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Effect of Cytolethal Distending Toxin on F-Actin Assembly and Cell Division in Chinese Hamster Ovary Cells

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Cytolethal distending toxin (CDT) is a newly described toxin produced by a number of enteropathogens, including *Campylobacter jejuni*, various *Escherichia coli* strains, and a few *Shigella* species. CDT induces distension and eventual death of a number of transformed cell lines. Here, we extend previous studies by demonstrating that morphological changes in CDT-treated Chinese hamster ovary cells are coincident with changes in cytoskeletal structure and an inhibition of cell proliferation. CDT-treated cells underwent a progressive accumulation of F-actin assemblies which microscopically resembled actin stress fibers. Accumulation of the stress fiber-like structures in CDT-treated cells was accompanied by an apparent blockage of cell division. Multinucleation was detected in some cells but did not constitute a significant feature of CDT action. Although toxin-treated cells failed to divide, cell viability remained high for the first 4 days following toxin treatment, as evidenced by trypan blue exclusion and neutral red uptake. [^{3}H]thymidine incorporation studies on CDT-treated cells were consistent with a blockage of cell proliferation without a direct inhibition of DNA synthesis. Although the progression of toxin action developed slowly, a 2-min exposure to CDT resulted in an irreversible development of toxicity. Together, our data indicate that CDT affects F-actin assembly within target cells and may interrupt the regulation or function of cell cycle-dependent events leading to cytokinesis.

Cytolethal distending toxin (CDT) is a newly described heat-labile protein cytotoxin produced by a growing list of diarrheal disease-causing enteropathogens. CDT-producing bacteria include some *Escherichia coli* isolates from at least three of the major pathogenic groups (enterotoxigenic, enteropathogenic, and enteroagglutinating) (11), nearly all *Campylobacter jejuni* isolates, a number of non-*C. jejuni* species (10, 23), and a few *Shigella* spp., including *Shigella dysenteriae* types 1 and 2 and *S. boydii* type 7 (12, 19). In addition to the reports of CDT production by enteropathogens, Cope et al. have recently reported cloning the genes responsible for production of a HeLa cell cytotoxin from *Haemophilus ducreyi* which upon sequence analysis proved to be a CDT homolog (4). Culture filtrates of CDT-producing strains cause progressive cellular distension and finally death in some cultured cell lines, including Chinese hamster ovary (CHO), Vero, HeLa, and HEp-2 cells (10, 11, 13). Morphological changes associated with CDT toxicity are evident by 48 h and progress until cell death in 72 to 96 h following toxin treatment. The gross cellular changes associated with CDT activity are clearly different from those caused by other known toxins, including the heat-labile (LT), heat-stable (STa and STb), and Shiga-like (Stx1 and Stx2) toxins of *E. coli* (11). Although CDT induces transient CHO cell elongation, like that associated with LT, the progression to cellular distension and eventual cell destruction is unique to CDT. Further, Y-1 adrenal cells are responsive to LT but not to CDT (11, 13). By comparison, *E. coli* Stx1 causes rapid cell death without distension and is poorly active on CHO cells (17). There are some similarities between CDT and cytotoxic necrotizing factor (CNF), but while CNF causes cell enlargement and a general multinucleation in CHO cells (3), CDT-induced cell distension is more striking and only limited multinucleation is observed (11, 13). In addition to the unique cytotoxic properties of CDT, gene cloning and sequence analysis of CDTs from various sources have confirmed that the CDT-associated polypeptides are unique and unrelated to other known proteins (23, 26–28).

CDT genes characterized to date include those from two different *E. coli* clinical isolates (22, 24), *C. jejuni* (23), and a type 1 *S. dysenteriae* strain (19). Based on these reports, 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 to 35 kDa (CdtA), 28 to 30 kDa (CdtB), and 20 to 21 kDa (CdtC) (22–24). Genetic and molecular cloning evidence suggests that all three polypeptides are required for toxicity (26–28); however, the nature of the CDT holotoxin and its subunit arrangement are unknown.

In addition to the effects of CDT on cultured cells, recent evidence from the use of a suckling mouse model suggests that the toxin causes secretory diarrhea and necrosis of colonic epithelium (18). CDT-mediated intestinal secretion in the mouse model was rapid, with diarrhea being evident within 4 h of toxin administration (18). The relationship of in vitro effects of CDT to the changes seen in the mouse model and their potential role in human disease are unknown.

In this report, we investigate some of the cell biological events surrounding CDT action to better understand the effects of this toxin on eukaryotic cells. We show that CDT produces radical morphological changes in cultured CHO cells accompanied by the accumulation F-actin assemblies which resemble stress fibers. We present additional evidence to support the hypothesis that CDT inhibits cellular cytokinesis without directly blocking DNA synthesis.

MATERIALS AND METHODS

Strains, plasmids, and bacteriological media. *E. coli* 9142-88 (O128:H$^-$), initially isolated from a child with diarrhea, was kindly provided by Nancy Strockbine, Centers for Disease Control and Prevention, Atlanta, Ga. Strain 9142-88 was used as the source of the *cdt* genes. *E. coli* XL-1 Blue (Stratagene, La Jolla, Calif.) and BL21(DE3) (Novagen, Madison, Wis.) were used for cloning and expression, respectively, of the CDT genes. Plasmid pGEM-7Zf(+) (Pro-

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mega Inc., Madison, Wis.) was used as a cloning vector. A recombinant clone containing the 6.6-kb *Cla*I fragment and encoding the CDT genes (see below) was designated pCDT32-6. Bacteria were grown on L-agar and L-broth media (15), containing carbenicillin (100 μg/ml) and isopropylthiogalactopyranoside (IPTG; 0.5 mM) when appropriate.

CDT gene cloning. Chromosomal DNA, isolated from *E. coli* 9142-88 by standard methods, was used as the template for PCR amplification of a CDT gene probe (15). Synthetic oligonucleotide primers based on the published *cdt* sequence of Pickett et al. (22) (5′ primers, 5′-AAGTGGAAGGAGGACCA ACC-3′; 3′ primer, 5′-ATAACGATGAACCAGCTCCG-3′) were used to amplify a 188-bp DNA fragment of *cdtA*. The amplified DNA fragment was purified by using Gene Clean (Bio 101, Inc., Vista, Calif.) and radiolabeled by using [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont NEN, Wilmington, Del.) and a random priming DNA labeling system (GIBCO BRL, Grand Island, N.Y.). Radiolabeled DNA was separated from unincorporated [α-^{32}P]dCTP by gel permeation chromatography over a disposable NAP-5 column (Pharmacia Biotechnology, Inc., Piscataway, N.J.). The labeled CDT gene probe was used to screen Southern blots of *E. coli* 9142-88 DNA digested with various restriction enzymes which do not cut within the *cdt* operon (22). Based on this analysis, *Cla*I was chosen to prepare a genomic library of *E. coli* 9142-88. Chromosomal DNA was digested to completion with *Cla*I, extracted with phenol-chloroform (1:1, vol/vol), ethanol precipitated, dissolved in water, and quantified by agarose gel electrophoresis compared to known standards. *Cla*I-digested pGEM-7Zf(+) DNA was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), followed by phenol-chloroform extraction and ethanol precipitation. Chromosome and plasmid DNAs were mixed in a 2:1 molar ratio, ligated with DNA ligase (Promega), and used to transform *E. coli* XL-1 Blue by electroporation. Transformants, selected by plating on L-agar plates containing carbenicillin, were screened by colony hybridization (15) using the ^{32}P-labeled gene probe. Positive clones were picked, and plasmid DNA was analyzed by restriction analysis and Southern blot. All positive clones identified contained the same 6.6-kb *Cla*I fragment. Restriction analysis and partial DNA sequencing was used to verify the presence of the *cdtABC* operon on the 6.6-kb *Cla*I fragment.

Preparation of crude CDT. For cytotoxicity assays, CDT-containing and control culture filtrates were prepared by growing overnight 25-ml L-broth cultures of *E. coli* BL21(pCDT32-6) and *E. coli* BL21(pGEM-7Zf+), respectively. At the time of inoculation, IPTG was added as described above. After 18 h of culture with vigorous shaking at 37°C, the bacterial cells were removed by centrifugation, and the culture supernatants were then filter sterilized through a 0.22-μm-pore-size filter and stored in aliquots at −80°C until used.

Toxin assay in cultured cells. CHO cells (CHO-K1 subclone; ATCC CCL-61) were used for in vitro testing of CDT activity. Cells were grown at 37°C in an atmosphere of 5% CO_2 (95% air) in Ham's F-12 medium containing 5% fetal bovine serum, 100 μg of streptomycin per ml, and 100 IU of penicillin per ml. For toxicity assay, cells were seeded in 24-well plates (Falcon 3047; Becton Dickinson and Co., Paramus, N.J.) at a concentration of 2×10^4 cells/ml (500 μl/well). After 18 h at 37°C, cells were treated with dilutions of the filter-sterilized toxin and control culture supernatants (described above); an additional set of control wells received no culture filtrate. Starting at 24 h following the addition of culture filtrates and at 24-h intervals for up to 5 days posttreatment, cells were observed for the characteristic cellular distension caused by CDT (13). In addition, at each time point, cells were removed from the plates by trypsin treatment and counted with the aid of a hemocytometer. Cell viability was assessed by trypan blue exclusion and neutral red uptake (2). To avoid interference of starvation in the assay, the medium was changed on the second day following treatment. In one set of experiments where the time course of toxin action was determined, CDT and control culture supernatants were removed at various times from 0 min to 48 h following their addition and replaced with fresh medium.

SEM. Cells seeded on glass coverslips were treated with bacterial supernatants and processed for scanning electron microscopy (SEM) at 24-h intervals. Toxin- and control filtrate-treated cells were fixed by immersion of the coverslips in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 3 h. After rinsing, twice with each of 0.1 M sodium cacodylate buffer (pH 7.4) and distilled water, the cells were serially dehydrated in increasing concentrations of ethanol (33%, 15 min; 67%, 15 min; 85%, 15 min; 95%, two times for 15 min each; 100%, two times for 30 min each). Following dehydration, the cells were treated with hexamethyldisilazane for 1 h, dried, mounted, coated with gold-palladium, and observed by scanning electron microscopy.

Staining of F-actin with fluorescein-phalloidin. The effect of toxin on actin assemblies was assessed by fluorescence labeling of F-actin filaments with fluorescein-phalloidin (Molecular Probes, Eugene, Oreg.). After 24 h of toxin or control supernatant treatment and at 24-h intervals thereafter, CHO cells seeded on 12-mm-diameter coverslips were washed twice with phosphate-buffered saline (PBS), fixed for 10 min with 3.7% formaldehyde in PBS, and then permeabilized by treatment with Triton X-100 (0.5% in PBS) for 10 min at room temperature. After two more washes with PBS, fluorescein-phalloidin conjugate (50 μg/ml in PBS containing 1% dimethyl sulfoxide) was added to each well, and the coverslips were incubated for 50 min in the dark at room temperature. Following this incubation, the coverslips were washed several times with PBS to remove unbound phalloidin. Stained coverslips were then observed and photographed with a camera-equipped Olympus BX50 incident fluorescence microscope.

Ethidium bromide staining of CHO cell nuclei. CHO cells were grown on coverslips and treated with bacterial supernatants as described above. At different times, cells were washed with a hypotonic salt solution, fixed in acetone-methanol (2:1) for 5 min, and incubated for 2 min with 5 ng of ethidium bromide per ml in water (6). After several washes with PBS, the coverslips were mounted and observed under the fluorescence microscope.

[^{3}H]thymidine incorporation. DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into ethanol-precipitable DNA. Cells were seeded at a concentration of 4×10^4/ml (200 μl/well) in 96-well plates and incubated for 18 h prior the addition of bacterial supernatants. At 24-h intervals after the addition of the toxin, cells were washed twice with PBS, and then serum-free medium (100 μl) containing 2.5 μCi of [^{3}H]thymidine per ml was added to the wells. The plates were incubated for an additional 16-h period, after which the cells were washed, treated with 50 μl of trypsin (2.5 mg/ml), and transferred to a MultiScreen-HV filtration plate (Millipore Corporation, Marlborough, Mass.). Cold ethanol (200 μl) was added to each well, and the wells were allowed to stand 10 min before vacuum filtration using a MultiScreen filtration apparatus (Millipore). After one wash with 200 μl of ethanol, the membranes were dried and punched directly into scintillation vials containing 500 μl of 0.42% sodium hypochlorite. The vials were incubated for 30 min with gentle shaking prior to the addition of 5 ml of scintillation cocktail (Ecolume; ICN, Costa Mesa, Calif.). Samples were then counted in a liquid scintillation spectrometer. Parallel control experiments were performed in which aphidicolin (5 μg/ml; Boehringer Mannheim Biochemicals), an inhibitor of DNA synthesis (8), was added to control and CDT-treated cells 3 h before the addition of [^{3}H]thymidine. The counts obtained in the presence of aphidicolin represent the background of the assay. All experiments were performed in sets of six, and the results were expressed as the average of the six replicates after correcting for the counts obtained in the corresponding experiment with aphidicolin.

RESULTS

Effect of CDT on CHO cell morphology. Previous reports have described the effect of CDT on susceptible cells as inducing progressive cellular distension and death. Preliminary observations in our laboratory confirmed these features of CDT action. We also noted that as CDT-treated cells become increasingly more distended and flattened in appearance, the boundaries between cells became less apparent, and the morphology of CDT-treated cells could not be fully visualized under phase-contrast microscopy. We therefore used SEM to examine the progression of cellular changes mediated by CDT. Figure 1 shows SEM images of CHO cells 2, 3, and 4 days after treatment with supernatants from *E. coli* BL21(pGEM-7Zf+) (control) or the CDT-producing strain BL21(pCDT32-6). A slight but detectable increase in cell size could be seen 2 days following CDT treatment compared to control cells; however, the general appearances of control- and toxin-treated cells were similar (Fig. 1A and D). Three days following CDT treatment, CHO cells appeared greatly distended and enlarged, with diameters that were approximately three times that of control cells, and possessed a more rounded cellular contour (Fig. 1B and E). The size of the CDT-treated cells continued to increase until day 4, when the morphological changes and enlargement stabilized; the cellular diameters, in some cases, were more than five times that of control cells (Fig. 1C and F). In addition to an expansion of total cell size, the nuclei of CDT-treated cells enlarged proportionally (Fig. 1F). Some cells appeared to be multinucleated (Fig. 1F), although this was not a general feature of CDT action. Increased nuclear size was also observed in CDT-treated cells when CDT-treated and control cells were stained with ethidium bromide and examined by fluorescence microscopy (not shown).

The massive cellular distension induced by CDT prompted us to examine the cytoskeleton of toxin-treated cells. Following CDT treatment, cells grown on glass coverslips were fixed, stained with fluorescein-phalloidin, and observed by both phase-contrast and fluorescence microscopy. After 48 h of toxin treatment, actin stress fibers were readily visible (not shown). After 3 or 4 days of treatment, a time when CDT-treated cells displayed obvious cellular distension, actin stress fibers were abundant throughout the toxin-treated cells (Fig.

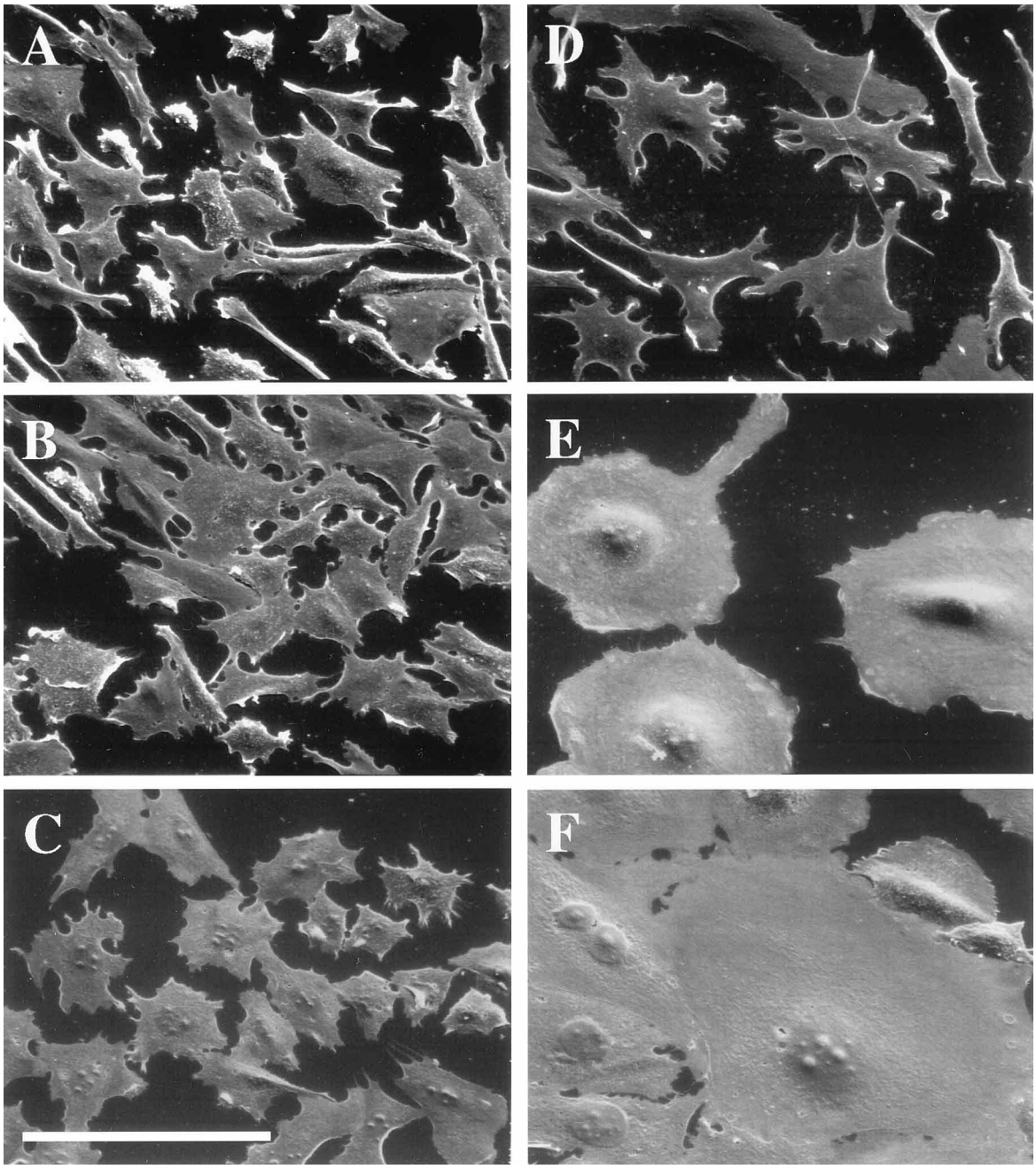

FIG. 1. Scanning electron micrographs of CDT-treated CHO cells. Cells treated with 1/16 dilutions of supernatant from *E. coli* BL21(pGEM-7Zf+) (A to C) or *E. coli* BL21(pCDT32-6) (D to F) were prepared for SEM as described in Materials and Methods and observed. Photographs were taken of representative fields of view after 2 (A and D), 3 (B and E), and 4 days (C and F) of toxin treatment. All panels are at a magnification of ×500; the scale bar in panel C represents 100 μm.

2A4). In contrast to these observations, control cells treated with the *E. coli* BL21(pGEM-7Zf+) culture supernatant displayed normal actin fibers localized at the cell periphery (Fig. 2A2), as did untreated control cells (not shown). After 5 days, actin stress fibers were still pronounced in toxin-treated cells (Fig. 2B4), while control cells maintained their normal morphology (Fig. 2B1 and 2). The morphological changes and the promotion of actin stress fiber polymerization observed in CHO cells treated with BL21(pCDT32-6) were identical to those produced by a supernatant fraction of *E. coli* 9142-88 (not shown).

Effect of CDT on CHO cell proliferation. Johnson and Lior (13) reported that sparsely seeded cells displayed more dramatic and reproducible changes than did cells seeded at nearly confluent densities. Although cytological changes could be observed at higher cell densities, the massive cellular distension, previously reported and shown here, was seen only when cells were seeded at a low cell density. These observations prompted us to assess the effect of CDT on cell proliferation and viability. The effects of culture supernatants from the CDT-producing strains 9142-88 and *E. coli* BL21(pCDT32-6) on CHO cell proliferation are shown in Fig. 3. The number of viable cells in

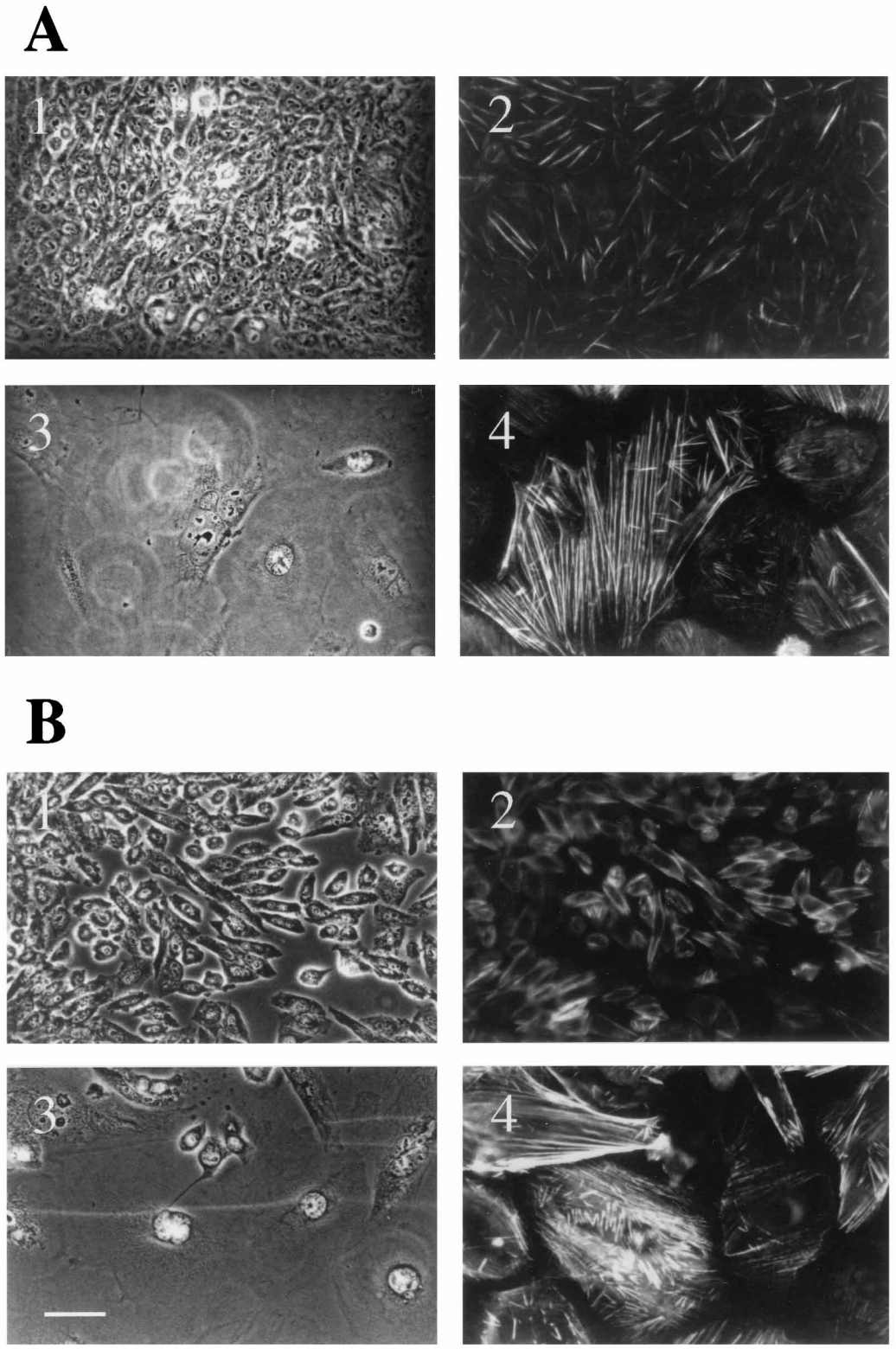

FIG. 2. F-actin assemblies in CDT-treated and control cells. Shown are phase-contrast (1 and 3) and fluorescent (2 and 4) micrographs of control (1 and 2) or CDT-treated (3 and 4) CHO cells at days 4 (A) and 5 (B) following the addition of 1/16 dilutions of culture filtrates. Scale bar in panel B3 represents 50 μm; all panels are shown at the same magnification.

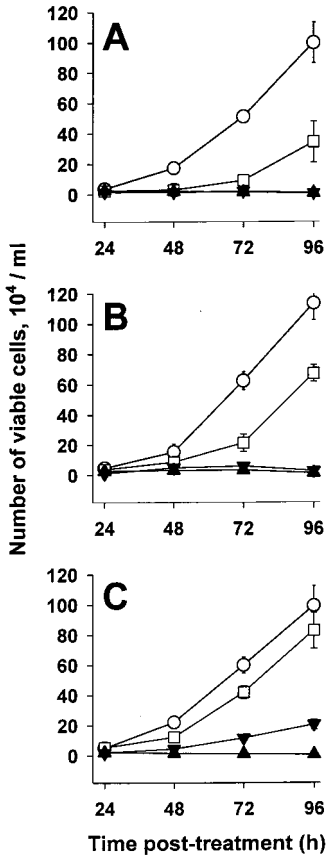

FIG. 3. Effect of CDT treatment on CHO cell growth and viability. Numbers of viable CHO cells after treatment with 1/4 (A), 1/16 (B) and 1/64 (C) dilutions of bacterial supernatant from *E. coli* BL21(pGEM-7Zf+) (squares), *E. coli* BL21(pCDT32-6) (upright triangles), or strain 9142-88 (inverted triangles) are shown. Cells with no treatment (circles) are shown as an additional control. Each data point represents the mean ± standard deviation of four samples taken from duplicate wells.

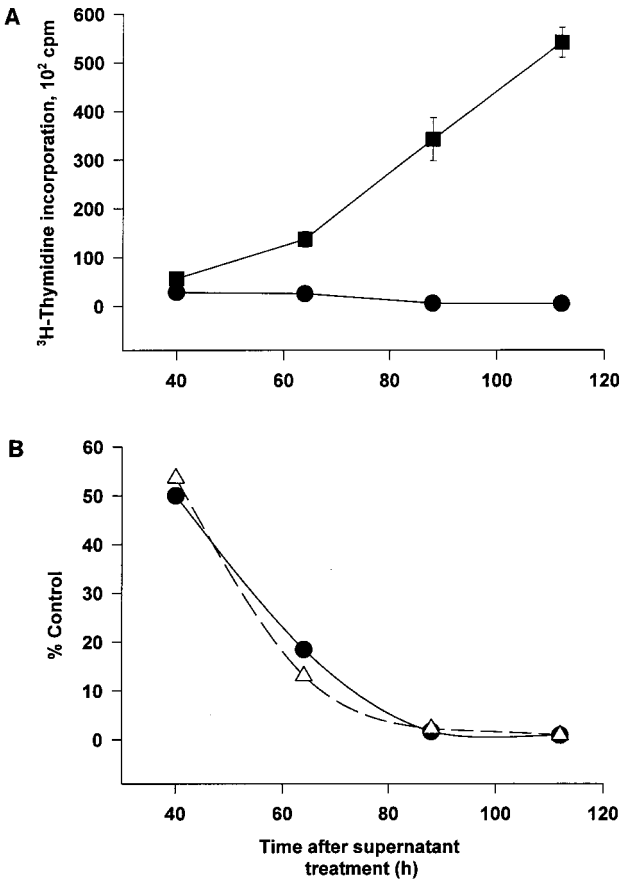

FIG. 4. Effect of CDT on [^{3}H]thymidine incorporation. [^{3}H]thymidine incorporation was measured in CHO cells treated with a 1/16 dilution of culture supernatant from *E. coli* BL21(pGEM-7Zf+) (squares) or *E. coli* BL21(pCDT32-6) (circles). Results are expressed as the net counts per minute after subtraction of the corresponding values obtained with aphidicolin to account for trapped but unincorporated ^{3}H (A). The relationship between [^{3}H]thymidine incorporation (circles) and viable cell counts (triangles) in CDT-treated cells relative to control values at various times is shown (B). The apparent decline in thymidine incorporation and viability in CDT-treated cells reflects the increase in control cell numbers with time.

CDT-treated wells remained almost constant through the 4 days of treatment (only a slight decrease was observed at the end of the observation period), while cells in the control (no treatment) and BL21(pGEM-7Zf+) supernatant-treated wells increased exponentially during the same time period (Fig. 3). Although cell growth was clearly inhibited by a low dilution of the BL21(pGEM-7Zf+) supernatant (Fig. 3A), cytopathic changes including elongation and distension were not observed; this inhibition of cell growth by the *E. coli* BL21(pGEM-7Zf+) culture supernatant was reduced and barely observed at the 1/16 and 1/64 dilutions, respectively (Fig. 3B and C).

The viability of the cells, as assessed by trypan blue exclusion, was corroborated by examining neutral red uptake. Despite the fact that CDT treatment induced dramatic structural changes in cells (Fig. 1 and 2) and appeared to arrest cell division (Fig. 3), toxin-treated cells incorporated neutral red dye through the 4 days of treatment (not shown). Unfortunately, the quantitative aspect of this assay (e.g., correlation between viable cell number and the level of neutral red uptake) could not be used, since the CDT-induced giant cells incorporated neutral red at levels which appeared to correspond to increased cell volume rather than viable cell number.

Effect of CDT on DNA synthesis. To further investigate the apparent cessation of cell division caused by CDT, we examined the effect of toxin treatment on [^{3}H]thymidine incorporation by CHO cells. Thymidine incorporation by control cells, treated with an *E. coli* BL21(pGEM-7Zf+) culture supernatant fraction, increased each day following treatment (Fig. 4A and Table 1), corresponding to the increase in cell numbers. In contrast to the linear increase observed in control cells, thymidine incorporation in CDT-treated cells remained constant over the first 3 days of the observation period (Fig. 4A and Table 1). After that time, a decrease in thymidine incorporation, presumably due to cell death, was detected (Table 1). Compared on the basis of percent thymidine incorporation and percent viability, CDT-treated cells displayed superimposable curves (Fig. 4B), indicating that the level of incorporation reflects the number of viable cells rather than a cessation of DNA synthesis. In fact, when the levels of [^{3}H]thymidine incorporation of CDT-treated and control cells were normalized for viable cell numbers, the values were nearly identical (Table 1). In the presence of aphidicolin, a DNA synthesis inhibitor, [^{3}H]thymidine incorporation was 11, 25, 40, and 42% of that of CDT-treated cells without the inhibitor at days 2, 3, 4, and 5, respectively. This result indicated that a significant level of

TABLE 1. Effect of CDT on [^{3}H]thymidine incorporation in CHO cells

Time[a]	BL21		BL21(pCDT32-6)	
	cpm[b]	cpm/10^3 cells[c]	cpm[b]	cpm/10^3 cells[c]
40	5,640 ± 305	589 ± 32	2,827 ± 255	550 ± 50
64	13,727 ± 1,605	511 ± 60	2,537 ± 628	725 ± 180
88	34,206 ± 4,447	421 ± 54	557 ± 303	311 ± 169
112	54,090 ± 3,062	466 ± 26	455 ± 95	455 ± 95

[a] Hours after treatment with BL21 or BL21(pCDT32-6) supernatant and addition of [^{3}H]thymidine.

[b] Mean of six experiments ± standard deviation expressed as counts per minute after subtraction of the values obtained in parallel experiments with aphidicolin.

[c] Mean of six experiments ± standard deviation expressed as counts per minute per 10^3 cells after subtraction of the values obtained in parallel experiments with aphidicolin.

synthesis continued in CDT-treated cells up to the time of cell death.

Minimum exposure resulting in CDT intoxication. Previous reports (11, 13) and the experiments described here have assessed the action of CDT on cells that were constantly exposed to the toxin. Our observations regarding the action of CDT on the cessation of cell division and the formation of actin stress fibers prompted us to examine the minimum time of CDT exposure that would result in these effects. Cells were incubated with the BL21(pCDT32-6) culture supernatant for various times ranging from 0 min to 48 h, washed twice with PBS, and then fed with complete medium without CDT. The cells were observed microscopically for 3 days, after which time cells in replicate wells were enumerated. After 2 days of incubation, toxicity was clearly discernible in all wells that received CDT. In addition, enumeration of viable cells at the end of 3 days indicated that a 2-min exposure to CDT was sufficient to result in a nearly complete cessation of cell growth compared to controls (Fig. 5). We did note that the viable cell counts resulting from wells treated with CDT for either 24 or 48 h were statistically lower than those resulting from CDT treatment for 8 h or less. Microscopic examination of cells throughout this experiment suggested that at the shorter treatment times, more cells had a normal appearance at 24 and 36 h posttreatment than did cells exposed to CDT for longer time periods.

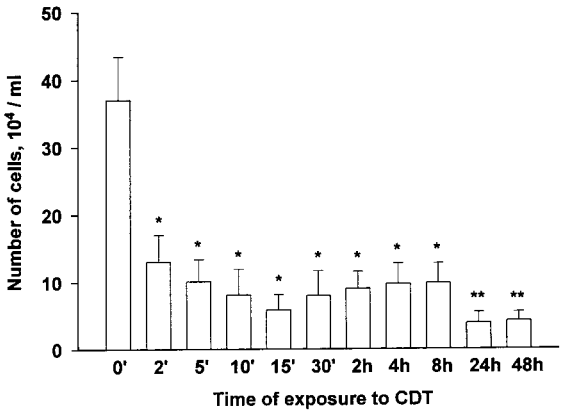

FIG. 5. Effect of exposure time to CDT on cell proliferation. Cells were treated with a 1/16 dilution of the *E. coli* BL21(pCDT32-6) culture filtrate for the times shown. Following toxin treatment, the culture wells were washed, the medium was replaced, and viable cell numbers were determined 3 days after the addition of CDT. Results are from a representative experiment performed twice. The datum for each time point is the mean of four samples from duplicate wells ± standard deviation. Significant values are given at $P < 0.001$ (*) relative to CDT treatment for 0 min and $P < 0.001$ (**) relative to times from 2 min through 8 h.

DISCUSSION

The characteristic morphological changes and cell target range associated with CDT action are distinct from those observed with other bacterial toxins. Cloning and sequencing of the genes encoding CDT from various sources, including *E. coli* (22, 24), *C. jejuni* (23), and *S. dysenteriae* (19), have confirmed that CDT is a unique toxin unlike other previously described gene products. However, despite the fact that CDT has been recognized for over a decade, the nature of toxin action remains obscure. Early reports on CDT action and our preliminary studies indicated that cellular distension due to CDT resulted in eventual cell death on or after days 4 to 5 following toxin treatment. It was not clear, however, if death was a function of toxin action over the 4- to 5-day period of time or if cells began dying immediately after the addition of toxin. This uncertainty, coupled with the observation that the effects of the toxin were greatest when target cells were sparsely seeded, suggested to us that the toxin may have been blocking cell division. That is, what on day 4 or 5 appeared to be the result of massive cell death (i.e., low cell numbers compared to control wells) was actually the result of the failure of toxin-treated cells to divide. By stripping cells from control and toxin-treated wells at daily intervals following the addition of CDT and enumerating viable cells, it became clear that CDT arrested cell division without immediately killing the cells. Further, the inhibition of cell division was not a function of cessation of DNA synthesis, although incorporation of [^{3}H]thymidine in CDT-treated cells did not increase over the 4-day observation period. The fact that CDT did not directly affect DNA synthesis was indicated by the fact that the levels of [^{3}H]thymidine incorporation per cell in control and toxin-treated CHO cells were nearly identical. This was confirmed by the observation that aphidicolin reduced the level of thymidine incorporation below that of CDT treatment.

Clues from previous reports describing the cellular distension mediated by CDT action suggested that the toxin may affect the cytoskeleton, since toxin-treated cells have lost the capacity to regulate cell shape and volume. Our results indicate that CDT causes an accumulation of actin stress fibers, coincident with cellular distension and cessation of cell division. Although the primary action of the toxin is not known, all of these events may be accounted for by an effect of CDT on actin assemblies (14, 16, 20).

A number of bacterial products are known to interact with elements of the eukaryotic cytoskeleton and affect F-actin assemblies (5). CNF1 and CNF2 both induce actin stress fiber formation following covalent modification and activation of RhoA (7, 21). Modification of RhoA by CNF appears to occur at or near the site of ADP-ribosylation by the *Clostridium botulinum* C3 transferase (7). The similarities between the action of CDT and that of CNF suggest that CDT may affect the function of RhoA, either through direct modification, as is the case for CNF, or through interaction with control elements which modulate the guanine nucleotide exchange rate of RhoA (9). However, the dissimilarities between CDT and CNF action, which include massive cellular distension, limited multinucleation, and slow progression to cell death mediated by CDT but not CNF, suggest that these toxins possess different modes of action.

Somewhat surprising was the fact that only a brief exposure

to CDT was necessary to initiate the cytological events associated with toxin action. Since CDT-treated cells were washed extensively following the appropriate time period, the action of CDT is apparently irreversible. This might imply that CDT binds very tightly to a surface receptor and initiates a cell signalling cascade or that CDT is rapidly internalized by target cells. In light of the dramatic cell morphological changes that accompany CDT action, including the formation of actin stress fibers and the cessation of cell division, it is perhaps more likely that CDT is internalized by CHO cells and imparts its action directly on the cellular components regulating actin assembly and cytokinesis.

The role of CDT in pathogenesis is presently unclear; however, CDT is widely distributed in *C. jejuni* isolates and in some clinical isolates where CDT was the only potential virulence factor observed. In one report, a CDT-producing *E. coli* strain (O55:K59:H4) was isolated from the stool of a child suffering from gastroenteritis and encephalopathy (1). No other viral or bacterial pathogens were isolated from the stool of the 3-year-old, and no other known toxin or virulence factor was associated with the *E. coli* isolate (1). In addition, a recent report (22) indicates that the *S. dysenteriae* CDT (and presumably other CDTs) induces watery diarrhea in a suckling mouse experimental model. In this experimental model, CDT also caused rapid necrosis and mucosal sloughing in the descending colon of treated animals. Although the relationship between in vitro toxin action and a potential role in pathogenesis is presently unclear, our findings reported here and those of others suggest that CDT may be an important factor in intestinal pathogenesis.

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