids that express various combinations of the cdt genes and demonstrate that maximum toxin activity is dependent upon the availability of all three cdt genes. Indeed, a holotoxin comprised of CdtABC was >50,000-fold more active than toxins composed of CdtAB or CdtBC. It is noteworthy that the levels of Cdt peptides were comparable in extracts derived from pUCAAcdtABC and pUCAAcdtAB; hence, the >50,000-fold difference in activity cannot simply be explained by variations in protein expression. Furthermore, pUCAAcdtBC, which expresses CdtB and CdtC, exhibited twice the activity of pUCAAcdtAB (expressing CdtA and CdtB), yet the former plasmid expressed approximately one-fifth the amount of CdtB protein. This suggests that CdtB and CdtC form a more active toxin than CdtA and CdtB. Finally, purified rCdtB was significantly more active (>100-fold) in the presence of exogenous CdtA and CdtC, although the toxic activity did not reach levels observed in pUCAAcdtABC extracts. This could reflect the requirement for processing of Cdt peptides.

The requirement for all three cdt genes for the production of a maximally active toxin is consistent with observations on the Cdt toxins of H. ducreyi (28) and C. jejuni (31). However, it is not clear whether toxic activity arises from the independent action of the three Cdt proteins or whether CdtA, CdtB, and CdtC associate into an active heterotrimer. The physical composition of the Cdt holotoxin has remained elusive, primarily because of the difficulty in purifying the putative holotoxin or the individual native proteins. To investigate the structure of the Cdt holotoxin, plasmids derived from pUCAacdtABC and pUCAacdtAB; hence, the presence of exogenous CdtA and CdtC, although the toxin activity did not reach levels observed in pUCAAcdtABC extracts. This could reflect the requirement for processing of Cdt peptides.

It is noteworthy that, although pUCAAcdtAABC, pUCAAcdtABCC, and pUCAAcdtABBC contained the complete nucleotide sequence for each of the cdt genes, N-terminal sequencing of the purified peptides showed that each polypeptide was truncated. This was not surprising for CdtB and CdtC, because their deduced amino acid sequences clearly contain consensus signal sequences and the N-terminal sequence was identical with the predicted mature protein sequence (12). However, a signal sequence has not been previously identified for CdtA. It is particularly relevant that all plasmids containing the cdtA gene expressed two immunoreactive CdtA bands corresponding to 25 and 18 kDa. Frisk et al. (28) also observed similar CdtA peptides when H. ducreyi cdtA
was expressed in *E. coli*. Interestingly, only the 18-kDa fragment was associated with the tripeptide Cdt complex isolated from both *E. coli* and *Actinobacillus actinomycetemcomitans* extracts. The N terminus of the 18-kDa peptide (LLSSSKN) corresponds to residues 59–66 in the full-length protein, confirming that the molecular mass of this peptide was ~6000 Da smaller than the complete CdtA protein. A secondary sequence was also detected at much lower concentration (LSSSKNG) corresponding to residues 60–66. The mechanism of posttranslational modification of CdtA is not known. Moreover, it is likely that the complexes of truncated peptides represent the active holotoxin. Our observations demonstrate not only that formation of the Cdt holotoxin requires all three Cdt peptides but also that the active complex may require processing of CdtA. Preliminary studies using isogenically expressed full-length peptides and truncated peptides confirm this requirement.

Flow-cytometric analysis of Jurkat cells treated for 2 h with His-tagged toxin complexes demonstrated that CdtB and CdtC could be detected on the surface of cells. Longer exposure times (up to 4 h) did not result in increased immunofluorescence. CdtA was not detected by immunofluorescence with the anti-His mAb. However, CdtA was detected by Western blot analysis, suggesting that all three Cdt peptides associate with cells. These findings differ from those of Mao and DiRienzo (32). One possible explanation for these differences is that they used full-length Cdt peptides that contained an N-terminal His tag, whereas we used mature proteins that are similar to that expressed by *A. actinomycetemcomitans*. Our failure to detect CdtA by flow cytometry could be due to inaccessibility of the CdtA-His tag when either the complex or peptide is associated with cells. Alternatively, it is possible that CdtA is either rapidly released, internalized, or modified, so that it is no longer immunologically reactive or available. Future experiments will focus on these possibilities as well as address whether these peptides are internalized.

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 appears to be composed of a tripeptide complex composed of CdtA, CdtB, and CdtC, and furthermore, each of the proteins is present in the active complex as truncated peptides. At this point, it is premature to speculate as to whether the toxin simply acts at the cell surface to trigger a signal transduction cascade or enters the cell and interacts with specific subcellular targets. Clearly, further investigation is required to identify the cellular target(s) and molecular events by which CdtDs induce cell cycle arrest. Finally, although the purified holotoxin is capable of inducing G2 arrest in a number of cell types such as HeLa cells, it is most potent on normal human lymphocytes and lymphoid cell lines, suggesting that, from a pathogenic perspective, the toxin most likely acts as an immunoinhibitory agent.

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References

22. Shenker, B. J., and I. Gray. 1976. Enrichment of PHA transformed lymphocytes from those of Mao and DiRienzo (32). One possible explanation for these differences is that they used full-length Cdt peptides that contained an N-terminal His tag, whereas we used mature proteins that are similar to that expressed by *A. actinomycetemcomitans*. Our failure to detect CdtA by flow cytometry could be due to inaccessibility of the CdtA-His tag when either the complex or peptide is associated with cells. Alternatively, it is possible that CdtA is either rapidly released, internalized, or modified, so that it is no longer immunologically reactive or available. Future experiments will focus on these possibilities as well as address whether these peptides are internalized.

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