Expression of the Cytolethal Distending Toxin (Cdt) Operon in Actinobacillus actinomycetemcomitans: Evidence That the CdtB Protein Is Responsible for G2 Arrest of the Cell Cycle in Human T Cells

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*J Immunol* 2000; 165:2612-2618; doi: 10.4049/jimmunol.165.5.2612

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Expression of the Cytolethal Distending Toxin (Cdt) Operon in *Actinobacillus actinomycetemcomitans*: Evidence That the CdtB Protein Is Responsible for G₂ Arrest of the Cell Cycle in Human T Cells

Bruce J. Shenker,\(^2\) Roselle H. Hoffmaster,\(^*\) Terry L. McKay,\(^*\) and Donald R. Demuth\(^\dagger\)

We have previously shown that *Actinobacillus actinomycetemcomitans* produces an immunosuppressive factor that is encoded by the cdtB gene, which is homologous to a family of cytolethal distending toxins (Cdt) expressed by several Gram-negative bacteria. In this study, we report that the cdt locus in *A. actinomycetemcomitans* is composed of five open reading frames, designated orf1, orf2, cdtA, cdtB, and cdtC. The deduced amino acid sequences of the five open reading frames are highly conserved among *A. actinomycetemcomitans* strains 652, Y4, 29522, and HK1651. There is also strong homology with the Cdt proteins of *Haemophilus ducreyi* (87–91%), but only partial homology with that of *Campylobacter jejuni* and *Escherichia coli* (29–48%). Analysis of *A. actinomycetemcomitans* mRNA by RT-PCR suggests that the two small open reading frames upstream of cdtA are expressed with cdtA, cdtB, and cdtC. We next utilized a series of plasmids that express various combinations of the cdt genes to determine their requirement for expression of immunoinhibitory activity. Cell extracts of *E. coli* transformed with each of the plasmids were tested for their capacity to induce G₂ arrest in the cell cycle of PHA-activated human T cells. These experiments suggest that expression of cdtB alone is sufficient to induce G₂ arrest in human T cells, but do not exclude the possibility that cdtC also contributes to cell cycle arrest. The implications of our results with respect to the function of the individual Cdt proteins are discussed. *The Journal of Immunology*, 2000, 165: 2612–2618.

*Actinobacillus actinomycetemcomitans*, a nonmotile, Gram-negative coccobacillus, is associated with several human diseases. These include endocarditis, meningitis, osteomyelitis, s.c. abscesses, and periodontal disease (1–6). Although the pathogenic mechanism(s) by which *A. actinomycetemcomitans* acts to cause disease is not known, it does produce several potential virulence factors capable of facilitating colonization, destroying host tissue, inhibiting tissue repair, and interfering with host defenses (reviewed in Ref. 5). With respect to the latter, several studies suggest that impaired host defense mechanisms may contribute to infectious diseases associated with *A. actinomycetemcomitans* (reviewed in Refs. 5 and 7). In this regard, we have previously shown that *A. actinomycetemcomitans* produces a heat-labile immunosuppressive factor (ISF)\(^3\) that is capable of inhibiting both human T and B cell function (8–11). Furthermore, we have recently shown that ISF-mediated immunosuppression is due to interference with the normal progression of lymphocytes through the cell cycle. Specifically, pretreatment of mitogen-activated human T cells results in their arrest in the G₂ phase of the cell cycle; these effects are associated with the failure to activate the cyclin-dependent kinase, cdk1 (12).

In addition to shedding new light on mode of action, our most recent studies have indicated that the *A. actinomycetemcomitans* ISF is a product of the cdtB gene, one of three genes encoding the cytolethal distending toxin (Cdt). The Cdt family includes the newly described family of heat-labile protein cytotoxins produced by several Gram-negative bacterial species. These include diarrheal disease-causing enteropathogens such as some *Escherichia coli* isolates, *Campylobacter jejuni*, and *Shigella dysenteriae* (13–16). More recently, related toxins have been identified in *Haemophilus ducreyi*, a human pathogen responsible for the formation of chancroid ulcers and buboes, and *A. actinomycetemcomitans* strains Y4 and 652 (17, 18). The Cdt proteins cause cell cycle arrest and in some cell lines a progressive cellular distension and finally death; it should be noted that the gross cellular change associated with Cdt activity is clearly different from those caused by other known toxins that induce rapid morphological alterations culminating in cell death (17, 19). There is now clear evidence that the Cdt is encoded by three genes, designated cdtA, cdtB, and cdtC, which are arranged in an apparent operon. These three genes specify polypeptides with predicted or apparent molecular masses of approximately 25–35 kDa.

To date, limited information is available that addresses the functional relationship(s) between the three Cdt proteins. In this regard, we have shown that highly purified CdtB from *A. actinomycetemcomitans* is not only capable of causing cell cycle arrest in human T cells, but is also able to induce all of the biological properties previously associated with the Cdt family: cell cycle arrest and morphological alterations in cell lines such as HeLa cells. In contrast, Stevens et al. (20) have reported contradictory findings and propose that CdtC is the biologically active component of *H. ducreyi* Cdt. Indeed, the possibility exists that the highly purified preparation of CdtB could have been contaminated with small amounts of CdtC (or CdtA). Therefore, the focus of this investigation was to further analyze the toxic activity of CdtB. In this

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\(^2\) Abbreviations used in this paper: ISF, immunosuppressive factor; Cdt, cytolethal distending toxin; orf, open reading frame.

\(^3\) This work was supported by U.S. Public Health Service Grant DE06014.

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study, we show that the *A. actinomycetemcomitans* *cdtA*, *cdtB*, and *cdtC* genes are coexpressed with two small open reading frames that reside upstream of *cdtA*. Furthermore, recombinant expression of *cdtB* alone generates an active toxin that is capable of inducing G2 arrest in human lymphocytes.

**Materials and Methods**

**Cell isolation, culture, and analysis of cell cycle**

Human PBMC were prepared by buoyant density centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ), and purified T cells were obtained by E-rosette formation, as described previously (8). Briefly, sheep erythrocytes were washed and treated with 0.14 M 2-aminooxyethylisothiouronium bromide at pH 9 for 15 min. After four washes, the erythrocytes were incubated with human PBMC as a cell pellet for 60 min. The cells were then gently resuspended; nonrosetted cells were separated from rosetted cells on Ficoll-Hypaque, as described above. The rosetted cells found in the pellet were lysed to remove erythrocytes and found to contain >98% T cells when stained with anti-CD3 mAb (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed by flow cytometry.

To measure Cdt-induced cell cycle arrest, 1-ml cultures of T cells (1 × 10⁶ cells/ml) were activated with PHA (1 μg/ml; Abbott Laboratories, Abbott Park, IL), following pretreatment for 45 min with appropriate bacterial cell extract; the cells were incubated in RPMI 1640, antibiotics, and 2% heat-inactivated human AB sera. Flow cytometric analysis was used to analyze cell cycle distribution 72 h later, as previously reported (10). Briefly, T cells were washed and fixed for 60 min with cold 80% ethanol. After washing, the cells were stained with propidium iodide (10 μg/ml containing 1 mg/ml RNase) for 30 min. Samples were analyzed on a Becton Dickinson FACStarPLUS flow cytometer. Propidium iodide fluorescence was excited by an argon laser operating at 488 nm and fluorescence 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).

**Sequencing of *A. actinomycetemcomitans* cdt gene cluster**

We previously isolated the complete cdt locus from an *A. actinomycetemcomitans* strain 652 library in EMBL3 by screening with the *cdtB* gene as a probe (12). One clone with a 23-kbp insert containing the entire cdt operon was chosen for further study; restriction analysis of this clone indicated that the cdt gene cluster could be localized to a 9-kb fragment following digestion with EcoRI. Further digestion of the 9-kb fragment with Smal produced a 4-kb fragment that was isolated and cloned into pUC19 to generate pUCAacd1. The sequences of the orf1, orf2, cdtA, and cdtC were determined from DNA fragments flanking the cdtB gene, which were derived from this clone. The *A. actinomycetemcomitans* strain 652 cdt gene sequence has been submitted to the GenBank database (AF102554).

**Construction of plasmids expressing cdt genes**

Several of the *cdt* gene constructs used in this study were derived from the pUCAacd2 plasmid, which contains the 4-kb insert described above (also see Table I). This DNA fragment contains cdtA, cdtB, cdtC, a small upstream open reading frame (orf2), and an additional 2.5 kb of sequence downstream of the *cdtC* gene. To assess the role of individual genes in generating biologically active toxin, a series of plasmids were constructed that lacked the region downstream of *cdtC* and/or one or more of the open reading frames present in pUCAacd2. These plasmids were prepared by first digestig pUCAacd2 with NheI, which cleaves within the cdtB gene, and EcoRII, which cleaves in the pUC multiple cloning region, as shown in Fig. 1. The resulting 3.2-kb DNA fragment contains the plasmid vector, orf2, and the first 250 residues of the cdtA gene (Fig. 1). PCR products were then generated from primer pairs P3/P4, P3/P5, and P3/P6 (see Table I) to generate products that possess an upstream NheI site and a downstream EcoRII site. The resulting fragments were subsequently ligated to the 3.2-kbp product from the restriction digestion above. Ligation with the 1946-bp P3/P4 product yielded pUCAacd3, which contains orf2, cdtA, cdtB, and cdtC, but lacks the 2.5-kbp sequence downstream of the *cdt* operon. Plasmid pUCAacd4, which contains orf2, cdtA, and cdtB, was produced by ligation with the 1350-bp P3/P5 product, and pUCAacd7, containing only orf2 and cdtA, resulted from ligation with the 430-bp P3/P6 product.

Plasmid pUCAacd3 was further modified by the addition of orf1 sequences to generate pUCAacd1. pUCAacd3 was digested with *XmnI* and *PstI*, which cleave within orf1 and in the vector multiple cloning region, respectively (Fig. 1). The resulting plasmid was ligated with the 716-bp PCR product generated from primer pair P1/P2, which introduced the complete orf1 sequence along with 466 bp of additional upstream sequence and regenerated an intact copy of orf2.

Two additional plasmids, pUCAacd5 and pUCAacd6, in which *cdtB* and *cdtA*, respectively, were inactivated, were also prepared. To construct pUCAacd5, a 273-bp segment near the end of the gene was excised from *cdtB* by digestion of pUCAacd3 with *NcoI* and *PstI*. The resulting plasmid contains orf2, cdtA, the truncated cdtB gene, and cdtC. pUCAacd6 was prepared by digesting pUCAacd3 with NheI, then blunting with Klenow polymerase followed by religation. The resulting plasmid thus contains a frameshift mutation in the *cdtA* gene.

All ligation mixtures were transformed into *E. coli* DH5α. For analysis of immunosuppressive activity, 10-ml cultures were grown in LB media supplemented with 100 μg/ml ampicillin to an OD₆₅₀ of 0.4. Samples of the culture supernatant and cell pellet were collected for analysis. The cell pellet was sonicated following a wash in 50 mM Tris (pH 8). Immunosuppressive activity was defined as growth arrest of PHA-activated cells and was assessed as an accumulation of cells in the G2 phase of the cell cycle over that observed in control (PHA alone) cultures (12). It should be noted that cells were exposed to the same amount of each cell extract based upon total protein content, and the plasmids were classified based upon their ability to express immunoinhibitory activity (G2 accumulation). Relative differences in immunosuppressive potency are difficult to determine since it was not possible to account for variations in the levels of expression between cultures of *E. coli* transfected with each of the plasmids.

**Expression of individual cdt genes and isolation of recombinant proteins**

Plasmids that contain only cdtA, cdtB, or cdtC were constructed as follows. pUCAacdB-his encodes CdtB with a C-terminal histidine tag, but lacking

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>PCR Product Size (bp)</th>
<th>Genes Expressed by Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUCAacd1</td>
<td>P1</td>
<td>CTCAGTCGAGCTGTTTACGCGAAGG</td>
<td>182-198</td>
<td>716</td>
<td>orf1, orf2, cdtA, cdtB, cdtC</td>
</tr>
<tr>
<td>pUCAacd3</td>
<td>P2</td>
<td>GGGGGGAGGTTTTAGCCGGTG</td>
<td>335-350</td>
<td>726</td>
<td>orf2, cdtA, cdtB, cdtC</td>
</tr>
<tr>
<td>pUCAacd4</td>
<td>P3</td>
<td>CAGAGGTTCAGCTGTTTACGCGAAGG</td>
<td>1391-1414</td>
<td>1946</td>
<td>orf2, cdtA, cdtB, cdtC</td>
</tr>
<tr>
<td>pUCAacd7</td>
<td>P4</td>
<td>CCACTTCTCTGTTTACGCGAAGG</td>
<td>1391-1414</td>
<td>1946</td>
<td>orf2, cdtA, cdtB, cdtC</td>
</tr>
<tr>
<td>pUCAacdB-his</td>
<td>P5</td>
<td>GCGGCGGAGGTTTTAGCCGGTG</td>
<td>1391-1414</td>
<td>1946</td>
<td>orf2, cdtA, cdtB, cdtC</td>
</tr>
<tr>
<td>pUCAacdC-GST</td>
<td>P6</td>
<td>GGGCGGCGGTTTTAGCCGGTG</td>
<td>1391-1414</td>
<td>1946</td>
<td>orf2, cdtA, cdtB, cdtC</td>
</tr>
</tbody>
</table>

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the signal sequence. The plasmid was prepared by ligating the 801-bp P7/P8 PCR product (see Table I; Fig. 1) into pGEM-T. The resulting plasmid was digested with Psfl and BamHl to remove the insert, which was subsequently ligated into pUC19 and used to transform E. coli DH5α. A plasmid that directs the expression of the CdtC protein was constructed in pGEX-6p-2 to generate a GST fusion protein. pUCAcadC-GST was prepared by ligation of the 511-bp P9/P10 PCR product (see Table I) into pGEM-T. The insert was then isolated from the resulting plasmid by digestion with EcoRI and BamHl, ligated into pGEX-6p-2, and transformed into E. coli DH5α.

Cultures of transformed E. coli pUCAcadB-His were grown in 500 ml LB broth and induced with 0.1 mM IPTG; bacterial cells were harvested, washed, and resuspended in 50 mM Tris (pH 8) containing 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, and 0.4 mg/ml lysozyme. The cells were frozen overnight, thawed, and sonicated. The expressed protein was contained in inclusion bodies that were isolated, solubilized, and refolded, as described by Li et al. (21). Briefly, the inclusion bodies were isolated by centrifugation (10,000 × g) and washed in 50 mM Tris (pH 8) containing 2 M urea. The inclusion bodies were solubilized in 50 mM Tris (pH 8) 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 refolded by transferring the sample (1/100 dilution) into 100 mM Tris (pH 8) containing 0.5 M l-arginine and 1 mM glutathione disulfide; the refolded protein was then transferred to 20 mM Tris, pH 8, containing 0.5 M NaCl by passage through a Sephadex G25 column. The recombinant protein was then isolated on a histidine-binding column (His-Bind Quick Columns; Novagen, Madison, WI), and eluted from the column with 60 mM imidazole.

E. coli pUCAcadC-GST was also found to produce inclusion bodies upon induction of expression. The inclusion bodies were isolated as described above. The refolded protein was then transferred to PBS by passage through a Sephadex G25 column. The fusion protein was then purified using a commercial kit (Amersham Pharmacia Biotech Bulk Purification Module), and the recombinant protein separated from GST by digestion with PreScission Protease (Amersham Pharmacia Biotech), according to the manufacturer’s recommendations.

**RNA extraction and RT-PCR**

Total RNA was isolated from 200-ml cultures of A. actinomycetemcomitans strain 652, JP-2, and Y4 (22). Briefly, the bacteria were harvested by centrifugation and resuspended in cold 200 mM Tris (pH 8) containing 20 mM EDTA, 20 mM sodium azide, and 20 mM aurothioglucose (Sigma, St. Louis, MO). The cells were centrifuged at 9000 × g for 5 min and resuspended in 50 mM Tris, pH 7, containing 8% sucrose, 5% Triton X-100, 5 mM EDTA, and 10 mM vanadyl ribonucleoside complex (Life Technologies, Grand Island, NY). RNA was then isolated by phenol-chloroform extraction. Following precipitation in 3 M sodium acetate (pH 6), the pellets were resuspended in diethyl pyrocarbonate (DEPC)-water and the RNA isolated by centrifugation in CsCl. The RNA pellet was suspended in TE buffer, treated with RNase-free DNase (Promega, Madison, WI), and precipitated with ethanol at −70°C.

RT-PCR was conducted using a commercial kit (Perkin-Elmer RNA PCR kit; Foster City, CA), according to the manufacturer’s protocol. Briefly, 1.5 μg RNA was converted to single-strand cDNA with primers derived from the cdt operon sequence determined above. A series of oligonucleotide primers were synthesized (see Table II) to generate products that spanned all of the intergenic regions between orf1 and cdtC. PCR reactions were performed on the cDNA template after the addition of Taq DNA polymerase, deoxyribonucleoside triphosphates (1 mM), and each primer (0.15 μM) with a Perkin-Elmer Thermocycler under the following conditions (1 min, 45 s at 95°C, 50 cycles, 15 s at 95°C, 15 s at 50°C, 1 min at 72°C; 7 min at 72°C). DNA products were visualized by ethidium bromide staining after electrophoresis in 1% agarose. Controls included samples either lacking the reverse transcriptase or the RNA template.

**Table II. Summary of oligonucleotide primers used for RT-PCR analysis**

<table>
<thead>
<tr>
<th>Region</th>
<th>Oligonucleotide Primers</th>
<th>Residue Range</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1 + orf2</td>
<td>CGGACGCAATCAAGCCGTACG</td>
<td>695–716</td>
<td>327</td>
</tr>
<tr>
<td>orf1 + orf2</td>
<td>GATATGCTTGATACTAACCG</td>
<td>695–716</td>
<td>264</td>
</tr>
<tr>
<td>orf2 + cdtA</td>
<td>GCAAAGCCACTAAACC</td>
<td>1197–1215</td>
<td>265</td>
</tr>
<tr>
<td>orf1 + orf2 + cdtA</td>
<td>CCGAGCAATCAAGCCGTACG</td>
<td>695–716</td>
<td>521</td>
</tr>
<tr>
<td>cdtA + cdtB</td>
<td>GAAACAAGCCACTAAACC</td>
<td>1197–1215</td>
<td>518</td>
</tr>
<tr>
<td>cdtA + cdtB</td>
<td>GATGGGACAAAATGGGGC</td>
<td>1401–1418</td>
<td>772</td>
</tr>
<tr>
<td>cdtB + cdtC</td>
<td>CATGGGGAAACGGCAATTT</td>
<td>2094–2112</td>
<td>157</td>
</tr>
<tr>
<td>cdtB + cdtC</td>
<td>AGTGGGAAGCGCAATTT</td>
<td>2679–2696</td>
<td>157</td>
</tr>
</tbody>
</table>
Results

Analysis of the A. actinomycetemcomitans strain 652 cdt gene cluster

Previously, we isolated and sequenced the A. actinomycetemcomitans cdtB gene (12). Subsequent sequencing upstream and downstream from the A. actinomycetemcomitans strain 652 cdtB gene identified genes exhibiting homology to known cdtA and cdtC genes. In addition, two short open reading frames, designated orf1 and orf2, were identified upstream of the cdtA gene. As shown in Fig. 2, the size of these genes are 263 bp (orf1), 258 bp (orf2), 669 bp (cdtA), 852 bp (cdtB), and 589 bp (cdtC). Each of the genes is preceded by a ribosome binding site, and relatively short intergenic domains lie between each gene. The entire nucleotide sequence of the A. actinomycetemcomitans strain 652 cdt locus was submitted to GenBank (accession no. AF102554).

As summarized in Table III, the deduced amino acid sequence of the five A. actinomycetemcomitans strain 652 open reading frames is highly conserved in A. actinomycetemcomitans strains, Y4, 29522, and HK1651. One major difference was noted: the nucleotide sequence upstream of orf1 in strain 652 diverges from the corresponding sequence in strain Y4. In addition, the deduced peptide sequences of CdtA, CdtB, and CdtC from A. actinomycetemcomitans strain 652 are highly similar to the corresponding Cdt proteins of H. ducreyi (87–91% identity), but share only partial homology with the Cdt proteins of C. jejuni (36–48%) and E. coli (29–49%).

To determine whether the A. actinomycetemcomitans cdt genes are coexpressed with orf1 and orf2, mRNA samples from A. actinomycetemcomitans strains 652, Y4, and JP-2 were analyzed by RT-PCR using a series of oligonucleotide primers designed to generate products that spanned all of the intergenic regions between orf1 and CdtC (see Table II). The results are shown in Fig. 2. For example, as shown in lanes 1–3, primers that span regions of orf1, orf2, and cdtA generated the predicted product of 327 bp encompassing the intergenic region between orf1 and orf2, the predicted 264-bp product spanning the intergenic region between orf2 and cdtA, and the 521-bp product of both of the above intergenic sequences, respectively. Two sets of primers were used to link cdtA and cdtB (see Table II). The first set of primers spanned the downstream half of cdtA through the first 35 residues of cdtB and generated a RT-PCR product of 518 bp, as predicted (Fig. 2, lane 4). A second set of primers generated a 772-bp product that spanned the region, including the last 53 residues of cdtA and approximately the first half of cdtB (Fig. 2, lanes 5 and 6). Finally, two sets of primers were used to show linkage of cdtB and CdtC. These primer sets generated the expected products of 772 and 157 bp (Fig. 2, lanes 6 and 7). As shown in Fig. 2A–C, similar results were obtained from all three of the A. actinomycetemcomitans strains examined. Control reactions consisted of samples that lacked the RNA template or were not treated with the reverse transcriptase. None of the controls produced detectable products (not shown). These observations suggest that the five open reading frames are coexpressed in a manner consistent with that of an operon.

<table>
<thead>
<tr>
<th>Bacterial Species/Strain</th>
<th>% Amino Acid Sequence Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orf1</td>
</tr>
<tr>
<td>A. actinomycetemcomitans strain Y4</td>
<td>100</td>
</tr>
<tr>
<td>A. actinomycetemcomitans strain 29522</td>
<td>100</td>
</tr>
<tr>
<td>A. actinomycetemcomitans strain HK1651</td>
<td>100</td>
</tr>
<tr>
<td>H. ducreyi</td>
<td></td>
</tr>
<tr>
<td>C. jejuni</td>
<td>36</td>
</tr>
<tr>
<td>E. coli strain 91428</td>
<td>29</td>
</tr>
</tbody>
</table>
However, we could not exclude the possibility that the CdtB preparations also contained other Cdt proteins such as CdtC. To determine the contribution of each cdt gene in generating toxic activity, plasmids containing defined deletions of cdtA, cdtB, and/or the cdtC genes were constructed (see Fig. 3), and cell extracts derived from recombinant E. coli cells transformed with each of the plasmids were analyzed for toxicity, as described in Materials and Methods. As shown in Fig. 4A, control cultures exposed to medium alone do not proliferate and exhibit a single peak of propidium iodide fluorescence representing cells in the G0/G1 phase. In contrast, activation of cells with PHA alone elicits a cell cycle profile consisting of cells not only in the G0/G1 phase (80.1%), but also in the S (14%) and G2/M (5.9%) phases as well (Fig. 5B). Plasmid pUCAcad1, which contains all five of the cdt open reading frames, expresses immunosuppressive activity that is evident from the accumulation of T cells in the G2/M phase (14.7%) of the cell cycle (Fig. 5D); similar results were found using extracts of A. actinomycetemcomitans containing native CdtB (not shown) (12). Extracts from cells transformed with pUC alone had no effect on PHA-induced cell cycle progression (Fig. 4C).

Deletion of orf1 had little effect upon Cdt-induced cell cycle arrest. As shown in Fig. 4E, treatment of T cells with extracts from E. coli transformed with pUCAcad2, which lacks orf1, results in the accumulation of G2/M cells (18.3%) over that observed in the PHA control cultures. A third plasmid, pUCAcad3, which lacks orf1 as well as the 2.5-kb sequence downstream of cdtC (see Fig. 3), also induces a G0/G1 arrest (10.7% G2/M cells) (Fig. 4F). These results strongly suggest that the entire functional cdt gene cluster is contained in a fragment comprising only orf2, cdtA, cdtB, and cdtC, but does not exclude a possible role for orf1 in the expression of toxin activity in A. actinomycetemcomitans.

To determine the contribution of the individual cdtA, cdtB, and cdtC genes, three additional plasmid constructs were tested. Plasmid pUCAcad4 contains the complete sequence for cdtA and cdtB, but lacks the cdtC gene. As shown in Fig. 4G, T cells pretreated with cell extracts from E. coli transformed with pUCAcad4 exhibit cell cycle arrest; 13.8% of the cells are in the G2/M phase representing a 2-fold increase over that observed in the PHA control cultures (B). The requirement for cdtA was assessed with pUCAcad6, which contains the complete cdtB and cdtC genes, but in which cdtA has been functionally deleted by a frameshift mutation near its 5' end. As shown in Fig. 4H, T cells treated with pUCAcad6 extracts exhibit 17.4% G2/M cells. Another plasmid, pUCAcad7, containing only the cdtA gene and orf2, failed to produce toxin activity (I). Finally, the requirement for cdtB was probed with pUCAcad5, which contains intact cdtA and cdtC genes, but is capable of expressing a truncated cdtB gene that lacks the last 250 residues of the sequence. Surprisingly, this construct was also capable of inducing cell cycle arrest, as indicated by an accumulation of G2/M phase cells (23.2%; Fig. 4J).

The results above suggest that Cdt-induced cell cycle arrest in human T cells may be associated with both CdtB and CdtC. To further evaluate these proteins, two isogenic plasmids were constructed that express only cdtB or cdtC. The CdtB peptide was expressed as a GST fusion protein, while the cdtB gene was modified so that the peptide contained a his tag at the C terminus (see Materials and Methods). E. coli transformed with each of the plasmids contained inclusion bodies. However, solubilization of the inclusion bodies from each strain was effected in 8 M urea, and the protein obtained was subsequently refolded and purified over a nickel column (CdtB) or glutathione-Sepharose (CdtC). The isolation and subsequent purification of CdtB and CdtC from inclusion bodies are shown in Fig. 5A. The purified peptides were then assayed for their ability to cause cell cycle arrest in human T cells (see Fig. 5B–E). B and C, Represent cell cycle profiles from control (medium alone) and PHA-activated T cells, respectively. The latter contains cells in the G0/G1 (80.2%), S (12.9%), and G2/M (8.9%) phases, as shown in Fig. 5F. As shown in Fig. 5G, the CdtB peptide induces a G0/G1 arrest (10.7% G0/G1 cells) (Fig. 5H). Similar results were found using extracts of E. coli, treatment of T cells with extracts from A. actinomycetemcomitans (D). Extracts from cells transformed with pUC alone had no effect on PHA-induced cell cycle progression (Fig. 4C).

Deletion of orf1 had little effect upon Cdt-induced cell cycle arrest. As shown in Fig. 4E, treatment of T cells with extracts from E. coli transformed with pUCAcad2, which lacks orf1, results in the accumulation of G2/M cells (18.3%) over that observed in the PHA control cultures. A third plasmid, pUCAcad3, which lacks orf1 as well as the 2.5-kb sequence downstream of cdtC (see Fig. 3), also induces a G0/G1 arrest (10.7% G2/M cells) (Fig. 4F). These results strongly suggest that the entire functional cdt gene cluster is contained in a fragment comprising only orf2, cdtA, cdtB, and cdtC, but does not exclude a possible role for orf1 in the expression of toxin activity in A. actinomycetemcomitans.

To determine the contribution of the individual cdtA, cdtB, and cdtC genes, three additional plasmid constructs were tested. Plasmid pUCAcad4 contains the complete sequence for cdtA and cdtB, but lacks the cdtC gene. As shown in Fig. 4G, T cells pretreated with cell extracts from E. coli transformed with pUCAcad4 exhibit cell cycle arrest; 13.8% of the cells are in the G2/M phase representing a 2-fold increase over that observed in the PHA control cultures (B). The requirement for cdtA was assessed with pUCAcad6, which contains the complete cdtB and cdtC genes, but in which cdtA has been functionally deleted by a frameshift mutation near its 5' end. As shown in Fig. 4H, T cells treated with pUCAcad6 extracts exhibit 17.4% G2/M cells. Another plasmid, pUCAcad7, containing only the cdtA gene and orf2, failed to produce toxin activity (I). Finally, the requirement for cdtB was probed with pUCAcad5, which contains intact cdtA and cdtC genes, but is capable of expressing a truncated cdtB gene that lacks the last 250 residues of the sequence. Surprisingly, this construct was also capable of inducing cell cycle arrest, as indicated by an accumulation of G2/M phase cells (23.2%; Fig. 4J).
(6.9%) phases of the cell cycle. As shown in D, recombinant CdtB was capable of altering cell cycle progression; 14% of the cells were in the G2 phase, representing a 2-fold increase over the PHA control cultures. These results suggest that the CdtB protein is capable of inducing cell cycle arrest of human lymphocytes in the absence of CdtC. In contrast, cells treated with recombinant CdtC (Fig. 5E) exhibit a cell cycle profile similar to that observed with cells incubated with PHA alone; 5.3% of the cells were in G2/M. It should be noted that a wide range of concentrations was employed in preliminary studies; in all instances, extracts prepared from the isogenic construct containing the cdtn gene failed to alter the cell cycle progression of PHA-activated T cells.

**Discussion**

All known cdtn operons contain three genes, cdtnA, cdtnB, and cdtnC, encoding proteins with similar molecular masses (20–35 kDa). The *A. actinomycetemcomitans* cdtn operon appears to be unique in that it contains two small open reading frames, orf1 and orf2, upstream of cdtnA that are not present in other cdtn gene clusters. These open reading frames are well conserved in all four *A. actinomycetemcomitans* strains examined. The nucleotide sequence upstream of orf1 revealed no additional open reading frames within several hundred bases. However, the nucleotide sequence of this region diverges considerably between *A. actinomycetemcomitans* 652 and Y4. This observation is consistent with Mayer et al. (23), who find evidence for considerable genetic diversity around the cdtn locus of *A. actinomycetemcomitans*. RT-PCR analysis suggests that orf1 and orf2 are cotranscribed with cdtnA, cdtnB, and cdtnC. Thus, the primary transcript derived from the cdtn operon of *A. actinomycetemcomitans* appears to consist of five open reading frames. At present, it is not known whether orf1 and orf2 are translated in vivo, although each sequence is preceded by a suitable ribosome-binding consensus sequence. In addition, deletion of orf1 had little effect on the expression of toxic activity, suggesting that it is not essential for the expression of immunosuppressive activity in *E. coli*. Further studies will be required to assess the role of orf2 in toxin expression and export.

There are conflicting reports regarding whether a single gene or multiple genes of the *E. coli* or *H. ducreyi* cdtn operons encode the structural Cdt (reviewed in Ref. 24). Our previous study suggested that cdtnB encoded the biologically active immunosuppressive factor in *A. actinomycetemcomitans*. However, we could not completely rule out the possibility that small amounts of CdtnC were present in the CdtB preparations used for these studies. In contrast, Stevens et al. (20) suggest that cdtnC encodes the structural toxin of *H. ducreyi*, and other reports suggest that two or even all three cdtn genes are required for toxic activity in *E. coli* (24). Our results clearly show that the CdtB protein of *A. actinomycetemcomitans*, when expressed in a cdtnΔcdtnC background, induces G2 arrest in lymphocytes. Thus, cdtnB encodes a biologically active component of the *A. actinomycetemcomitans* Cdt that is capable of acting independently of the other gene products encoded by the cdtn operon.

Little information is available for any of the CdtNs regarding the nature of the holotoxin that is secreted by the bacterial cell. Our results show that the CdtnC protein, when expressed in a cdtnΔcdtnB background, had little effect on human lymphocytes. However, we cannot rule out a role for CdtnC in immunosuppression in vivo. Indeed, pUCAcodtn5, which contains the intact cdtnA and cdtnC genes as well as a truncated cdtnB gene, also induced G2 arrest in lymphocytes. One possible explanation for this result is that the truncated CdtB protein, which is presumably expressed from pUCAcodtn5, retains immunosuppressive activity. Alternatively, cdtnC may also encode a biologically active immunosuppressive factor that was unable to refold properly when isolated from inclusion bodies in *E. coli*. Thus, it is possible that the cytotoxic distending holotoxin of *A. actinomycetemcomitans* may be a heterodimer of CdtB and CdtnC, with one or both of the individual proteins being capable of inducing G2 arrest. This alternative is also consistent with the work of several investigators, demonstrating that Abs against the *H. ducreyi* Cdt polypeptide neutralize the Cdt activity of that organism (17, 25).

The role of the CdtNs in pathogenesis remains to be clarified. Our results suggest that these proteins may be involved in modulating the immune response of the host. Such immunosuppressive activity could lead to a state of hyporesponsiveness that may contribute to the disease process by favoring colonization by the organism itself or by that of other opportunistic organisms. Thus, we propose that infection by Cdt-producing microbial species would impair host defense mechanisms and that this disturbance would, in turn, adversely affect the development of normal immunologic defense
mechanisms. In addition to our demonstration that Actinobacillus actinomycetemcomitans CdtB is a potent immunosuppressive factor, Gelfanova et al. (26) have recently shown that the H. ducreyi Cdt is also capable of impairing lymphoid function. It will be interesting to determine whether the Ctds of other Gram-negative organisms such as E. coli, Shigella, or Campylobacter are also capable of impairing lymphoid function. Demonstrating conservation of function in these cdt operons, which have diverged in sequence from the Actinobacillus actinomycetemcomitans and H. ducreyi genes, might highlight important regions of the Cdt proteins that are required for interactions with lymphocytes.

In conclusion, our results show that CdtB derived from Actinobacillus actinomycetemcomitans is active against human lymphocytes in the absence of other Cdt proteins and when expressed in a cdtA::tetr background. However, it is possible that the mature toxin is actually comprised of multiple subunits including CdtC. It is also important that any definition of biologic activity must be made in the context of the target cell. In this regard, our studies clearly define the Actinobacillus actinomycetemcomitans Cdt as an immunoinhibitory protein(s).

Acknowledgements
We acknowledge the technical expertise of Rose Espiritu and Ali Zekavat as well as the School of Dental Medicine Flow Cytometry Facility for their support of these studies.

References