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Resistance of Human Periodontal Ligament Fibroblasts to the Cytotoxic Distending Toxin of *Actinobacillus actinomycetemcomitans*

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Abstract

Background—The cytotoxic distending toxin (CDT) of *Actinobacillus actinomycetemcomitans* is a typical member of this Gram-negative bacterium holotoxin family that targets a wide spectrum of eukaryotic cells, typically causing cell cycle arrest at either the G₁ or G₂/M phase of the cell cycle. In view of the possible role of the CDT as a prominent *A. actinomycetemcomitans* virulence factor in periodontal diseases, we have examined the effects of the toxin on primary cultures of human periodontal ligament fibroblasts (HPLF).

Methods—HPLF and an immortalized human gingival epithelial cell line, GSM-K, were exposed to recombinant *A. actinomycetemcomitans* CDT. Effects of the toxin on cell proliferation and cell cycle were assessed by a cell viability assay and flow cytometry, respectively. Double-strand DNA damage was detected by pulsed field gel electrophoresis. Binding of the toxin and its individual subunits to HPLF was examined by immunofluorescence microscopy.

Results—Viability of HPLF was not reduced following prolonged exposure to the CDT. There was no indication of cell cycle arrest or double-strand DNA damage. GSM-K cells exhibited morphological alterations and a rapid decrease in cell viability within 6 and 12 hours, respectively, following exposure to the toxin for 5 minutes. These effects were dependent on toxin dose and age of the cultures and occurred more rapidly compared to CDT-treated HeLa cells. CDT-treated GSM-K cells displayed cell cycle arrest at the S phase of growth and double-strand DNA damage was observed by 6 hours post-intoxication. Holotoxin and the CdtA subunit were detected on the surface of both HPLF and epithelial cells.

Conclusions—These results demonstrate that HPLF are resistant to the cytotoxic effects of the *A. actinomycetemcomitans* CDT. The mechanism of resistance is not known but may be related to the inability of the toxin to cause DNA damage. The difference in sensitivities of HPLF and oral epithelial cells to the CDT has important implications for the role of this putative microbial virulence factor in periodontal pathogenesis.

Keywords

Actinobacillus actinomycetemcomitans; cell cycle; cells, epithelial; fibroblasts, periodontal; periodontal diseases/pathogenesis; toxin, cytotoxic distending

Cytolethal distending toxin (CDT) is a secreted bacterial protein holotoxin that induces growth arrest in a wide variety of eukaryotic cells. The holotoxin is the product of three genes expressed by a handful of facultative or microaerophilic Gram-negative pathogenic bacterial species. The species identified to date that express a biologically active CDT include select strains of enteropathogenic *Escherichia coli*,^{1,2} *Campylobacter jejuni*,³ *Campylobacter upsaliensis*,⁴ *Campylobacter coli*,⁵ *Shigella dysenteriae*,^{6,7} *Haemophilus ducreyi*,⁸ *Helicobacter hepaticus*,^{9,10} *Helicobacter flexispira*,¹¹ *Helicobacter bilis*,⁹ *Helicobacter canis*,⁹ and *Actinobacillus actinomycetemcomitans*.¹²⁻¹⁴ Organization of the genetic locus and the structure and biological activity of the holotoxin are fairly well conserved among the CDT produced by these genera.^{8,10,12,15,16} The *cdt* locus is a polycistronic operon containing three essential genes, *cdtA*, *cdtB*, and *cdtC*, which encode for polypeptides having sizes of 27 to 30, 29 to 32, and 20 kDa, respectively.

The various CDTs inhibit the proliferation of immortalized human cell lines, including HeLa,^{3,17-20} KB,³ HEP-2,^{3,21-23} Vero,³ HaCat,^{22,24} Jurkat,²⁵ and Caco-2,²⁰ as well as primary human cells such as lymphocytes^{4,13,25} and fibroblasts.²⁶⁻²⁸ The initial step that leads to cytotoxicity appears to be the introduction of double-strand DNA breaks, most likely due to the activity of the CdtB polypeptide.²⁹⁻³¹ Subsequent growth arrest results from a block in cell cycle progression. However, the location of the block is cell line or strain dependent. The epithelial cells lines HeLa,^{10,14,17-20} HEP-2,²¹ and Caco-2²⁰ are arrested at the G₂/M phase transition due to inhibition of dephosphorylation of the checkpoint protein kinase Cdc2 (Cdk1) by the protein phosphatase Cdc25. Cdc2 forms a complex with cyclin B which is made in S phase. In order to form this complex, Cdc2 is phosphorylated at residues threonine-161, tyrosine-15, and threonine-14. The Cdc2-cyclin B complexes are inactive and accumulate during S and G₂. Cdc25 dephosphorylates threonine-14 and tyrosine-15, which activate the Cdc2-cyclin B complexes, sending the cells into mitosis (summarized in reference³²).

In contrast, it was found that human foreskin and embryonic lung fibroblasts exposed to the *H. ducreyi* CDT were blocked at both the G₁ and G₂ phases.³³ Toxin-treated fibroblasts exhibited induced expression of the tumor suppressor gene p53 as well as significantly increased expression of p21, an inhibitor of cyclin-dependent kinase activity. Non-proliferating primary human fetal fibroblasts treated with *C. jejuni* toxin arrested in the G₁ phase once they were stimulated to proliferate.²⁸ Belibasakis et al.²⁶ reported that the *A. actinomycetemcomitans* CDT exhibits a non-lethal inhibition of proliferation of human periodontal ligament (HPLF) and gingival (HGF) fibroblasts. It has also been found that murine NIH 3T3 fibroblasts are resistant to the *H. ducreyi* CDT.²²

Actinobacillus actinomycetemcomitans is the only indigenous member of the human oral flora identified to date that expresses the CDT. This bacterium is a facultative Gram-negative pathogen that is strongly associated with the development of localized and generalized forms of aggressive periodontitis. This species produces a variety of products that can be classified as potential virulence factors (see reference³⁴ for review), but is most notable for the expression of several multi-gene toxins including a leukotoxin and the CDT. A large percentage of strains of *A. actinomycetemcomitans* isolated from the human oral cavity express biologically active CDT.^{12,35} However, it has been difficult to demonstrate a clear link between the presence of strains of the bacterium that produce an active CDT and active periodontal lesions.^{35,36} The greatest potential for involvement of *A. actinomycetemcomitans* in oral disease is through interactions with the various cellular components of periodontal tissues. Fibroblasts are the predominant cell type within the periodontal ligament (PDL). This heterogeneous population of cells plays an important role in the normal maintenance, repair, and regeneration of not only the PDL but also the cementum and investing alveolar bone.³⁷ To assess the potential role of the *A. actinomycetemcomitans*

CDT in periodontal pathogenesis, it is essential to characterize specific effects on cells making up the human periodontium including PDL (HPLF) fibroblasts.

In this study we examine the effects of recombinant *A. actinomycetemcomitans* CDT on the proliferation and cell cycle of HPLF in primary cultures. The results are compared to those obtained with an immortalized human oral epithelial cell line.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Preparation of CDT

Recombinant clones containing all three *A. actinomycetemcomitans* *cdt* genes (*E. coli* DH5 α [pCDT1], *E. coli* BL-21[DE3] [pET15bcdt]), combinations of genes (*E. coli* BL-21[DE3] [pET15bcdtAB], *E. coli* BL-21[DE3] [pET15bcdtBC]), and the individual genes (*E. coli* BL-21[DE3] [pET15bcdtA], *E. coli* BL-21[DE3] [pET15bcdtC], *E. coli* BL-21[DE3] [pET15bcdtB]) were previously constructed and characterized.^{12,38} For preparation of CDT-containing extracts, single colonies of the appropriate clone were inoculated into 10 ml of LB medium containing 50 μ g/ml ampicillin and incubated overnight at 37°C with vigorous shaking. Overnight cultures were added to 100 ml of the same medium and grown until late log phase (OD₆₀₀ = 0.8 to 1.0) was reached. IPTG was added to a final concentration of 1 mM, and the cells grown for an additional 4 to 5 hours. The bacteria were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and suspended in PBS. The bacterial suspensions were sonicated[‡] in an ice bath using three 30-second pulses separated by 30-second rest. The lysed cells were centrifuged at 12,000 $\times$ g for 10 minutes to remove unbroken cells and sterilized by passage through a 0.22 μ m pore size filter.[§] Total protein concentration was determined with a protein assay kit. The TD₅₀ concentration was determined as described previously.¹² Expression of the *cdt* genes was assessed by analysis of total cell protein on 10% to 20% polyacrylamide Tris-HCl gels.^{||} An aliquot of the lysed bacterial suspension was boiled in gel loading buffer (2% SDS, 0.05 M Tris-HCl, pH 6.8, 10% glycerol) for 5 minutes. Apparent molecular size was determined by comparison to prestained molecular weight standards.^{||} For some experiments, His₆-tagged CdtA, CdtB, and CdtC were purified and the holotoxin reconstituted as previously described.³⁸

Cells and Tissue Culture Conditions

Human epithelial-like (HeLa) cells were used as a standardized cell line because they exhibit a well-characterized response to the CDT. These cells were grown in Eagle's minimal essential medium[¶] supplemented with 10% fetal bovine serum (FBS).

Human periodontal ligament fibroblasts (HPLF) were derived from periodontal ligament explants using a technique similar to that described by Somerman et al.³⁹ Clinically healthy third molars were obtained from five patients (three males and two females; age range 23 to 28 years) who presented to the Oral Surgery Clinic of the University of Pennsylvania School of Dental Medicine. Immediately following extraction, the teeth were placed into modified Eagle's medium (DMEM),[¶] supplemented with penicillin G (10,000 U/ml), streptomycin (25 μ g/ml), and fungizone (0.85%). Within 1 hour of extraction the teeth were washed twice with DMEM and the attached gingival tissue removed. The teeth were then placed in a petri dish containing DMEM and the periodontal ligament dissected away from the mid-third of the root surface by gentle scraping with a disposable surgical scalpel. The collected tissue was centrifuged at 1,000 rpm for 10 minutes at 4°C. The tissue was suspended in 5 ml of growth

[‡]Braun-Sonic 2000, B. Braun Biotech, Inc., Allentown, PA.

[§]Millipore Corporation, Billerica, MA.

^{||}Ready-Gels, Bio-Rad Laboratories, Hercules, CA.

[¶]Invitrogen, Carlsbad, CA.

medium supplemented with 10% fetal calf serum (FCS) and incubated at 37°C in 5% CO₂. Monolayers of HPLF were typically observed 2 to 3 weeks after the initial seeding of cultures. Upon reaching confluence, the primary cultures were treated with trypsin (1 mg/ml in PBS) to detach the cells for passaging. Cells were stored in 2 ml of 10% dimethylsulfoxide (DMSO) and 90% DMEM in liquid nitrogen. Cells in the second through seventh passages were used for CDT assays since they typically became senescent in later passages.

Multiple attempts to isolate and maintain pure primary cultures of human gingival epithelial cells were inconsistent. Therefore, an immortalized human oral epithelial cell line was used to compare the effects of the CDT to those obtained with the oral fibroblasts. This cell line, GSM-K, was grown in keratinocyte serum-free medium[†] as reported.⁴⁰ Cells in the second through fifth passages were used for CDT assays.

All protocols for obtaining and handling human oral tissues were reviewed and approved by the Institutional Review Board of the University of Pennsylvania.

Microscopy

HeLa cells, HPLF, and GSM-K cells were examined by bright field microscopy. Cells were seeded in T-75 flasks and incubated for 24 hours. Filter sterilized sonicate from *E. coli* BL-21 (DE3) (pET15bcdt), prepared as described above, was added to cultures at a concentration of 18.4 µg total protein per ml of medium (TD₅₀). The cultures were then incubated up to 96 hours. A second set of cultures received the same concentration of bacterial extract. After incubation for 5 minutes at 37°C, the cells were washed to remove unbound toxin and fresh medium was added. The cultures were then incubated for up to 96 hours post-intoxication. A third set of control cultures did not receive bacterial extract. Cultures were viewed using an inverted microscope.[#] Images were captured^{**} at 6, 12, 24, 48, 72, and 96 hours after addition of the extracts.

Kinetics of Response to the CDT

HPLF and GSM-K cell suspensions were seeded in 96-well tissue culture plates (15,000 cells/well) in 0.1 ml/well of appropriate medium. Cultures were incubated overnight at 37°C with 5% CO₂ to allow the cells to attach to the plates. For dose response kinetic assays, 1 to 1,000 ng of total soluble protein from *E. coli* BL-21(DE3) (pET15bcdt) and *E. coli* BL-21 (DE3) (pET15b) were added to triplicate wells. One set of triplicate wells did not receive any bacterial extract. The cultures were incubated for 48 hours after addition of the extracts. Rate kinetics were performed by adding 5 µg of total soluble protein from *E. coli* BL-21(DE3) (pET15bcdt) and *E. coli* BL-21(DE3) (pET15b) to 15,000 cells/well in triplicate. The cultures were incubated for 5 minutes at 37°C with 5% CO₂. One set of cultures did not receive bacterial extract. All cultures were then washed to remove unbound toxin and fresh medium was added. The cultures were incubated an additional 0.5 to 36 hours.

Following the various CDT treatments, cell viability was assayed using a cell proliferation assay kit.^{††} The assay was performed according to the manufacturer's instructions and absorbance of each well was read at 492 nm in a microplate reader. Reduction in cell viability was recorded as a decrease in the A₄₉₂ relative to untreated cells grown under the same conditions. Cell culture experiments were repeated a minimum of three times and all samples were run in triplicate in each replicate experiment. Standard curves of absorbance versus cell number were prepared to determine appropriate cell concentrations for use in the kinetic

[#]Nikon TMS-F inverted microscope, Nikon Instrument, Inc., Melville, NY.

^{**}Nikon N70 camera, Nikon Corporation, Melville, NY.

^{††}CellTiter96, AQueous Non-Radioactive Cell Proliferation Assay Kit, Promega Corporation, Madison, WI.

experiments. A linear increase in the A_{492} of the cell cultures over the range of 5,000 to 15,000 cells/well was observed (data not shown).

Cell Cycle Analysis

Cell cycle distribution was determined for untreated and CDT-treated cell populations by flow cytometry. One set of overnight cultures was treated with 18.4 μg of total soluble protein from *E. coli* BL-21(DE3) (pET15bcdt)/ml of medium. Cultures were incubated 3 to 72 hours post-intoxication. Nuclei from washed cells were isolated and stained by the Vindelov procedure.⁴¹ Briefly, cells (1×10^6) were suspended in 1 ml of filter sterilized Tris-buffered saline (pH 7.6) containing (10 $\mu\text{g}/\text{ml}$ ribonuclease A,^{††} 7.5 $\mu\text{g}/\text{ml}$ propidium iodide,^{††} and 0.1% Nonidet P-40^{††} for 1 to 2 hours. In other experiments HPLF and HGF were fixed in 70% cold ethanol overnight at -20°C , treated with RNase, and stained with propidium iodide. Stained nuclei and cells were analyzed on a flow cytometer^{§§} at the University of Pennsylvania Cancer Center Flow Cytometry and Cell Sorting Shared Resource facility. The data from 30,000 events were analyzed with a software package.^{|||}

Pulse-Field Gel Electrophoresis (PFGE)

HeLa, GMSM-K cells, and HPLF cultures (5×10^6 cells) were incubated overnight. One set of cultures received an LD₅₀ equivalent dose (4.5 $\mu\text{g}/\text{ml}$ of medium) of total soluble protein from *E. coli* BL-21(DE3) (pET15bcdt). A second set of cultures did not receive any extract. Cells were collected after incubation for 3 to 72 hours post-intoxication and suspended in 500 μl of 1% agarose^{¶¶} made up in F12 medium. Plugs (35 μl) were formed and the cells lysed by suspension in a solution containing 1 mg/ml proteinase K,^{††} 1% sarcosyl,^{††} and 0.5 M EDTA (pH 8.0). The suspended plugs were shaken overnight at 50°C . The plugs were washed with 0.5 M EDTA and treated with 0.1 mg/ml DNase-free RNase^{††} for 30 minutes at 37°C . The plugs were applied to wells cast in a 0.8% agarose gel, containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide, and electrophoresis was performed at 175 V (starting voltage) for 40 hours at 4°C in a PFGE apparatus. The ratio of DNA in the well and in the gel was estimated from digitized images using a software program.^{##}

Immunofluorescence

Detection of binding of the Cdt polypeptides to the surface of HeLa, HPLF, and GMSM-K cells was performed as described previously.³⁸ Briefly, cells were suspended in the appropriate growth medium and added to each well (1×10^4 cells per well) of an eight-well chamber slide^{***} and incubated for 48 hours at 37°C in a moist atmosphere containing 5% CO₂. Slides used for GMSM-K cells were precoated with 0.2% gelatin to aid cellular attachment. Slides were washed and purified individual His₆-tagged proteins or reconstituted holotoxin were added at a concentration of 1 to 5 μg per well. The slides were then incubated on ice for 15 to 30 minutes, washed twice with PBS, and fixed in 10% formalin for 5 minutes at room temperature. The slides were washed, blocked with 3% BSA in PBS, and then incubated with a 1:1,000 dilution of anti-His•Tag monoclonal anti-body^{†††} for 1 hour at room temperature. The slides were then washed and incubated in the dark with a 1:1,000 dilution of goat anti-mouse IgG^{†††} (heavy and light chain) conjugate. The slides were thoroughly washed, followed by the addition of mounting solution.^{§§§} Coverslips were placed on the slides, which were

^{††}Sigma Chemical, St. Louis, MO.

^{§§}FACSCalibur, Becton Dickinson, Franklin Lakes, NJ.

^{|||}ModFit 3.0, Verity Software House, Topsham, ME.

^{¶¶}InCert agarose, FMC Corporation, Philadelphia, PA.

^{##}ImageJ Version 1.32a, available at <http://rsbweb.nih.gov/nih-image/index.html>.

^{***}Nalgene, Nunc International, Rochester, NY.

^{†††}Novagen, EMD Biosciences, San Diego, CA.

^{§§§}Alexa Fluor 488, Molecular Probes, Inc., Eugene, OR.

viewed under a fluorescent microscope.^{||||} Binding experiments were repeated a minimum of three times.

Statistical Methods

Mean values and standard deviations were plotted. The paired *t* test ($P = 0.05$) was used to evaluate the data in some experiments.

RESULTS

Characterization of *cdt* Gene Products Produced by *E. coli* BL-21(DE3) (pET15b *cdt*)

The *A. actinomycetemcomitans cdt* genes were previously cloned in *E. coli* to enhance expression, provide a method for isolation of the gene products, and express the CDT free from a background of other reported cytotoxic proteins produced by this oral pathogen (see reference 19 for review). Two recombinant strains *E. coli* DH5 α (pCDT1)¹² and *E. coli* BL-21(DE3) (pET15b*cdt*)²⁸ contain the *cdt* locus cloned in two different vectors (pBluescript II SK+ and pET15b). Both constructs yielded active holotoxin based on the inhibition of proliferation of control HeLa cells 48 hours post-intoxication (Fig. 1A). The pET15b*cdt* construct produces toxin with a His-tag on the amino terminus of the CdtA polypeptide.³⁷ It is possible that the His-tag alters the activity of the toxin. However, both forms of the recombinant toxin had equivalent cytotoxic activities.

The relative cytotoxic activity of the recombinant extracts was significantly greater than that of an extract from *A. actinomycetemcomitans* Y4 ($P < 0.05$). There was no difference between cultures of cells exposed to toxin at the time of plating or after attachment of cells to tissue culture plates overnight (data not shown). Protein extract from bacteria carrying vector plasmid alone (pBluescript II SK+) had no effect on the HeLa cells. CDT-containing extract from *E. coli* BL-21(DE3) (pET15b*cdt*), rather than the affinity purified recombinant proteins, were used in the following experiments so that assays could test the effects of holotoxin assembled *in situ* by the bacterium rather than artificially reconstituted *in vitro*. The three *cdt* genes were previously cloned separately in pET15b.³⁸ Expression of the *cdt* genes by the clones in the pET15b collection is shown in Figure 1B.

Microscopic Evaluation of Human Oral Cells Treated With Recombinant *A. actinomycetemcomitans* CDT

To determine if the *A. actinomycetemcomitans* CDT has the potential to perturb cell types indigenous to the human periodontium, primary cultures of HPLF were exposed to CDT-containing extract from the *E. coli* BL-21(DE3) (pET15b*cdt*). The effect of the toxin on HPLF proliferation was compared to the effect on the GSM-K and HeLa cell lines. Microscopic inspection revealed that cultures of HPLF did not appear to be altered by exposure to the toxin reaching confluence by 96 hours of growth (Fig. 2). By 96 hours post-intoxication, the appearance of the fibroblast culture was identical to that of the untreated culture with no discernable decrease in cell numbers. In comparison, the proliferation of CDT-treated epithelial cells was significantly reduced. Reduction in proliferation of CDT-treated GSM-K cells occurred more rapidly than that of the HeLa cells. This did not appear to be a consequence of the absence of serum in the GSM-K growth medium. Addition of 10% FCS to the growth medium did not alter the response of the GSM-K cells to the toxin. A change in morphology of the GSM-K cells and apparent reduction in cell numbers was observed by 12 hours after exposure to the CDT-containing extract. Cultures of HeLa cells did not show the same effects until 48 hours post-exposure. Both the GSM-K and HeLa cell cultures manifested the toxic

§§§Prolong Anti-Fade, Promega Corporation.

||||Nikon Eclipse E600, Nikon Instruments, Inc.

effects after exposure to CDT-containing extract for a minimum of 5 minutes. The appearance of these cultures following intoxication was identical to that of cultures which contained the toxin extract over the course of the experiment (data not shown).

Kinetics of Response of Human Oral Cells to the CDT

Dose response and rate kinetics were performed to further characterize the differential response of the oral epithelial cells and fibroblasts to the CDT. CDT-containing extract from *E. coli* BL-21(DE3) (pET15bcdt) exhibited a dose-dependent effect on the viability of GSM-K cells (Fig. 3A). One nanogram of total soluble protein from the recombinant clone was enough to inhibit cell viability when compared to extract from *E. coli* carrying the vector plasmid (pET15b) alone (inset). There was a 50% decrease in the A_{492} of 15,000 cells with 1 ng of total soluble protein from *E. coli* BL-21(DE3) (pET15bcdt). A maximum decrease in the A_{492} of 15,000 cells was obtained with 125 ng of total soluble protein. The average A_{492} of the GSM-K cell culture that was not exposed to bacterial extract was 0.60. This CDT-induced reduction in A_{492} was statistically significant when compared to controls ($P < 0.05$).

One thousand times more total soluble protein from *E. coli* BL-21(DE3) (pET15bcdt) had no effect on primary cultures of HPLF (Fig. 3B). The average A_{492} of HPLF cultures not exposed to bacterial extract was 1.19. The difference in baseline absorbance values for GSM-K cells and HPLF was consistent and appeared to be due to differences in the size of the cells. Being the larger of two cell types, the HPLF likely have more mitochondria and are, therefore, more metabolically active on a per cell basis. This would affect the magnitude of the response in the cell viability staining assay.

Rate kinetic experiments showed that exposure of GSM-K cells to CDT-containing extract in culture for a minimum of 5 minutes was enough to illicit a cytotoxic response. GSM-K cells exposed to the CDT-containing extract from *E. coli* BL-21(DE3) (pET15bcdt) for 5 minutes, washed, and then incubated for various lengths of time showed a decrease in A_{492} by 12 hours post-intoxication, indicating a significant reduction in viability (Fig. 4A, unfilled circles). However, initial effects on cell viability were detected by 3 hours post-intoxication (Fig. 4A, inset). The cultures continued to exhibit a decrease in A_{492} up to 36 hours post-intoxication. Cultures not exposed to CDT-containing extract (Fig. 4A, filled triangles) and those receiving extract from *E. coli* BL-21(DE3) (pET15b) (Fig. 4A, unfilled squares) showed an increase in the A_{492} up to 12 hours, which was indicative of normal cell growth. The A_{492} of these cultures increased only slightly from 12 to 36 hours, indicating that the cultures were approaching confluence.

A reduction in cell number was not detected in HeLa cell cultures exposed to CDT-containing extract until at least 36 hours post-intoxication (Fig. 4B, unfilled circles). HeLa cell cultures not exposed to CDT-containing extract (Fig. 3B, filled triangles [only at 0 concentration]) and those receiving extract from *E. coli* BL-21(DE3) (pET15b) (Fig. 4B, unfilled squares) exhibited a steady increase in the number of viable cells up to 26 hours. These values remained constant up to 36 hours. The kinetic data support the visual data (Fig. 2) of the cell-specific effects of the CDT-containing extract on proliferation. The toxin appears to rapidly and irreversibly affect the oral epithelial cell line.

Effect of the CDT on Cell Cycle of HPLF and Oral Epithelial Cells

The differential effects of the recombinant *A. actinomycetemcomitans* CDT on the proliferation of HPLF and oral epithelial cells prompted an assessment of the ability of the toxin to perturb the cell cycle of these various cell types. The effect of recombinant CDT on the cell cycle was examined by flow cytometry (Fig. 5). In untreated cultures, the number of HeLa cells with a $4n$ DNA content represented less than 1% of the total cell population (Table 1). HeLa cells

exposed to CDT-containing extract for 12 and 24 hours were arrested at the G₂/M phase transition. By 12 hours and 24 hours post-intoxication the percentage of cells exhibiting a 4*n* DNA content increased to 16% and 81%, respectively. There was no significant difference in the percentage of arrested HeLa cells whether cultured in the continuous presence of CDT for 24 hours or for 24 hours following exposure for 5 minutes (Fig. 5, inset and Table 1).

The DNA content of a population of CDT-treated GSM-K cells was very different than that of HeLa cells. A small percentage (8%) of GSM-K cells in untreated cultures consistently had a DNA content of 4*n*. There was little difference between the DNA content of untreated cells and those treated with CDT-containing extract for 3 hours. By 6 hours post-intoxication the percentages of cells with both 2*n* and 4*n* DNA contents decreased. By 12 hours, less than 1% of the cells in a population exposed to CDT extract had a DNA content of 4*n* and those with a 2*n* content were reduced to 13%. This trend continued up to 24 hours post-intoxication. Most of the cells in the CDT-treated population had a DNA content between 2*n* and 4*n* represented by the large S peak. Unlike HeLa cells, the proliferation of GSM-K cells may be arrested in the S phase (Fig. 4). These data indicate that the CDT may block DNA replication in the GSM-K cell line.

Primary cultures of HPLF treated with CDT extract failed to show evidence of cell cycle arrest. There was no significant accumulation of cells having a DNA content of 4*n* nor was there a decrease in the percentage of cells with a 2*n* DNA content associated with increased time of exposure (72 hours maximum) to the CDT (Fig. 5 and Table 1). Unlike the GSM-K cells, there was no indication that CDT-treated fibroblasts were arresting in the S phase. The cell cycle analysis data support the observation that there is no inhibition of proliferation of CDT-treated HPLF as determined by microscopic examination and by evaluation of growth kinetics.

CDT-Induced DNA Damage

An early step in the intoxication process is the uptake of the CdtB polypeptide and its targeting to the cell nucleus resulting in DNA double-strand breaks.³⁰ Pulsed field gel electrophoresis (PFGE) was used to determine if the recombinant *A. actinomycetemcomitans* CDT induced DNA damage in HPLF. DNA extracted from HeLa cell cultures contained an extensive size range of DNA fragments only when the cells were treated with CDT-containing extract from *E. coli* BL-21(DE3) (pET15bcdt) (Fig. 6). DNA fragmentation was observed by 15 hours of growth following exposure to the toxin. Approximately 60% of the ethidium bromide-stained DNA migrated into the gel. Double-strand DNA damage was not observed in cells treated with extract from *E. coli* that does not express the CDT. We previously showed that *E. coli* BL-21 (DE3) does not contain DNA nicking activity.³⁸ GSM-K cells were very sensitive to the DNA-damaging effects of the CDT. Approximately 75% of the ethidium bromide stained DNA from cells treated for 6 hours entered the gel following electrophoresis. The small amount of damaged DNA (16%) observed in untreated GSM-K cells may have resulted from cell lysis during the preparation of the agarose plugs. No fragmentation of DNA from CDT-treated HPLF was observed, up to 72 hours post-exposure, following analysis by PFGE. The absence of evidence of CDT-induced DNase activity further supports our observation that HPLF are resistant to the CDT.

Binding of Specific CDT Polypeptides to Human Oral Cells

It was previously shown that reconstituted CDT holotoxin and CdtA could be detected on the surface of Chinese hamster ovary cells by immunofluorescence.³⁸ Resistance of human oral fibroblasts to the CDT could be due to the lack or alteration of a CDT receptor on the cell surface. To test this possibility, reconstituted recombinant holotoxin and the individual recombinant Cdt polypeptides were examined for their ability to bind to HeLa, HPLF, and GSM-K cells. Both reconstituted toxin and CdtA were detected on the surface of all three

cell types (Fig. 7). The GSM-K cells attach poorly to the glass slides typically used for immunofluorescence. Consequently, gelatin coated slides had to be used. The gelatin coating reduced the clarity of the immunofluorescence and most likely is the reason for the apparent weak detection of CdtC on the GSM-K cells. However, these data indicate that the apparent resistance of HPLF to the CDT is not due to the inability of the cells to bind the toxin.

DISCUSSION

The CDT is an unusual cytotoxin because it is expressed by a group of bacterial species that are pathogens in disparate diseases and colonize dissimilar environments. Strains of *E. coli*,² *Campylobacter* spp.,^{3,42} and *S. dysenteriae*^{1,43} that express the CDT participate in diarrheal diseases. *Haemophilus ducreyi* causes chancroid ulcers,⁸ while *Helicobacter* spp.¹⁰ are involved in the formation of stomach ulcers. *Actinobacillus actinomycetemcomitans* is thought to be a prominent pathogen in multiple forms of periodontal disease.^{34,44} It is not clear at the present time if the CDT is a virulence factor in all of these diseases. Since *A. actinomycetemcomitans* is the only member of the human oral flora to carry *cdt* genes and express active toxin, we are interested in its potential role as a virulence factor in periodontal disease. It is assumed that the CDT has an important role in disease-associated activities of the bacterium since a proportionately high number of fresh clinical isolates express *cdt* genes.^{12, 35,36} This is a good indication that there is selective pressure to maintain the *cdt* operon in *A. actinomycetemcomitans*.

Periodontal ligament fibroblasts have a key role in maintaining the integrity of the ligament that is responsible for supporting the teeth within the adjacent alveolar bone. Epithelial cells represent another abundant cell type in the oral cavity that are morphologically and functionally distinct from HPLF. Thus, our aims were primarily to examine the effects of the CDT on HPLF and secondarily to compare and contrast these with the effects of the CDT on oral epithelial cells. Difficulties in the routine culturing of primary oral epithelial cells persuaded us to use a recently immortalized epithelial cell line (GSM-K) for our studies. The comparative data should be viewed with caution since the epithelial cells are immortalized and this may influence the response of the cells to the CDT. However, the most intriguing finding was that primary cultures of HPLF failed to exhibit typical cellular responses to the CDT. There was no effect on proliferation or cell cycle. This is in contrast to results obtained by others^{28,33} who worked with fibroblasts derived from non-oral tissue compartments. Several studies examining the activities of the *H. ducreyi* CDT have used fibroblasts from non-human or non-oral sites. It was found that Chinese hamster lung (Don) fibroblasts were irreversibly sensitive to the CDT but required a relatively long exposure time to achieve intoxication.²² The Don fibroblasts became distended following 24 hours of continuous treatment and this was accompanied by a decrease in their rate of proliferation. In contrast, neither the morphology nor proliferation of mouse 3T3 fibroblasts were altered by the *H. ducreyi* CDT. The growth of human foreskin fibroblasts appeared to be inhibited by *H. ducreyi* CDT but the cells were not killed.²⁴ We did not see an effect of the *A. actinomycetemcomitans* CDT on proliferation of HPLF up to 96 hours of exposure with relatively high levels of toxin. It has been reported that both human foreskin and human embryonic lung fibroblasts had a CDT-induced pattern of cell cycle arrest different from that of epithelial cells and keratinocytes which arrested in the G₂ phase.³³ The CDT-treated fibroblasts had an incomplete block in G₂ phase up to 48 hours post-intoxication and were arrested in the S and G₁ phases. Cdc2 was down-regulated with a corresponding accumulation of phospho-Cdc2. The human lung fibroblasts also exhibited increased expression of p53 and upregulation of p21 upon exposure to the toxin. Collectively, these studies established that the response to CDT was cell specific and not necessarily associated with a G₂ arrest. We failed to detect cell cycle arrest at either G₁ or G₂/M phase transition of HPLF. These opposing results could be attributed to differences between HPLF and fibroblasts from other tissue compartments or differences between the CDT from *A.*

actinomycetemcomitans and *H. ducreyi*. However, the deduced amino acid sequences of the Cdt polypeptides from these two species are more than 90% identical.^{8,12}

Several studies have reported a growth inhibitory effect of *A. actinomycetemcomitans* on HPLF and human gingival fibroblasts (HGF).^{26,45} One of these studies directly implicated the CDT.²⁶ However, the conditions under which these studies were conducted were not particularly stringent. Helgeland and Nordby⁴⁵ reported a cell cycle inhibition in HGF with uncharacterized secreted material from *A. actinomycetemcomitans*. The fibroblasts accumulated in G₂/M phase between 28 to 40 hours post-exposure to the *A. actinomycetemcomitans* material. The cytotoxic activity in the inhibitory fraction was not identified and this crude fraction was most likely heavily contaminated with lipopolysaccharide (LPS) and other cytotoxic proteins. This work was published prior to the identification of the CDT in *A. actinomycetemcomitans*. The strain used by these investigators was *A. actinomycetemcomitans* ATCC 33384,¹⁸ which is the type strain (NCTC 9710) of this species. We previously showed that strain NCTC 9710 produces biologically active CDT.¹² It is not clear if the reported effects on HGF were due to other factors produced by the bacterium. This is one reason why recombinant *A. actinomycetemcomitans* CDT was used in our study.

In another study, a crude extract of whole cells of *A. actinomycetemcomitans* was used to treat cultures of HPLF and HGF.²⁶ There was a rapid inhibition of DNA synthesis. The fibroblasts became distended by 72 hours but remained viable. It was reported that these effects were abolished when extract from a CDT knockout mutant was used. However, it is extremely difficult to evaluate these results because: 1) these investigators did not attempt to identify either *cdt* genes or Cdt polypeptides in their test strain *A. actinomycetemcomitans* HK 1519; 2) their bacterial extract was a crude preparation contaminated with significant levels of LPS (6.5 µg/ml); 3) the concentration of extract used in their assays was not reported; and 4) effects on cell cycle and evaluation of DNA damage, standard measures of CDT activity, were not determined.

Our data clearly show that HPLF do not undergo the classic double-strand DNA damage and cell cycle arrest associated with the CDT. It appears that a combination of factors may contribute to this resistance. Results of the PFGE indicate that either the CDT does not get into the HPLF nucleus, that the chromatin is protected from the DNase I-like nicking activity of the toxin, or that the DNase I-like activity is neutralized in the cells.^{4,29,30} Immunofluorescence detection of both whole toxin and CdtA on the surface of HPLF cells suggest that failure of toxin binding to the cells is not a likely reason for resistance. If this is the case then resistance of HPLF to the toxin is most likely related to a direct or indirect inhibition of the DNase I-like activity of the CdtB polypeptide. Preliminary data from our studies also indicate that HGF exhibit the same pattern of resistance to the CDT (unpublished observations). This suggests that human oral fibroblasts, in general, may be resistant to the *A. actinomycetemcomitans* toxin.

In addition to the finding that HPLF are resistant to the CDT, it was also found that at least one type of oral epithelial cell is exceptionally sensitive to the toxin. Furthermore, the growth inhibitory effect of the CDT on these epithelial cells is atypical since arrest at G₁ or G₂/M was not observed. Results of FACS (fluorescence-activated cell sorter) analyses indicate that a block in DNA replication may lead to the inhibition of proliferation of these cells. The arrest of cells in the S phase has not been previously reported for the CDT.

These findings have important implications for the potential role of the CDT and *A. actinomycetemcomitans* in the pathogenesis of periodontal disease. Epithelial cells, by virtue of their forming a physical barrier, provide an innate form of host defense against microbial infection. The epithelial cells of the gingiva thereby protect the underlying supporting

structures of the periodontium from the deleterious effects of bacterial colonization. The ability of the CDT to inhibit the growth of oral epithelial cells may lead to disruption of this protective barrier allowing *A. actinomycetemcomitans*, along with other putative periodontal pathogens, access to the underlying gingival connective tissues. In this tissue compartment other virulence factors as well as the concomitant activation of a host inflammatory response are likely to play more direct roles in destruction of the tooth-supporting structures, including the periodontal ligament. Thus, the *A. actinomycetemcomitans* CDT may have a role in the initial stages of developing periodontal lesions.

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Figure 1.

Characterization of *cdt* gene products produced by *E. coli* BL-21(DE3) (*pET15bcdt*). **A**) No bacterial extract or total soluble protein extract (10 ng of protein/ μ l of medium) from *A. actinomycetemcomitans* Y4, *E. coli* DH5 α (*pBluescript II SK+*), *E. coli* DH5 α (*pCDT1*), or *E. coli* BL-21(DE3) (*pET15bcdt*) was added to cultures of HeLa cells. *Statistically significant differences, $P < 0.05$. **B**) SDS-PAGE of total soluble protein from recombinant strains of *E. coli* containing combinations or individual *cdt* genes cloned in *pET15b*. A, B, C: native polypeptides CdtB and CdtC; His: His-tagged polypeptides determined by Western blotting; *truncated CdtA.

Figure 2. Microscopic examination of time course exposure of HPLF, GSM-K, and HeLa cell cultures to the CDT. Top row: untreated (–CDT) cell cultures after incubation for 0, 48, or 96 hours. Remaining rows: cell cultures treated (+CDT) with 18 μ g protein/ml of CDT-containing extract from *E. coli* BL-21(DE3) (pET15bcdt).

Figure 3.

Dose-dependent response of GSM-K cells and HPLF to recombinant CDT -containing extract. A) Increasing concentrations of extract from E. coli BL-21(DE3) (pET15bcdt) (open circles), E. coli BL-21(DE3) (pET15b) (closed squares), and no extract (closed triangles). The absorbances of cultures not receiving bacterial extract were measured only at the 0 concentration point. B) Cultures of HPLF treated as for the GSM-K cells. Regions of the curves showing the lower concentrations of extract are expanded for clarity (insets).

Figure 4.

Rate kinetics of the response of GSM-K and HeLa cells to recombinant CDT containing extract. A) Cultures of GSM-K cells received 50 ng total protein extract sonic extract from E. coli BL-21(DE3) (pET15bcdt) (open circles) and E. coli BL-21(DE3) (pET15b) (open squares) per ml of medium. B) Cultures of HPLF treated as for the GSM-K cells except incubation was extended to 50 hours. The region of the GSM-K curve showing the lower concentrations of extract is expanded for clarity (inset).

Figure 5.

Effects of CDT on the cell cycle of HeLa, GSM-K cells, HPLF, and HGF. Approximately 1×10^6 cells from cultures untreated (top row) or treated with 18.4 $\mu\text{g/ml}$ protein extract from *E. coli* BL-21(DE3) (pET15bcdt) per ml of medium were incubated for 12 to 72 hours post-exposure. Cell cycle profiles obtained by flow cytometry are shown. The solid line (PI) is the DNA profile determined by propidium iodide staining. G1, G2/M (solid filled) and S (diagonal line filled) peaks were determined by computer analysis.

Figure 6.
Pulse-field gel electrophoresis of CDT-treated oral cells. The numbers represent the relative percent of DNA in the well versus the gel as determined by scanning densitometry of the ethidium bromide stained gel.

Figure 7.
Immunofluorescent detection of recombinant holotoxin and Cdt polypeptides on the surface of HeLa, HPLF, and GSM-K cells. Individual His6-tagged polypeptides and reconstituted toxin were incubated with each cell type on chamber slides for 15 minutes at 4°C. Bound protein was detected with His•Tag monoclonal antibody (1:1,000 dilution) and fluorescein-conjugated second antibody (1:1,000 dilution).

Table 1
Cell Cycle Analysis of HPLF and Oral Epithelial Cells Treated With CDT

Cell Type	CDT Exposure (hours)	Diploid G1 (2n) (%)	Diploid G2 (4n) (%)	Diploid S (%)	Coefficient of Variance (%)
HeLa	0	72.86	0.91	26.24	5.00
	12	31.81	15.42	52.77	3.37
	24	0.60	80.54	18.86	8.37
GMSM-K	24 (5 minutes)	0.46	95.19	4.35	6.13
	0	39.22	8.00	52.78	3.94
	3	32.02	8.00	59.98	4.29
	6	25.40	4.16	70.44	3.31
	12	12.84	0.18	86.98	3.87
	24	9.58	0.15	90.27	4.05
HPLF	0	85.84	1.97	12.19	7.53
	24	63.49	0.31	36.20	6.78
	48	77.36	1.16	21.49	7.07
	72	90.15	1.23	8.62	9.01