Cytolethal Distending Toxin from Shiga Toxin-Producing *Escherichia coli* O157 Causes Irreversible G_2/M Arrest, Inhibition of Proliferation, and Death of Human Endothelial Cells

Martina Bielaszewska,[1]* Bhanu Sinha,[2] Thorsten Kuczius,[1] and Helge Karch[1]

Institut für Hygiene[1] and Institut für Medizinische Mikrobiologie,[2] Universitätsklinikum Münster, Münster, Germany

Recently, cytolethal distending toxin V (CDT-V), a new member of the CDT family, was identified in Shiga toxin-producing *Escherichia coli* (STEC) O157 and particular non-O157 serotypes. Here we investigated the biological effects of CDT-V from STEC O157:H⁻ (strain 493/89) on human endothelial cells, which are believed to be major pathogenetic targets in severe STEC-mediated diseases. CDT-V caused dose-dependent G_2/M cell cycle arrest leading to distension, inhibition of proliferation, and death in primary human umbilical vein endothelial cells (HUVEC) and two endothelial cell lines, EA.hy 926 cells (HUVEC derived) and human brain microvascular endothelial cells (HBMEC). The cell cycle effects of CDT-V were cell type specific. In HUVEC and EA.hy 926 cells, CDT-V caused a slowly developing but persistent G_2/M block which resulted in delayed nonapoptotic cell death. In contrast, in HBMEC, CDT-V induced a rapidly evolving but transient G_2/M block which was followed by progressive, mostly apoptotic cell death. In both HBMEC and EA.hy 926 cells, G_2/M arrest was preceded by the early accumulation of a phosphorylated inactive form of cdc2 kinase. Significant G_2/M arrest and inhibition of proliferation in both HUVEC and each of the endothelial cell lines were induced by 2 to 15 min of exposure to CDT-V, indicating that the effects of the toxin are irreversible. CDT-V-treated HBMEC and EA.hy 926 cells displayed fragmented nuclei and expressed phosphorylated histone protein H2AX, indicative of DNA damage followed by a DNA repair response. Our data demonstrate that CDT-V causes irreversible damage to human endothelial cells and thus may contribute to the pathogenesis of STEC-mediated diseases.

Cytolethal distending toxins (CDTs), a family of proteins which interfere with the cell cycle control machinery through their genotoxic activity (12, 22, 47), are produced by multiple pathogens, such as *Escherichia coli* (30, 44, 45, 51, 57), *Campylobacter* spp. (31, 46), *Shigella* spp. (41), *Salmonella enterica* serovar Typhi (24), *Haemophilus ducreyi* (11), *Actinobacillus actinomycetemcomitans* (37), and *Helicobacter* spp. (9, 59). CDTs are tripartite toxins encoded by three adjacent or slightly overlapping genes, *cdtA*, *cdtB*, and *cdtC* (11, 30, 35, 44, 45, 51). All *cdt* genes are required for arrest of the eukaryotic cell cycle in the G_1 phase (12, 13, 26) and/or the G_2 phase (12, 13, 35, 44, 45, 58); this arrest characteristically distends cells and eventually causes cell death (12, 34, 35, 44, 47). Recent studies suggest that within the CDT holotoxin, CdtB is the enzymatically active (A) subunit (34, 35), which is transported into the nucleus (40). In the nucleus, CdtB damages through DNase I-like activity (18, 19, 34) host cell DNA (18, 34), thereby triggering DNA damage checkpoint responses (12, 22) that arrest the cell cycle (12, 18, 19, 34, 35). The CdtA and CdtC polypeptides constitute the heterodimeric (B) subunit (15, 35, 36), which is required for CDT binding to target cells (15, 36) and for the intracellular delivery of CdtB (15, 35). This mechanism of

action is supported by the crystal structure of CDT holotoxin from *H. ducreyi* (HdCDT) (38). The cellular response to CDT is associated with the accumulation of serine-20-phosphorylated (stabilized) tumor supressor protein p53, which is the key regulator of G_1 arrest (12, 13, 22), or of a tyrosine-15- and threonine-14-phosphorylated, inactive form of cyclin-dependent kinase cdc2, which accounts for G_2 arrest (10). Some cells, such as B lymphocytes (13) and T lymphocytes (52), undergo apoptosis after exposure to CDT.

Shiga toxin (Stx)-producing *E. coli* (STEC) strains, including *E. coli* O157:H7 (56) and several non-O157 serotypes (3, 20, 42), cause diarrhea and hemolytic-uremic syndrome (HUS) worldwide. Stx is considered to be the cardinal virulence factor of STEC, but several other bacterial products, including toxins (49), adhesins (8, 29, 43, 55, 60), and proteases (7, 50), have been implicated as putative virulence factors of these strains. Recently, a new member of the CDT family, CDT-V, was identified in sorbitol-fermenting (SF) STEC O157:H⁻ strain 493/89 (30). This toxin was shown to be produced by the majority (87%) of SF STEC O157:H⁻ strains and a subset (6%) of STEC O157:H7 strains (30). Subsequently, it was demonstrated that CDT-V is also frequently found in non-O157 STEC strains of serotypes O91: H21 and O113:H21 (4), which cause serious human diseases, including HUS (4, 27, 42), despite the fact that these strains lack the *eae* gene encoding intimin (4, 42), a STEC factor associated with virulence and HUS (6). Thus, we have spec-

* Corresponding author. Mailing address: Institut für Hygiene, Universitätsklinikum Münster, Robert-Koch Str. 41, 48149 Münster, Germany. Phone: 49-251-980-2849. Fax: 49-251-8355341. E-mail: mbiela @uni-muenster.de.

ulated that CDT-V may contribute to the pathogenicity of these organisms (4).

Injury to microvascular endothelial cells in the renal glomeruli, large intestine, and brain is a key histopathological event underlying the development of severe STEC-mediated diseases, such as HUS (48). Recently, Svensson and colleagues (54) reported that HdCDT, another member of the CDT family, has antiproliferative effects on microvascular endothelial cells from human skin and hypothesized that these effects may play a role in the pathogenesis of the disease caused by *H. ducreyi* (54). The earlier finding of CDT-V in STEC of serotypes frequently associated with HUS, combined with the results of that report (54), prompted us to investigate the effects of CDT-V on human endothelial cells in vitro.

MATERIALS AND METHODS

Reagents. Cell culture materials and their sources were as follows: medium 199 and Dulbecco minimal essential medium (MEM)–F-12 medium (1:1) containing GlutaMAX-I, Gibco Invitrogen (Paisley, United Kingdom); RPMI 1640, fetal calf serum (FCS), L-glutamine, sodium pyruvate, MEM nonessential amino acids, MEM vitamins, penicillin-streptomycin, trypsin, and phosphate-buffered saline (PBS), Cambrex Bioscience (Verviers, Belgium); Nu-Serum, Becton Dickinson Biosciences (Bedford, Mass.); human serum and endothelial cell growth supplement, PromoCell (Heidelberg, Germany); heparin, Roche (Mannheim, Germany); collagenase and fibronectin, Sigma-Aldrich (Taufkirchen, Germany); and cell culture flasks (25 and 75 cm^2) and cell culture plates (6, 12, and 96 wells), Corning Inc. (Corning, N.Y.). The antibodies used were as follows: anti-p34^{cdc2} mouse monoclonal antibody (clone A17), Sigma-Aldrich; anti-phospho-histone H2AX (serine-139) mouse monoclonal antibody (clone JBW301), Biomol (Hamburg, Germany); and horseradish peroxidase-conjugated affinity-purified goat anti-mouse immunoglobulin G antibody, Dianova (Hamburg, Germany). Other reagents and their sources were as follows: irreversible broad-spectrum caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(methyl ester)-fluoromethylketone (zVAD-fmk), Enzyme Systems (Livermore, Calif.); propidium iodide (PI), nocodazole, RNase A, diamidinophenylindole (DAPI), Triton X-100, sodium citrate, and Tween 20, Sigma-Aldrich; skim milk powder, Roth (Karlsruhe, Germany); and mounting medium for fluorescence microscopy, DakoCytomation (Hamburg, Germany).

Cell cultures. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by collagenase treatment (28). These cells were grown on fibronectin-coated (50 µg/ml) dishes with medium 199 containing 10% FCS, 10% human serum, 0.2% endothelial cell growth supplement, 0.1% heparin, penicillin (100 U/ml), and streptomycin (100 µg/ml). EA.hy 926 cells, a permanent endothelial cell line derived from HUVEC by fusion with the lung carcinoma cell line A549 (17), were kindly provided by Volker Gerke (University Hospital of Münster, Münster, Germany). These cells were maintained in Dulbecco MEM–F-12 (1:1) containing GlutaMAX-I and supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Human brain microvascular endothelial cells (HBMEC) (53) were a generous gift from Kwang Sik Kim (School of Medicine, Johns Hopkins University, Baltimore, Md.). These cells were grown in RPMI 1640 supplemented with 10% FCS, 10% Nu-Serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential amino acids, 1% MEM vitamins, penicillin (100 U/ml), and streptomycin (100 µg/ml).

CDT-V preparation. CDT-V was produced from *E. coli* XL1-Blue MR hosting a cosmid (SuperCos I; Stratagene, Heidelberg, Germany) which contains the three CDT-V open reading frames (*cdtA*, *cdtB*, and *cdtC*) cloned from SF STEC O157:H$^-$ strain 493/89 (32) as described previously (30). The CDT-V preparation was a filter-sterilized (0.22-µm-pore size; Schleicher & Schuell GmbH, Dassel, Germany) supernatant of a 24-h-aerated (180 rpm) culture of the CDT-V open reading frame-containing cosmid clone (clone 13–18) in cell culture medium. The protein concentration was 3.7 mg/ml. The 50% cytotoxic doses (CD$_{50}$) for HUVEC, EA.hy 926 cells, and HBMEC, which were defined as the highest dilutions of the CDT-V preparation that caused distension in 50% of the cells after 5 days of incubation, corresponded to dilutions of 1:64, 1:512, and 1:512, respectively, and to protein concentrations of 57.8, 7.2, and 7.2 µg/ml, respectively. In the experiments, 1, 2, or 8 CD$_{50}$/ml were used. The control preparation was a sterilely filtered supernatant of *E. coli* XL1-Blue MR hosting the SuperCos I cosmid without the CDT-V insert. In all experiments, it was used at a dilution corresponding to that of the CDT-V preparation which contained 8

CD$_{50}$/ml. Cells treated with the control preparation did not display any significant changes in their morphology, cell cycle, viability, or proliferation compared to untreated cells. Therefore, in this article the term "control cells" refers to cells exposed to the control preparation. Both the CDT-V and the control preparations were stored in aliquots at $-20°C$ until use.

CDT bioassay. The morphological effect of CDT-V on human endothelial cells was assayed by adding 1-ml portions of twofold dilutions of the CDT-V preparation to HUVEC, EA.hy 926 cells, and HBMEC freshly seeded in six-well tissue culture plates in amounts of 1×10^6, 1.5×10^5, and 1×10^5 cells/well, respectively. The assay mixtures were incubated for 5 days at 37°C in 5% CO$_2$ and examined daily by using a light microscope (Axiovert 100; Zeiss, Jena, Germany) at a final magnification of ×100.

Cell cycle analysis and apoptosis assay. HUVEC, EA.hy 926 cells, and HBMEC were seeded in 12-well plates in amounts of 1×10^5, 1.4×10^5, and 7×10^4 cells/well, respectively, and grown (37°C, 5% CO$_2$) until 70 to 80% confluence. The CDT-V preparation (1, 2, or 8 CD$_{50}$/ml) or the control preparation was added to the cells, and the mixtures were incubated for 24, 48, 72, 96, and 120 h. At each time point, detached cells that were free within the culture medium and adherent cells harvested by trypsinization were pelleted together and stained with Nicoletti buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 µg of PI/ml, 20 µg of RNase/ml) (39). After incubation for 30 min on ice, the DNA content of the nuclei was determined by flow cytometry (FACScalibur; Becton Dickinson, Heidelberg, Germany) with red (PI) emission (FL-2 channel, 570 nm). After forward scatter-side scatter gating for the exclusion of debris, the data from at least 10^4 nuclei were collected and analyzed by using CellQuest software (Becton Dickinson). In a set of experiments in which the irreversible effect of CDT was studied, cells were exposed to CDT for various times, CDT was removed, and cells were washed twice with PBS, incubated in fresh medium for up to 24 h (HBMEC) or 120 h (EA.hy 926 cells), and then analyzed as described above. Apoptosis was measured by flow cytometric determination of the proportion of hypodiploid nuclei (39) as described earlier (2, 25). In some experiments, the polycaspase inhibitor zVAD-fmk (50 µM) was added to the cells 30 min before the other stimuli.

Cell viability and cell proliferation. Cell viability and proliferation were tested by trypan blue (0.2%) exclusion and two different commercial assays (Roche). The WST-1 metabolic viability assay is based on the mitochondrial reduction of the tetrazolium salt WST-1 to formazan, which is quantified spectrophotometrically. In the DNA synthesis assay (cell proliferation enzyme-linked immunosorbent assay [ELISA] of bromodeoxyuridine [BrdU]), the incorporation of pyrimidine analog BrdU into newly synthesized DNA of replicating cells is measured by an ELISA. The WST-1 and BrdU assays were performed with 96-well plates seeded with EA.hy 926 cells at 2×10^3/well and 3×10^3/well, respectively, and with HBMEC at 6×10^2/well and 2×10^2/well, respectively. Overnight monolayers were treated with the CDT-V preparation (1, 2, or 8 CD$_{50}$/ml) or the control preparation for 24, 48, 72, 96, and 120 h and subsequently processed according to the manufacturer's instructions. The absorbance was measured at 450 nm and reference wavelength 650 nm by using an ELISA reader (*E*max precision microplate reader; MWG Biotech, Ebersberg, Germany). To avoid interference of starvation with cell proliferation, fresh medium was added to cells after 48 h. The irreversible effect of CDT-V on cell proliferation was tested by exposing cells to CDT-V for various times, washing them with PBS, incubating them in fresh medium for up to 96 h, and then processing them in a proliferation assay.

Immunoblot analysis. EA.hy 926 cells and HBMEC (1.4×10^5 and 7×10^4/well, respectively) were grown in 12-well plates until 70 to 80% confluence and stimulated with the CDT-V preparation (8 CD$_{50}$/ml), the control preparation, or nocodazole (100 nM) for various times. Subsequently, the cells were washed with PBS, harvested by trypsinization, resuspended in electrophoresis sample buffer (33), lysed by being heated to 99°C for 10 min, and subjected to sonication (Sonopuls; Bandelin Electronic, Berlin, Germany) for 90 s. Total cellular proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (33) in a mini-slab gel apparatus (Bio-Rad, Munich, Germany) with 13% separation gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Roth) by using a semidry blotting system (Roth). After blocking was done for 1 h in PBS containing 0.1% Tween 20 (PBS-T; pH 7.4) and 1% skim milk powder, membranes were incubated for 2 to 16 h with specific antibodies diluted 1:2,000 (anti-p34^{cdc2}) or 1:10,000 (anti-phospho-histone H2AX) in PBS-T. Membranes then were washed and incubated with horseradish peroxidase-conjugated antimouse immunoglobulin G secondary antibody diluted 1:1,000 in PBS-T for 1 h. After washing was done, the membranes were developed by using a chemiluminescence enhancement kit (Perbio Science, Bonn, Germany), and signals were
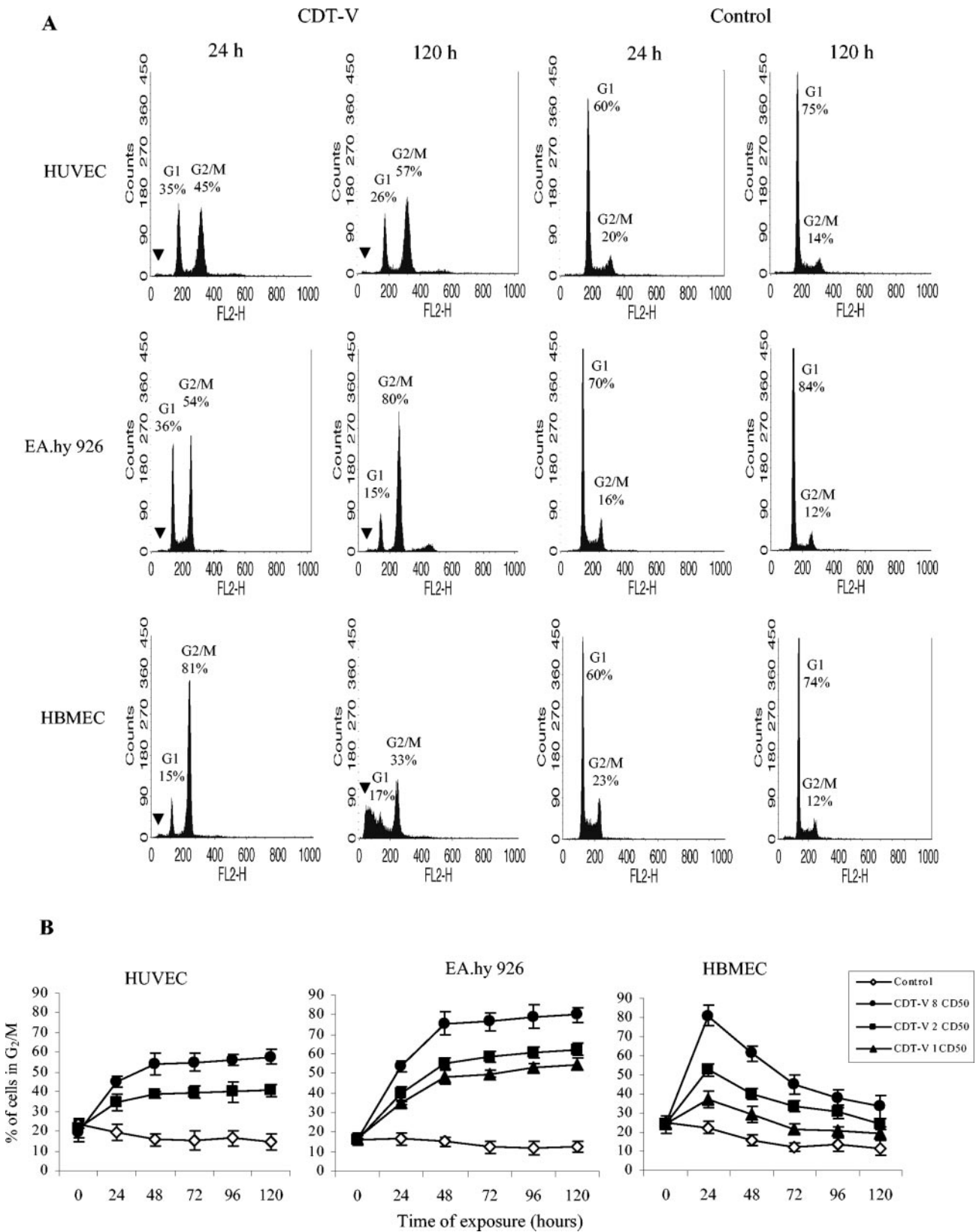
FIG. 1. CDT-V causes G_2/M arrest in human endothelial cells. (A) Flow cytometric analysis of HUVEC, EA.hy 926 cells, and HBMEC after 1 and 5 days of treatment with 8 CD_{50} of CDT-V/ml or with the control preparation. The proportions of cells in G_1 (2n DNA) and G_2/M (4n DNA) are indicated. Black arrowheads indicate the sub-G_1 population. Data represent one of three independent experiments. (B) HUVEC, EA.hy 926 cells, and HBMEC were treated with 1 (triangles), 2 (squares), or 8 (circles) CD_{50} of CDT-V/ml or with the control preparation (diamonds) for the indicated times. The proportions of cells in G_2/M were determined by flow cytometry. Time zero indicates basal numbers of cells in G_2/M, which were obtained by exposing the cells to CDT-V or the control preparation for 5 min, followed by immediate flow cytometric analysis. Data are means and standard deviations from three independent experiments.

visualized on a chemiluminescence photoimager (Boehringer, Mannheim, Germany).

Fluorescence microscopy. EA.hy 926 cells (1.5×10^5/well) and HBMEC (1×10^5/well) were cultured with 8 CD_{50} of the CDT-V preparation/ml or the control preparation on cover slides placed in six-well plates. After 5 days, the cells were stained with DAPI (20 μg/ml) by the procedure described by De Rycke et al. (16). The slides were mounted in DakoCytomation mounting medium and examined by using a fluorescence microscope (Axiophot; Zeiss) with a ×40 objective lens.

Statistical analysis. Statistical analysis was performed by using the paired t test (two tailed) calculated with OpenStat2 software (W. Miller, Iowa State University; http://www.statpages.org/miller/openstat/OPENSTAT2.htm). P values of <0.05 were considered significant.

RESULTS

CDT-V interferes with the cell cycle of endothelial cells in a cell type-specific manner. To investigate the effect of CDT-V on the cell cycle of human endothelial cells, HUVEC, EA.hy 926 cells, and HBMEC were treated with the toxin for 1 to 5 days, and the DNA content was analyzed by flow cytometry. CDT-V caused G_2/M arrest, characterized by a 4n DNA content, in each of the cell cultures investigated (Fig. 1A). In contrast, control cells stimulated with the vector preparation demonstrated normal cycling, with the majority of cells being in the G_1 phase (2n DNA content) (Fig. 1A). The G_2/M arrest was dose and time dependent (Fig. 1B), and its time course differed among the cell types investigated. In HUVEC and EA.hy 926 cells, the proportion of cells arrested in G_2/M increased from day 1 to day 5 posttreatment, reaching a plateau on day 2 (Fig. 1B). However, at each time point, the proportion of HUVEC in G_2/M was lower than that of EA.hy 926 cells (Fig. 1), with a substantial proportion of the former cells remaining in G_1 even after 5 days of exposure to CDT-V (Fig. 1A). The same pattern of G_2/M arrest for HUVEC was observed after treatment with the G_2/M cell cycle inhibitor nocodazole (100 nM), which blocked between 46 and 58% of HUVEC in G_2/M between days 1 and 5, leaving between 36 and 26% of cells in G_1 (data not shown). In contrast to the findings for HUVEC and EA.hy 926 cells, the G_2/M block in HBMEC peaked 24 h after toxin exposure (81% of cells in G_2/M) and then decreased steadily during the next 4 days to 33% of cells in G_2/M on day 5 (Fig. 1).

As determined by trypan blue exclusion (Fig. 2A), the majority of HUVEC and EA.hy 926 cells arrested in G_2/M remained alive up to day 4, with cell death starting on day 5. In contrast, the number of viable HBMEC decreased sharply between day 1 and day 5, indicating that the observed decrease in the G_2/M block was caused by cell death. The latter finding was consistent with the rapid decrease in the HBMEC G_2/M block being accompanied by a progressive increase in the population of cells with hypodiploid nuclei (sub-G_1) (Fig. 1A), which are considered apoptotic. The proportion of cells with hypodiploid nuclei rose from 28% on day 1 to 83% on day 5, when it was more than threefold larger in CDT-V-treated cells than in control cells (data not shown). Neither HUVEC nor EA.hy 926 cells contained a sub-G_1 population (Fig. 1A).

To determine whether HBMEC in the sub-G1 population underwent caspase-mediated apoptotic cell death, we determined the proportions of cells containing hypodiploid nuclei with and without pretreatment with the polycaspase inhibitor zVAD-fmk (Fig. 3). The proportion of CDT-V-exposed cells
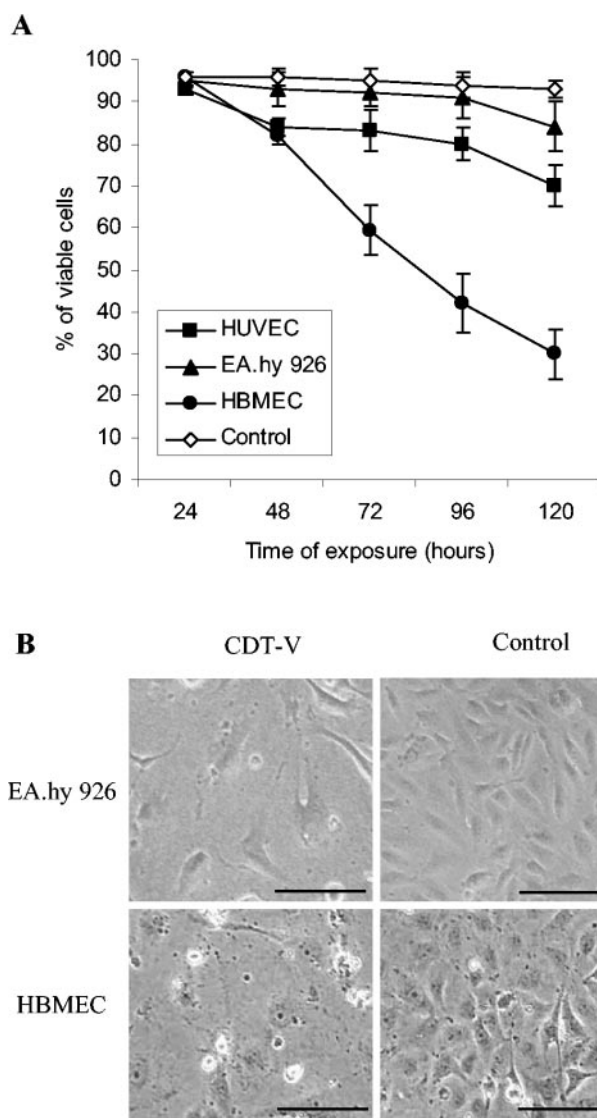

FIG. 2. G_2/M arrest of endothelial cells results in cell distension and ultimately cell death. (A) Viability of cells arrested in G_2/M. Cells analyzed for G_2/M arrest were stained with trypan blue. Proportions of viable cells were determined at each time point microscopically. Numbers of viable control cells did not differ significantly in the three cell cultures and are shown combined as "Control." Data are means and standard deviations from three independent experiments. (B) Photomicrographs of EA.hy 926 cell and HBMEC cultures exposed for 5 and 4 days, respectively, to 8 CD_{50} of CDT-V/ml or to the control preparation. CDT-V caused the formation of giant cells that were four- to eightfold larger than control cells and had nuclei that were two- to threefold larger. Magnification, ×100; bars, 200 μm.

with hypodiploid nuclei was largely reduced by preincubation with zVAD-fmk to the level seen in cells exposed to the vector (Fig. 3). This result suggests that caspase-dependent apoptotic cell death accounted for most of the observed death of G_2/M-arrested HBMEC.

Taken together, these data demonstrate that CDT-V displays cell type-specific patterns of interaction with the cell cycle of human endothelial cells. HUVEC and EA.hy 926 cells sustain a slowly developing but persistent G_2/M arrest, resulting in
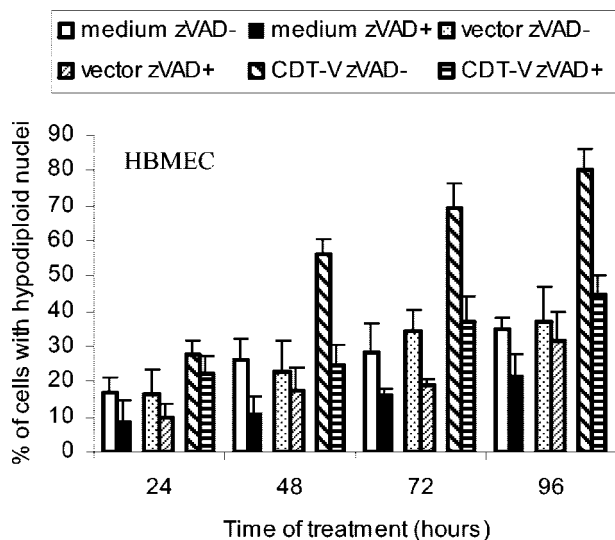
FIG. 3. CDT-V induces apoptotic cell death in HBMEC. Cells were incubated with 8 CD_{50} of CDT-V/ml, the vector preparation, or cell culture medium in the absence or the presence of zVAD-fmk (50 μM). Apoptosis was determined by flow cytometry as a proportion of hypodiploid nuclei. Data are means and ranges from two experiments.
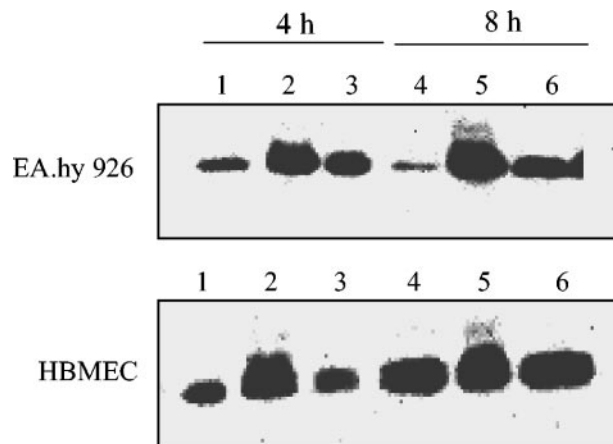

FIG. 4. CDT-V induces the accumulation of phosphorylated, inactive cdc2 kinase. EA.hy 926 cells and HBMEC were treated with 8 CD_{50} of CDT-V/ml (lanes 2 and 5), the vector preparation (lanes 1 and 4), or nocodazole (lanes 3 and 6) for 4 h (lanes 1 to 3) or 8 h (lanes 4 to 6). Isolated total cellular proteins were analyzed by immunoblotting with anti-cdc2 antibody. The strong signals displayed by cells treated with CDT-V (lanes 2 and 5) were double bands which were not completely separated on the gels.

delayed nonapoptotic cell death, whereas HBMEC experience a rapidly peaking but transient G_2/M block, followed by progressive cell death caused mostly by caspase-dependent apoptosis. In each of the cell cultures, cell death was preceded by cellular distension which was apparent from day 3 after CDT-V exposure and which was more pronounced in EA.hy 926 cells and HBMEC (Fig. 2B) than in HUVEC (data not shown).

CDT-V-mediated G_2/M arrest is preceded by the accumulation of the phosphorylated form of cdc2 kinase in endothelial cells. The phosphorylation status of cdc2 in EA.hy 926 cells and HBMEC treated with CDT-V or nocodazole (100 nM) for 4 or 8 h or in control cells was determined by immunoblot analysis of total cellular proteins with a monoclonal antibody against cdc2 (Fig. 4). At each time point, CDT-V-treated cells of both lines (Fig. 4, lanes 2 and 5) expressed two bands reacting with the anti-cdc2 antibody; these bands were consistent with the phosphorylated, inactive form of cdc2 (upper band) and the dephosphorylated, active form of cdc2 (lower band) (10). Dephosphorylation of cdc2 on threonine-14 and tyrosine-15 in late G_2 is a prerequisite for its activation and for cells to enter mitosis (10). The phosphorylated form of cdc2 was absent from control cells (Fig. 4, lanes 1 and 4) and from cells treated with nocodazole (Fig. 4, lanes 3 and 6), both of which contained only the dephosphorylated form of the kinase. The absence of the phosphorylated form of cdc2 in nocodazole-treated cells, which progress from G_2 to mitosis but are blocked in prometaphase (10), and its presence in cells treated with CDT-V suggest that the CDT-V-treated cells were unable to pass from G_2 to mitosis; these cells were therefore arrested in G_2 rather than in mitosis.

CDT-V inhibits the proliferation of human endothelial cells. To investigate the effect of CDT-V on the proliferation of endothelial cells, cells were treated with 1, 2, or 8 CD_{50} of toxin/ml for 1 to 5 days. Proliferation was measured at 24-h

intervals by quantifying cellular metabolic activity (WST-1 assay) and DNA synthesis (BrdU incorporation assay) and by light microscopy (trypan blue exclusion). Based on metabolic activity (Fig. 5A and B) and DNA synthesis (Fig. 5C and D), CDT-V inhibited the proliferation of EA.hy 926 cells (Fig. 5A and C) and HBMEC (Fig. 5B and D) in a dose- and time-dependent manner. In contrast, control cells proliferated linearly during the experimental period (Fig. 5A to D). The low levels of metabolic activity and DNA synthesis in EA.hy 926 cells and HBMEC treated with 8 CD_{50} of CDT-V/ml (Fig. 5A to D) corresponded to a nearly complete cessation of cell proliferation in each of the cell lines, as determined by trypan blue exclusion (data not shown). The latter test also demonstrated that the declines in metabolic activity and DNA synthesis observed on day 5 in EA.hy 926 cells (Fig. 5A and C) and throughout the entire experimental period in HBMEC (Fig. 5B and D) reflected cell death (data not shown). The effect of CDT-V on the proliferation of HUVEC, which did not grow reliably in the 96-well plates used for the WST-1 and BrdU assays, was determined by trypan blue exclusion performed on cells cultured in 12-well plates. The toxin inhibited the proliferation of these primary endothelial cells (Fig. 5E) in a manner similar to that observed in EA.hy 926 cells and HBMEC.

CDT-V-mediated G_2/M arrest and inhibition of proliferation are irreversible. All experiments described so far were performed with cells that were continuously stimulated with CDT-V. Therefore, we further investigated the minimum length of exposure to CDT-V which is required to induce a G_2/M block and inhibit cell proliferation. To this end, cells were exposed to CDT-V (8 CD_{50}/ml) for various intervals (2 min to 120 h) and then washed and incubated in fresh medium without CDT-V until the end of each experiment. Exposures of 5 and 15 min (Fig. 6A) were sufficient for CDT-V to cause G_2/M arrest in 50% of the total HBMEC and EA.hy 926 cells, respectively, which were arrested in G_2/M at the time when the block peaked upon continuous stimulation (24 h for HBMEC
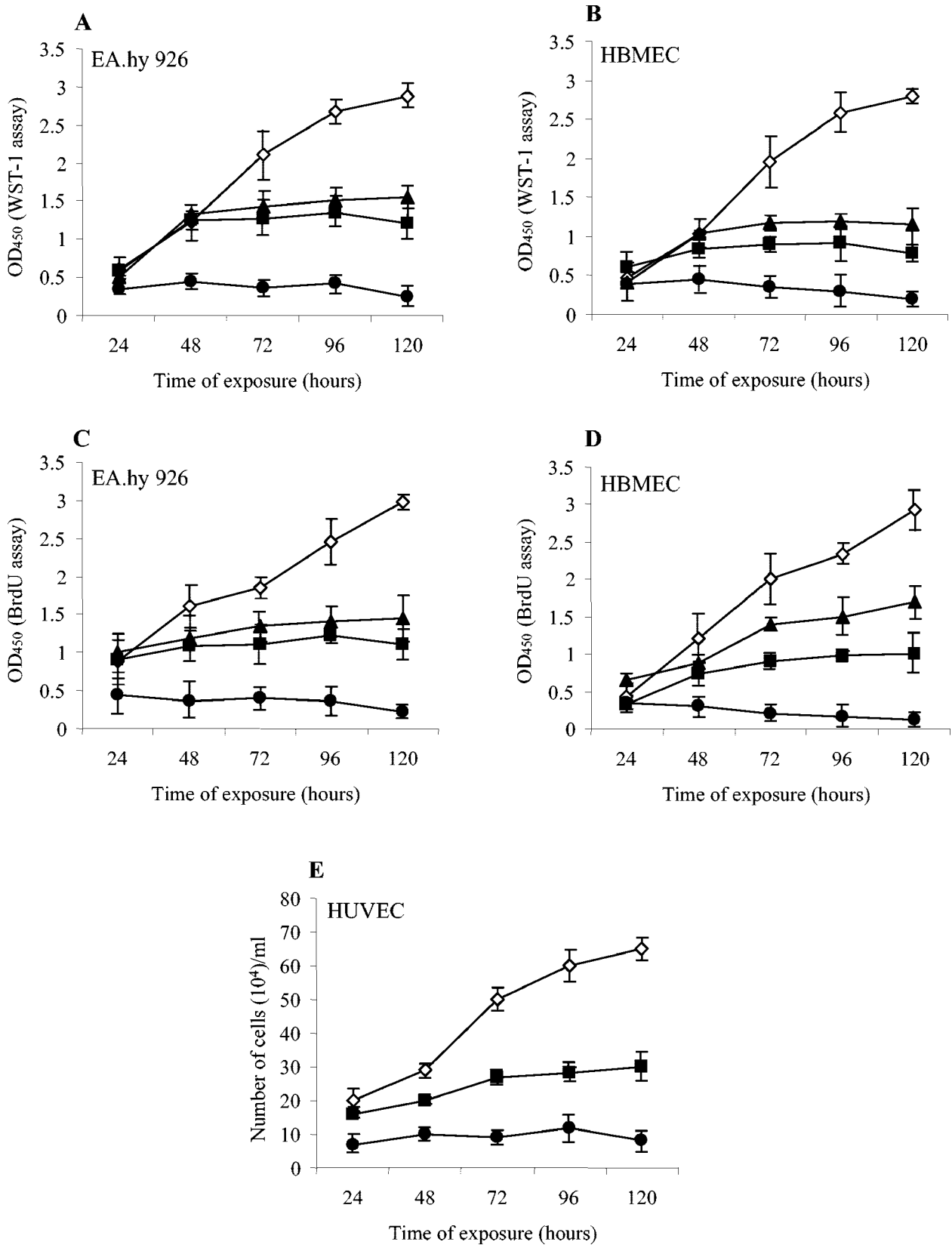
FIG. 5. CDT-V inhibits the proliferation of human endothelial cells. EA.hy 926 cells (A and C), HBMEC (B and D), and HUVEC (E) were treated with 1 (triangles), 2 (squares), or 8 (circles) CD_{50} of CDT-V/ml or with the vector preparation (diamonds) for the indicated times. Cells then were processed in the WST-1 (A and B), BrdU (C and D), and trypan blue (E) assays. Data are means and standard deviations from three independent experiments.
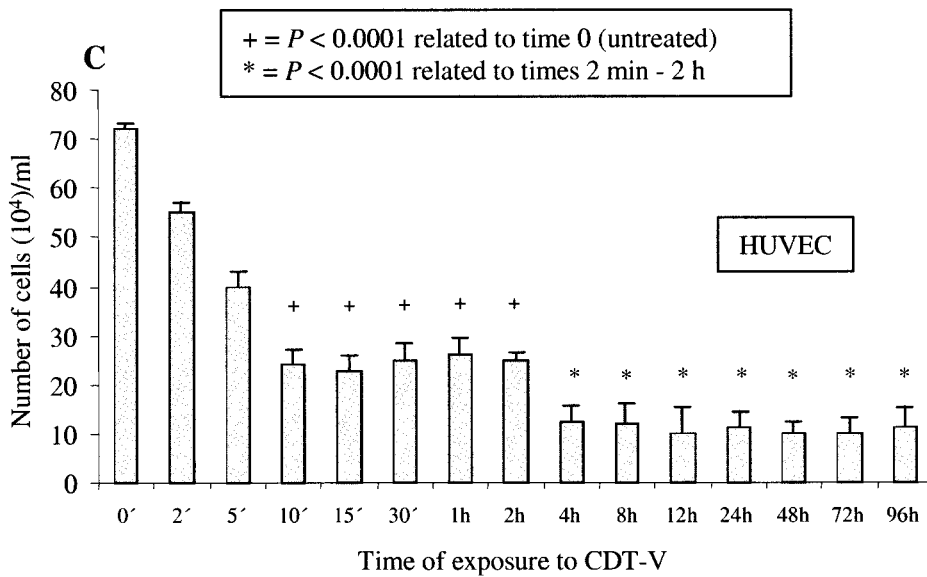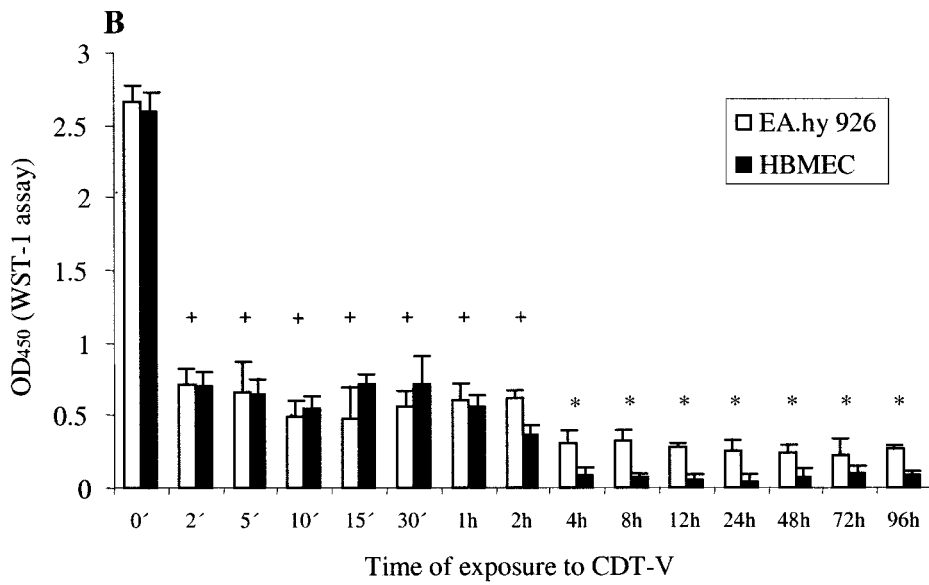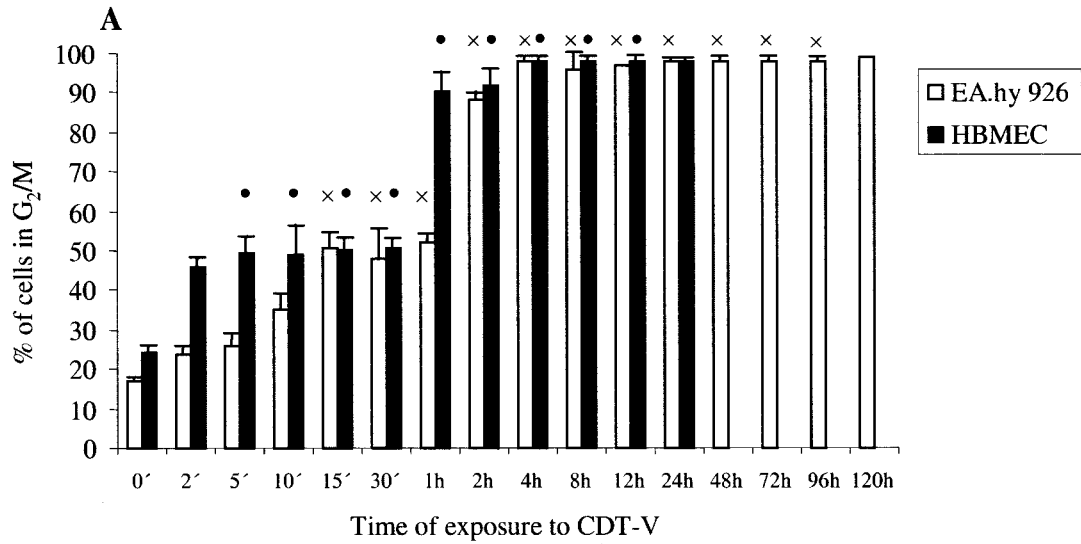
FIG. 6. Minimum length of exposure to CDT-V required for G₂/M arrest and inhibition of proliferation in endothelial cells. Cells were exposed to CDT-V (8 CD₅₀/ml) for the indicated times, the toxin then was removed, and cells were washed and incubated in medium without CDT-V. G₂/M arrest (A) was analyzed by flow cytometry 24 h (HBMEC) or 120 h (EA.hy 926 cells) after the addition of CDT-V. The percentages of the total

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and 120 h for EA.hy 926 cells). The plateau of G_2/M arrest in HBMEC and EA.hy 926 cell cultures was induced by exposures of 1 and 2 h, respectively (Fig. 6A). Exposure to CDT-V for as little as 2 min resulted in an almost complete cessation of proliferation of both HBMEC and EA.hy 926 cells, as determined by the WST-1 assay (Fig. 6B). In HUVEC, CDT-V significantly inhibited cell proliferation, as measured by trypan blue exclusion, after 10 min of exposure (Fig. 6C). In every instance, the inhibition of proliferation was significantly higher (P value determined by the paired t test, <0.0001) in cells treated with CDT-V for ≥ 4 h than in those treated for ≤ 2 h (Fig. 6B and C).

CDT-V causes DNA damage in human endothelial cells. The ability of CDT-V to cause DNA damage in endothelial cells was investigated by detecting DNA fragmentation by DAPI staining and the expression of phosphorylated histone protein H2AX (γ-H2AX), an indicator of double-stranded DNA breaks (26), by immunoblot analysis. As evidenced by DAPI staining, 11 and 14% of EA.hy 926 cells and HBMEC, respectively, treated with 8 CD_{50} of CDT-V/ml for 5 days had fragmented nuclei; examples are shown in Fig. 7A. No cells with fragmented nuclei were visible in any of the cell cultures after 5 days of treatment with the vector preparation (Fig. 7A). Protein immunoblot analysis (Fig. 7B) demonstrated the expression of γ-H2AX in EA.hy 926 cells and HBMEC treated with CDT-V for 21 h but not in control cells. γ-H2AX was not detected in CDT-V-treated or control cells after 3 h of exposure (data not shown).

DISCUSSION

We demonstrate for the first time that CDT-V from STEC O157:H$^-$ strain 493/89 has the potential to act on a variety of endothelial cells in vitro. This ability was previously shown only for HdCDT (54) and not for other members of the CDT family, although the latter CDTs have the capacity to interact with many other cell types, including epithelial cells, keratinocytes, and fibroblasts (1, 10, 12, 13, 26, 34, 44, 45, 51, 58). Moreover, our finding that CDT-V affects, in addition to primary cells, endothelial cell lines, including HUVEC-derived EA.hy 926 cells and HBMEC, extends a previous report (54) that HdCDT affects primary endothelial cells (HUVEC and microvascular endothelial cells from human dermal tissue [HMVEC-d]). Specifically, CDT-V induced, in a dose-dependent manner, irreversible G_2/M arrest, which led to cell distension, inhibition of proliferation, and ultimately lethality in HUVEC and in each of the cell lines. However, the responses to CDT-V differed among the cell cultures investigated. This phenomenon has not been reported for primary endothelial cells (54). Although both HUVEC and EA.hy 926 cells displayed slowly developing G_2/M arrest, they substantially differed in the proportions of cells arrested in G_2/M. In contrast
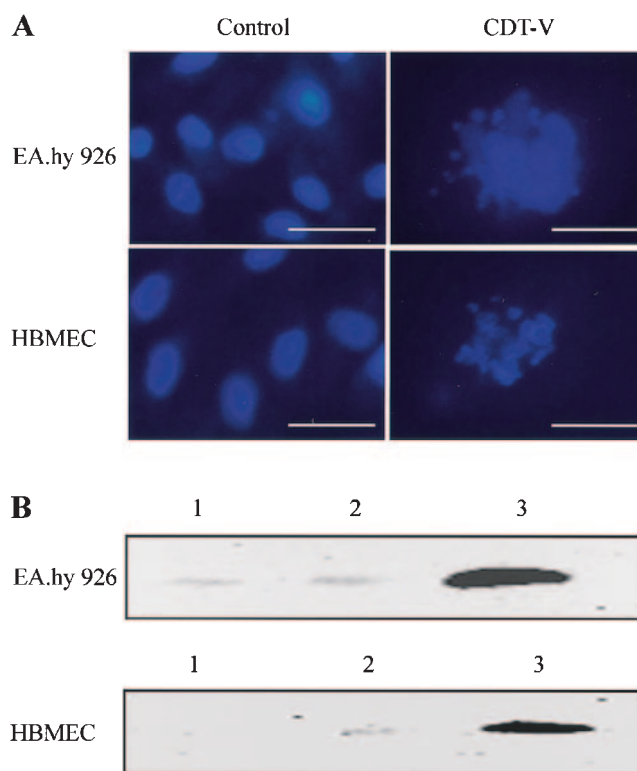

FIG. 7. CDT-V induces nuclear fragmentation and the formation of γ-H2AX in endothelial cells. (A) DAPI staining of EA.hy 926 cells and HBMEC treated for 5 days with the vector preparation or with 8 CD_{50} of CDT-V/ml. A Zeiss fluorescence microscope with a $\times 40$ objective lens was used; magnification, $\times 400$; bars, 50 μm. (B) Immunoblot analysis with a γ-H2AX-specific monoclonal antibody of EA.hy 926 cells and HBMEC treated for 21 h with 8 CD_{50} of CDT-V/ml (lanes 3) or the vector preparation (lanes 2) or left untreated (lanes 1).

to a nearly complete G_2/M block in EA.hy 926 cells 5 days posttreatment (Fig. 1A), only 57% of HUVEC were arrested in G_2/M at this time. The observation in HUVEC of the same pattern in the response to nocodazole, which synchronizes cells in mitosis (10, 13), suggests that this incomplete G_2 arrest probably reflects a finite life span of these cells, which divide relatively slowly (54), rather than an additional block in G_1; the latter scenario was previously reported for human foreskin and embryonic lung fibroblasts (13). The incomplete G_2/M block in HUVEC and HMVEC-d was also observed after treatment with HdCDT (54) and might be a consequence of a relatively low sensitivity of heterogeneous primary cell populations to CDT (54). Accordingly, the CD_{50} of CDT-V in our study is approximately 10-fold higher for HUVEC than for each of the cell lines.

In contrast to the findings for HUVEC and EA.hy 926 cells, the G_2/M arrest induced by CDT-V in HBMEC peaked 24 h

numbers of cells arrested in G_2/M after 24 h (HBMEC) or 120 h (EA.hy 926 cells) are shown, and the exposure times which resulted in G_2/M arrest in significant proportions ($\geq 50\%$) of EA.hy 926 cells ($\times$) and HBMEC ($\bullet$) also are shown. Cell proliferation was measured by the WST-1 assay (EA.hy 926 cells and HBMEC) (B) or trypan blue exclusion (HUVEC) (C) 96 h after the addition of the toxin. Cells at 0 min were not exposed to CDT-V and were cultured in medium only. Differences in mean optical densities at 450 nm (OD_{450}) (EA.hy 926 cells and HBMEC) and cell numbers (HUVEC) were compared with the paired t test, and the P values are shown. All data are means and standard deviations from three independent experiments.

after toxin exposure but rapidly declined during the next 4 days, apparently as a consequence of progressive cell death. The fact that the polycaspase inhibitor zVAD-fmk largely inhibited the formation of an HBMEC population with hypodiploid nuclei, which increased concomitantly with the decrease in the G_2/M block (Fig. 1A), suggests that the death of HBMEC was mostly due to caspase-mediated apoptosis. In addition, caspase-independent apoptosis (14) or necrosis might have contributed to the death of these cells. Interestingly, the pattern of response of HBMEC to CDT-V differed from that of human T cells to CDT from *A. actinomycetemcomitans*, which is characterized by slowly developing G_2 arrest followed by apoptotic cell death (52), and from that reported for B lymphocytes (13), which respond to HdCDT by the rapid development of apoptosis with only slight G_2 arrest. Notably, apoptotic cell death was not observed in HUVEC and EA.hy 926 cells, as evidenced by the absence of populations with hypodiploid nuclei in these cultures (Fig. 1A). The absence of an apoptotic cell population in HUVEC cultures in our study, which is in contrast to findings reported by Svensson et al. (54), might reflect either qualitative differences between HdCDT and CDT-V or different toxin doses used by those authors (100 cytopathic units/ml) and in our study (8 CD_{50}/ml).

Despite the observed differences in their cell cycle responses to CDT-V, the initial mechanisms underlying these responses appear to be similar in EA.hy 926 cells and HBMEC. This suggestion is based on the fact that the accumulation of phosphorylated cdc2 kinase, a mediator of G_2 arrest, preceded the development of the G_2/M block in both of these cell lines. Moreover, the mechanism which underlies the CDT-mediated G_2/M block in these cell lines is similar to that described previously for primary endothelial cells, including HUVEC and HVMEC-d (54). Thus, our present data do not allow us to determine a basis for the observed cell type-specific effects of CDT-V on the cell cycle of the endothelial cells investigated. Cortes-Bratti et al. (13) recently reported that human cells of other types, such as epithelial cells, keratinocytes, fibroblasts, and B cells, respond to HdCDT in a cell type-specific manner (arrest exclusively in G_2, arrest in both G_1 and G_2, or apoptosis). These responses were associated with different DNA damage checkpoint pathways and with different kinetics of expression of phosphorylated p53 protein (mediator of G_1 arrest) and phosphorylated cdc2 kinase during 24 h of intoxication (13). Because we determined the expression of phosphorylated cdc2 in CDT-V-treated endothelial cells only at early (after 4 and 8 h of exposure) and not at later time points, it is possible that similar differences in the kinetics of the cdc2-dependent checkpoint response might be one of the factors contributing to the cell type-specific responses of endothelial cells to CDT-V. However, the basis for this phenomenon is likely to be more complex and needs to be investigated in further studies.

To our knowledge, the irreversible effect of CDT on endothelial cells, as observed in this study for CDT-V on HUVEC, EA.hy 926 cells, and HBMEC, was not reported previously. In our study, this effect was demonstrated by the finding that as little as 2 to 15 min of exposure to the toxin resulted in significant G_2/M arrest and inhibition of proliferation in the endothelial cells investigated (Fig. 6). This finding of a rapid and irreversible interaction between CDT-V and endothelial cells extends earlier observations on similar interactions between CDT from *Campylobacter jejuni* (58) and *E. coli* CDT-II (1) and epithelial cells. Closer insight into an as-yet-unknown mechanism(s) underlying the irreversible interaction of CDT with host cells might result in a potentially therapeutically useful approach for preventing this CDT effect.

CDTs from several pathogens have been shown to degrade chromatin and to cause double-stranded DNA breaks with subsequent repair responses in various epithelial cells and fibroblasts (18, 22, 23, 34). Therefore, we investigated the ability of CDT-V to cause DNA fragmentation and to induce the expression of γ-H2AX, which plays a crucial role in the recruitment of DNA repair complexes to sites of double-stranded DNA breaks (26), in endothelial cells. The findings of nuclear fragmentation as well as γ-H2AX expression in HBMEC and EA.hy 926 cells exposed to CDT-V suggest that this toxin has the potential to induce DNA damage and DNA repair responses in these cells. To our knowledge, a similar effect of CDT was not reported for endothelial cells. The detection of γ-H2AX in EA.hy 926 cells and HBMEC at 21 h but not at 3 h postintoxication suggests that DNA lesions induced by CDT-V might be delayed but persistent. Accordingly, a persistent DNA lesion, as indicated by the expression of γ-H2AX 22 h posttreatment, was induced by CDT from *C. jejuni* in human fibroblasts (26). Because no apoptosis was detected in EA.hy 926 cells up to day 5 posttreatment, DNA fragmentation and γ-H2AX expression likely resulted from a direct effect of CDT-V. In contrast, because apoptosis was a prominent feature in HBMEC cultures after 5 days of CDT-V exposure, when DNA fragmentation was determined, our data do not allow us to differentiate between a direct DNA-damaging effect of CDT-V and DNA cleavage secondary to CDT-V-induced apoptosis.

Although Stxs are presumably the major virulence factors accounting for endothelial cell damage during STEC infection (5), several lines of evidence support a potential contribution of CDT-V to the pathogenesis of STEC-mediated diseases, in particular those caused by STEC lacking the intimin-encoding *eae* gene. These include (i) a significant association between CDT-V production by STEC isolates and clinical symptoms versus asymptomatic carriage in subjects infected with *eae*-negative STEC strains (4); (ii) the ability of *eae*-negative STEC of serotypes O91:H21 and O113:H21, which frequently produce CDT-V (4), to cause severe disease, including HUS (4, 27, 42), a feature which is otherwise rare among *eae*-negative STEC strains (6, 21); and (iii) the ability of recombinant CDT-V to irreversibly damage human endothelial cells in vitro, as demonstrated in this study. In any event, it is almost certain that non-Stx virulence factors contribute to host injury during STEC infection. On the basis of our data, CDT-V should be studied further in order to determine its role in augmenting or supporting the pathogenicity of STEC, particularly *eae*-negative strains.

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Editor: A. D. O'Brien