The *Haemophilus ducreyi* Cytolethal Distending Toxin Induces Cell Cycle Arrest and Apoptosis via the DNA Damage Checkpoint Pathways*.

The cytolethal distending toxins (CDTs) induce cell cycle arrest by a mechanism still not well characterized. We demonstrate that the effect of the *Haemophilus ducreyi* CDT (HdCDT) is cell type-specific: B cell lines underwent apoptosis, epithelial cells and keratinocytes arrested exclusively in G2, whereas normal fibroblasts arrested both in G1 and G2. We studied normal keratinocytes and fibroblasts, which are relevant for understanding the pathogenicity of *H. ducreyi*. The response to HdCDT resembles the checkpoint response activated by ionizing radiation. Both responses were characterized by an early induction of the p53 gene and the cyclin-dependent kinase inhibitor p21 in fibroblasts, and activation of the chk2 kinase in epithelial cells. In the Ataxia Telangiectasia-mutated gene (ATM)-deficient lymphoid cell lines, intoxication was significantly delayed compared with ATM wild type cells, and was associated with a slower kinetic of p53 stabilization, suggesting that the early response to HdCDT is ATM-dependent. Activation of ATM-dependent pathways was further confirmed by the ability of caffeine to partially override the HdCDT-mediated cell cycle arrest. Our data shed new light on the mechanism of action of this novel family of bacterial toxins, limiting the target candidates to DNA or molecules directly involved in activation of checkpoint responses.

The cytolethal distending toxins (CDTs) are newly discovered bacterial protein toxins with the unique ability to induce cell cycle arrest, thereby inhibiting cell proliferation. CDTs are produced by a number of bacterial pathogens, including *Escherichia coli*, *Haemophilus ducreyi*, *Campylobacter sp.*, *Actinobacillus actinomycetemcomitans*, *Shigella dysenteriae*, and *Helicobacter hepaticus*. Three linked genes, cdtA, cdtB, and cdtC, encode three polypeptides, which are responsible for the toxic activity. Coexpression of all three components is required to confer toxicity (reviewed in Ref. 1), but the functions of the individual gene products are still not known. It was reported that purified cdtB from *A. actinomycetemcomitans* could induce cell cycle arrest in human T lymphocytes and HeLa cells (2, 3). However, cdtC is absolutely required for the toxin activity in *H. ducreyi*, because culture supernatant fluid from a bacterial strain carrying a mutated cdtC did not display any cytotoxicity (4).

*H. ducreyi* is a Gram-negative cocobacillus, which causes chancroid, a sexually transmitted disease, characterized by genital tissue necrosis and retardation of healing. The pathogenesis of the disease remains poorly understood. The *H. ducreyi* CDT (HdCDT) may represent an important virulence factor, because it is produced by the majority of *H. ducreyi* strains from clinical samples and most patient sera contain HdCDT-neutralizing antibodies, whereas control sera do not (5, 6). Furthermore, the production of HdCDT is crucial for cell destruction after adhesion of *H. ducreyi* to cultured cells, whereas toxin-negative strains adhere but leave the cells intact (7).

Cells exposed to CDTs have been shown to arrest in the G2 phase of the cell cycle, and this is due to accumulation of the tyrosine-phosphorylated form of the cyclin-dependent kinase (cdk) 2 (reviewed in Ref. 1). A similar response is induced in HeLa cells after exposure to ionizing radiation (IR) (8). In proliferating cells, genotoxic stress activates checkpoint responses, which prevent progression through the cell cycle until the DNA damage has been repaired, thus avoiding genetic instability. Checkpoint arrest occurs at different stages of the cell cycle: the G1/S transition (G1 checkpoint), the S phase progression, and the G2/M boundary (G2/M checkpoint) (9, 10). The closely related protein kinases ATM and ATR are key molecules in sensing DNA damage. ATM responds mainly to DNA double stranded-breaks and is inactivated by mutations in ataxia-telangiectasia patients, who are extremely sensitive to IR and other genotoxic agents. ATM can activate all the different checkpoints in response to double stranded-breaks (reviewed in Ref. 11).

G2 arrest induced by DNA damage requires the tumor suppressor protein p53, which is stabilized via phosphorylation on serine 20 in an ATM-dependent manner by the chk2 protein kinase (12–14). Phosphorylated p53 dissociates from the Mdm2 protein that otherwise will target p53 for degradation via the ubiquitin-proteasome pathway (15, 16). The G2/M checkpoint involves the maintenance of cdc2 in an inactive hyperphosphorylated state. In the unperturbed cell cycle, cdc2 is kept inactive by phosphorylation on threonine 14 and tyrosine 15 by the
wee1 and myt1 kinases. In late G2, the cdc25C phosphatase dephosphorylates these residues, and thereby activates the cdc2/cyclin B1 complex (reviewed in Ref. 17). Recent data demonstrate that the protein kinases chk1 and chk2 are activated via phosphorylation in vivo in response to DNA damage, and both kinases are able to phosphorylate and inactivate cdc25C in vitro (18, 19).

In this study, we demonstrate that HdCDT induces cell growth arrest in a broad panel of human cell types, including normal keratinocytes and fibroblasts, which are the possible toxin targets in vivo. Intoxication of human cells with HdCDT did not exclusively induce G2 arrest, because human foreskin and embryonic lung (HL) fibroblasts were arrested also in G1, and B cells underwent apoptosis. HdCDT induced responses similar to IR in epithelial HEp-2 cells, fibroblasts, and lymphoblastoid cell lines (LCLs). Furthermore, ATM-deficient LCLs were much more resistant to the intoxication than ATM wild type cells. These data show that HdCDT induces cell cycle arrest by activating checkpoint responses similar to those induced by IR, and that rapid intoxication requires a functional ATM protein kinase.

EXPERIMENTAL PROCEDURES

Cell Lines

Human foreskin fibroblasts were obtained from the American Type Culture Collection (HTB-1, ATCC, Manassas, VA), and human embryonic lung fibroblasts (HL) were purchased from the Department of Microbiology, Sahlgrenska Hospital, Gothenburg, Sweden. Both lines were used between passages 15 and 25.

The Burkitt’s lymphoma (BL) cell line BL41 was established from an Epstein-Barr virus-negative tumor biopsy (20). The JAC-B1 and SN-B1 LCLs were obtained by in vitro infection of B lymphocytes from healthy donors with the B95.8 strain of Epstein-Barr virus as previously described (21).

The AT06LA, AT00LA, AT01LA, and AT13LA LCLs established from ataxia-telangiectasia patients were kindly provided by Dr. R. A. Gatti, Department of Pathology, UCLA, Los Angeles, CA.

Normal keratinocytes, Ad3 and Ad5, were established from human neonatal foreskin and used between passages 3 and 7.

The other cell lines were as follows: the human larynx carcinoma HEp-2 (ATCC no. CCL-23), the human cervix carcinoma HeLa (ATCC no. CCL-2), and the human keratinocyte line HaCaT (kindly provided by Dr. N. E. Fusenig, Heidelberg, Germany). All cell lines, except keratinocytes, were cultivated in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal calf serum, 5 mM t-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete medium) (Life Technologies, Inc., Gaithersburg, MD). Keratinocytes were cultivated in keratinocyte-SFM medium supplemented with bovine pituitary extract (25 μg/ml) and recombinant epidermal growth factor (0.2 ng/ml) (Life Technologies, Inc.).

Toxin and Treatments

HdCDT was purified by immunoaffinity chromatography using the neutralizing monoclonal antibody M4D4, as previously described (22); Western blot analysis with specific antibodies showed that the purified product contained both the B and C components, whereas A was not detectable. The protein concentration of the stock solution was 2 mg/ml.

Toxin Treatment—Cells were incubated for the indicated time periods in the presence of HdCDT (2 μg/ml) in complete medium. Irradiation—Cells were irradiated (20 Gy), washed once in phosphate-buffered saline (PBS), and incubated for the indicated time periods in complete medium.

Hydroxyurea and Nocodazole Treatment—Cells were incubated in complete medium supplemented with 2.5 mM hydroxyurea (Calbiochem, La Jolla, CA) or 100 nM nocodazole (Sigma Chemical Co., St. Louis, MO) for 24 h.

Caffeine Treatment—Cells were cooled on ice for 15 min before addition of the toxin (2 μg/ml for 15 min on ice). After the binding step, cells were washed three times with PBS to remove unbound toxin and were further incubated for 24 h in complete medium with or without 4 mM caffeine (Sigma).

Cell Cycle Analysis

Cells were trypsinized, centrifuged, and washed once with PBS. The cell pellet was resuspended and fixed on ice for 15 min with 1 ml of cold ethanol (70%). The cells were subsequently centrifuged and resuspended in 1 ml of propidium iodide (PI) solution (0.05 mg/ml PI; 0.02 mg/ml RNase; 0.3% Nonidet P-40; 1 mg/ml sodium citrate) for 1 h at 4 °C. Flow cytometry analysis was performed on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA). Data from 104 cells were collected and analyzed using the CellQuest software (Becton & Dickinson).

Western Blot

Cells were lysed in 300 μl of SDS electrophoresis sample buffer (23), and samples were boiled for 10 min. The amount of protein in the cell lysates was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Twenty micrograms of total cell lysate was fractionated by SDS-polyacrylamide gel (23), transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with the respective antibodies. To detect the electrophoretic mobility shift of the chk2 kinase, 10–20% linear gradient SDS-polyacrylamide gels were used (Bio-Rad). Blots were developed with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Sweden), using the appropriate horseradish peroxidase-labeled secondary antibody, according to the instructions of the manufacturer. The following antibodies were used: anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY); anti-cdc2, anti-Cip1/p21, and anti-Rip1/p27 (Transduction Laboratories, Lexington, KY); anti-p53 (Ab-6, Calbiochem, La Jolla, CA); anti-phospho-enzyme 15 p53 rabbit serum (kind gift of Dr. Yoichi Taya, University of Japan); anti-chk2 (H-300), anti-cdc25C (C-20), and anti-wee1 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-chk1 rabbit serum (kindly provided by Dr. Stephen J. Elledge, Verna and Marrs McLean Department of Biochemistry and Molecular Biology and Department of Molecular and Human Genetics, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX).

RESULTS

The effect of HdCDT is cell type-specific—We tested the activity of HdCDT in a broad panel of human cell lines, including two epithelial cell lines, three keratinocyte lines, two normal fibroblast lines, and three lines of B cell origin. As shown in Table I and Fig. 1A, HdCDT induced cell death or cell cycle arrest in all the lines, judged by propidium iodide (PI) staining and flow cytometry analysis 24 h after toxin treatment. Most of the cell lines tested were arrested exclusively in G2, as detected by accumulation of cells with a 4n DNA content. The HdCDT-induced G2 arrest was not associated with the absence of a functional p53 protein, because cells expressing either wild type or mutated p53 were arrested in G2 (Table I). A different pattern of cell cycle arrest was observed in two normal fibroblast lines derived from foreskin and lung. Intoxicated fibroblasts partially accumulated in G2 24 h after toxin treatment, however, the block in G2 was not complete, and, even 48 h post-intoxication, cells were arrested also in S and G1 (Fig. 1A). B cell lines showed a 50% decrease of the G1 peak, which was associated with a 2- to 4-fold increase of the apoptotic sub-G1 population 24 h after intoxication. A slight increase in the G2 population could be detected only in some experiments (Figs. 1A and 4A; Table II).

It has been previously shown that G2 arrest induced by HdCDT was associated with an accumulation of the hyperphosphorylated form of the cyclin-dependent kinase cdc2 (24). Consequently, we assessed whether the different pattern of cell cycle arrest observed in the fibroblasts was associated with a lack of accumulation of hyperphosphorylated cdc2. As shown in Table I and Fig. 1B, accumulation of tyrosine-phosphorylated cdc2 was detected by Western blot, using anti-cdc2 and anti-phosphophorynospecific monoclonal antibodies, only in HEp-2, HeLa, and HaCaT cells 24 h post-intoxication. At this stage, cdc2 was strongly down-regulated in normal keratinocytes and fibroblasts, and no hyperphosphorylation was observed. However, we detected accumulation of hyperphosphorylated cdc2 at
early time points after intoxication in a representative line of each cell type (Fig. 2, B and C, and data not shown).

**HdCDT-induced Cell Cycle Arrest Resembles the Response Induced by Ionizing Radiation**—To test whether HdCDT induced a response similar to other agents known to cause cell cycle arrest, we compared the effect of HdCDT and IR in HEp-2 cells and HL fibroblasts, which showed different patterns of cell cycle arrest. The cell cycle distribution of irradiated cells was similar to that observed upon intoxication in both cell types (Fig. 2A). The block in G2/S observed in fibroblasts is not an artifact due to the slow proliferation rate of these cells, because we did not detect a complete accumulation in G2 even 48 h post-intoxication (Fig. 1A) and treatment of cells with nocodazole, known to synchronize cells in mitosis, induced accumulation of the majority of fibroblasts in G2/M already 24 h after treatment (Fig. 2A). It is noteworthy that both cell types treated with 2.5 mM hydroxyurea, which blocks DNA synthesis by reducing the intracellular levels of deoxynucleoside triphosphates, were arrested in early S phase. Thus, HdCDT and IR induced a similar response, which differed from that caused by hydroxyurea. This issue was further analyzed in time kinetic experiments, where we tested the expression of known regulators of the cell cycle in toxin- and radiation-treated cells.

In HL fibroblasts, accumulation of phosphorylated cdc2 was detected only at early time points (4 and 6 h) after intoxication, and an identical response was observed in irradiated cells (Fig. 2B). Increased expression of the tumor suppressor protein p53 was observed in both intoxicated and irradiated fibroblasts 4 h post-treatment. This was associated with a strong up-regulation of the cyclin-dependent kinase inhibitor p21, and to a lesser extent p27, and their levels increased over time. IR-induced activation of p53 is mediated by the ATM kinase, which phosphorylates p53 on serine 15. Using a specific anti-phosphoserine-15 p53 rabbit serum, we showed that this event occurs with a similar kinetic both in intoxicated and irradiated cells 4 h after treatment (Fig. 2C).

A different pattern was detected in HEp-2 cells, consistent with the fact that the two cell types behaved differently when exposed to HdCDT or IR (Fig. 2A). Phosphorylation of cdc2 was observed 4 h after treatment and was maintained up to 24 h, confirming previous data (Table I, Fig. 2C, and Ref. 24). Increased expression of p53 and p21 was detected much later than the accumulation of hyperphosphorylated cdc2. The strongest accumulation of p53 and p21 was observed in HdCDT-treated cells 24 h post-intoxication (Fig. 2C). IR treatment is known to induce phosphorylation of the chk2 kinase in an ATM-dependent manner, detected as a shift of the chk2-specific band in Western blot analysis (19). The chk2 protein was shifted both in intoxicated and irradiated HEp-2 cells 4 h after treatment (Fig. 2C). We did not detect any relevant changes in the levels of expression or in electrophoretic mobility of other regulatory molecules of the cell cycle, such as chk1, chk2C, and wee1 (data not shown).

**Caffeine Partially Overrides the Cell Cycle Arrest Induced by HdCDT**—Caffeine is known to override the G2/M arrest induced by DNA damage in mammalian cells by indirectly releasing the inhibitory cdc2 phosphorylation (25–27). Because we have seen a strong correlation between the responses evoked by HdCDT and IR, we assessed whether caffeine could prevent the HdCDT-induced G2 arrest in HEp-2 cells. Cells were treated with HdCDT for 15 min on ice. After this binding step, cells were washed three times, to remove unbound toxin, and incubated in complete medium supplemented with 4 mM caffeine. The level of cdc2 phosphorylation and cell cycle progression was monitored 24 h post-intoxication by Western blot and flow cytometry analysis, respectively. The caffeine treatment completely prevented accumulation of hyperphosphorylated cdc2 (Fig. 3A), and this was associated with partial release of the HdCDT-induced G2 arrest, because 35% of the cells were found in G1 upon caffeine treatment compared with 8% in the untreated cells (Fig. 3B).

**HdCDT-induced Cell Cycle Arrest Is Delayed in ATM-deficient Cells**—The ATM protein is a key activator of cell cycle checkpoints in response to DNA-damaging agents (reviewed in Ref. 11). Furthermore, recent data have shown that caffeine exerts its activity by directly inhibiting the ATM kinase (26, 27). Therefore, we tested the response to HdCDT in two ATM wild type LCLs and four ATM-deficient cell lines. Cells were exposed to HdCDT for 24 h and subsequently stained with PI, followed by flow cytometry analysis. As shown in Fig. 4 and in Table II, a 2- to 4-fold increase of the sub-G1 population was detected in the ATM wild type LCLs. This was associated with a strong decrease of the G1 peak (50% reduction), although no major changes were observed in the S-G2-M population. The ATM-deficient cells were much more resistant to the toxin effect, because we could observe only minor changes in both the sub-G1 and the G1 populations 24 h after treatment. It has been previously shown that p53 levels increase with a much faster kinetic in ATM wild type LCLs than in ATM-deficient cells (12). A similar pattern was observed when the control SN-B1 and the ATM-deficient AT13LA lines were exposed to HdCDT (Fig. 5, A and B). A 20-fold increased p53 expression was observed in SN-B1 LCL compared with the 2-fold increase in

### Table I

| Cell line | Cell type | G2 arrest | Apoptosis | cdc2 phosph. | cdc2 decrease | p53 status |
|-----------|-----------|-----------|-----------|--------------|---------------|------------|
| HEp-2     | Epithelial| +         | –         | +            | –             | wt         |
| HeLa      | Epithelial| +         | –         | +            | –             | wt         |
| BL41      | B lymphocyte | –/+      | +         | ND           | ND            | Mutated    |
| JAC-B1    | B lymphocyte | –/+      | +         | –             | –             | wt         |
| SN-B1     | B lymphocyte | +        | –         | –             | –             | wt         |
| HaCdT     | Keratinocyte | +        | –         | –             | –             | wt         |
| Ad5f      | Keratinocyte | –        | –         | –             | –             | wt         |
| HFSf      | Fibroblast | –/+      | –         | –             | –             | wt         |
| HLf       | Fibroblast | –/+      | –         | –             | –             | wt         |

* Detection of the sub-G1 population by PI staining 24 h after treatment.

**D** Accumulation of the tyrosine-phosphorylated form of cdc2, detected by Western blot 24 h after treatment.

**C** Down-regulation of the cdc2 protein detected by Western blot 24 h after treatment.

**E** Burkitt’s lymphoma-derived cell line.

**B** Epstein-Barr virus-transformed B lymphocytes derived from healthy donors.

**H** Normal foreskin keratinocytes.

**F** Normal HFS and HL.

**ND** Not done.
the AT13LA line 5 h after treatment, and this trend was maintained throughout our experiment.

**DISCUSSION**

The aim of this study was to clarify the mechanism(s) by which the cytolethal distending toxin produced by *H. ducreyi* induces cell cycle arrest and cell death in human cells. The strategy was to investigate the early events occurring in intoxicated cells and compare the effects caused by HdCDT with those induced by ionizing radiation. This is the first report where CDT-intoxicated cells have been analyzed at short time points after toxin exposure, whereas all previous studies have been performed mainly at 24 or 72 h post-intoxication (2, 24, 28, 29). Furthermore, we have tested a broad panel of human cell types, including normal keratinocytes and fibroblasts, which are conceivable toxin targets *in vivo*.

Our data demonstrate that HdCDT activates a cell cycle

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**TABLE II**

**Effect of HdCDT on ATM wild type and ATM-deficient LCLs**

Data are presented as the ratio between the HdCDT-treated cells and the untreated control.

| Exp. | SN-B1<sup>a</sup> | JAC-B2<sup>a</sup> | AT06LA<sup>b</sup> | AT06LA<sup>b</sup> | AT01LA<sup>b</sup> | AT13LA<sup>b</sup> |
|------|------------------|------------------|------------------|------------------|------------------|------------------|
| Sub-G<sub>1</sub> | 2 | 2 | 2 | 2 | 2 | 2 |
| G<sub>1</sub> | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| S-G<sub>2</sub>-M | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |

<sup>a</sup> ATM wild type.

<sup>b</sup> ATM-deficient.

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**FIG. 1. Effect of HdCDT on human cell lines.** A, effect of HdCDT on human lung embryonic fibroblasts (HL), HEp-2 cells, and B cell lines. Cells were treated with HdCDT, and cell cycle distribution was assessed by DNA staining with PI and flow cytometry analysis at the indicated time points. The G<sub>1</sub> peak was arbitrarily set on the mean fluorescence intensity value of 50. B, lack of hyperphosphorylated cdc2 in HL fibroblasts. Cells were treated with HdCDT for 24 h, and Western blot analysis was performed from total cell lysate as described under “Experimental Procedures.” Polyvinylidene difluoride membranes were probed with anti cdc2 and anti-phosphotyrosine-specific monoclonal antibodies. Cdc2<sup>*</sup> indicates the hyperphosphorylated form. One out of three experiments is shown.
checkpoint, which resembles that induced by IR, and highlight a new mechanism of action for bacterial protein toxins. This conclusion is based on three lines of evidence: i) different cell types responded differently to HdCDT (Table I; Figs. 1A and 2A), suggesting that HdCDT does not specifically target the G2 phase of the cell cycle, as previously suggested (1); ii) similar profiles of cell cycle arrest and time kinetic responses were induced by HdCDT and IR (Fig. 2, A–C); iii) ATM-deficient cells were much more resistant than ATM wild type cells to HdCDT (Fig. 4).

Activation of Checkpoint Responses—We have demonstrated that the response to HdCDT was cell-specific and not exclusively associated with the induction of cell cycle arrest in G2. This observation suggested that HdCDT might target an upstream event that could subsequently activate several down-stream pathways leading to cell cycle arrest or cell death. This has prompted us to investigate whether the response of intoxicated cells was similar to any other agent causing activation of checkpoint responses. In normal fibroblasts, both HdCDT and IR induced a rapid stabilization of the tumor suppressor gene p53, which was phosphorylated on serine 15, and an increased expression of the cyclin-dependent kinase inhibitor p21 (Fig. 3C), suggesting that the checkpoint response in normal lung fibroblasts is p53-dependent. Functional p53 and p21 proteins are also required to sustain G2 arrest caused by DNA damage (30). However, this is unlikely to occur in HEp-2 cells, because p53 and p21 up-regulation was detected much later than the accumulation of hyperphosphorylated cdc2 (Fig. 2C). Furthermore, cell lines that carried a nonfunctional p53, such as HeLa and HaCaT, still arrested in G2 and did not up-regulate p21, suggesting the involvement of a p53-independent pathway (Table I and data not shown). In intoxicated HEp-2 cells, we could...
demonstrate phosphorylation of the chk2 kinase, which plays a major role in the induction of G2 checkpoint responses upon exposure to IR (19). Activation of chk2 can lead to cdc25C inactivation and accumulation of hyperphosphorylated cdc2. Indeed, inactivation of cdc25C was previously demonstrated in HeLa cells treated with the *Escherichia coli* CDT (29), and overexpression of cdc25B and cdc25C could prevent cell intoxication (31).

Increased expression of phosphoserine-15 p53 in human fibroblasts and phosphorylation of the chk2 kinase in HEp-2 cells strongly suggested that the response to HdCDT was mediated by the ATM kinase. Involvement of ATM for rapid cell intoxication was demonstrated by the delay in intoxication (Fig. 4) and in p53 stabilization (Fig. 5) observed in ATM-deficient cells compared with wild type LCLs. This notion was further supported by the ability of caffeine to alleviate the G2 block imposed by HdCDT in HEp-2 and HeLa cells (Fig. 3, A and B, and Ref. 29). Recent studies have demonstrated that caffeine abolishes the G2 block in mammalian cells upon DNA damage by inhibiting the activation of the ATM kinase and consequently blocking the downstream activation of the chk2 protein (26, 27). The delayed intoxication observed in ATM-deficient cells suggests that this protein is required for an early toxin response, but checkpoint responses can still be activated in the absence of functional ATM, probably by homologous molecules.

These data indicate that the response to HdCDT is similar to that induced by IR and therefore suggest that the toxin acts either at the level of a central regulator(s) of the cell cycle control machinery or at the level of DNA, activating a checkpoint response in the target cells. Sert and colleagues (29) did not detect DNA strand breaks in cells intoxicated with a CDT derived from *E. coli*, using the single cell gel electrophoresis assay ("comet assay"). However, two recent reports have demonstrated that cdtB from *E. coli* and *Campylobacter jejuni* has structural and functional homology to the mammalian type I DNase. DNase activity was associated with the *E. coli* cdtB, as detected by *in vitro* digestion of the coiled pGEM-Tzf+ plasmid (32), whereas marked chromatin disruption was observed in HeLa cells following transfection or microinjection of low amounts of cdtB from *C. jejuni* (33). In both cases, point mutations in conserved residues required for catalysis or magnesium binding abolished the DNase activity and the ability of the toxin to induce cell cycle arrest. It is therefore likely that CDTs induce subtle DNA damage leading to the activation of cell cycle checkpoint responses, in agreement with the data presented in our study.

**H. ducreyi Pathogenicity—**CDTs derived from *A. actinomycetemcomitans* and *H. ducreyi* inhibit T cell proliferation and induce apoptotic death in the Jurkat T cell line, respectively (2, 34). We have extended these findings demonstrating that also B cell lines are sensitive to HdCDT. Interestingly, among all the cell lines we have tested, the two LCLs and the two Burkitt's lymphoma-derived cell lines BL41 and Rael were very prone to apoptosis upon HdCDT treatment judged by accumulation of a sub-G1 population of PI stained cells (Figs. 1A and 4A, and data not shown).

These data, together with the observation that normal keratinocytes and fibroblasts are sensitive to HdCDT, suggest that the toxin contributes to the pathogenesis and immunodeficiency of chancroid by: 1) damaging the epidermal and dermal layers of the genital mucosa and causing retardation in heal-

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**Fig. 4.** HdCDT intoxication is delayed in ATM-deficient cells. Two ATM wild type LCLs (A) and four ATM-deficient LCLs (B) were incubated with HdCDT for 24 h, as described under “Experimental Procedures.” DNA was stained with PI, and cell cycle distribution was analyzed by flow cytometry as described in Fig. 1A.

**Fig. 5.** Different kinetic of p53 stabilization. A, the ATM wild type SN-B1 and the ATM-deficient AT13LA LCLs were treated with HdCDT as described under “Experimental Procedures.” At the indicated time points total cell lysates were prepared and expression of p53 was assessed by Western blot analysis. B, densitometric analysis was performed using the ImageQuant software (Molecular Dynamics). Data are presented as the ratio between the optical density of the specific band in HdCDT-treated cells and the optical density of the corresponding band in the untreated control.
ing; and 2) decreasing and delaying the host immune response by induction of apoptosis in B and T lymphocytes, allowing bacterial replication and enhancing tissue damage.

**CDTs as Biological Tool**—Because CDTs interfere with the cell cycle control machinery, they can offer a possible new tool to intervene in all cases of cell cycle deregulation, tumors being one of the major issues. It is noteworthy that the use of cytotoxic prodrugs has been considered in the field of cancer gene therapy. Attention has been focused on the herpes simplex virus-thymidine kinase (HSV-tk) gene, which induces single-strand breaks in DNA synthesized in the presence of the nucleoside analogue ganciclovir (35, 36). The murine melanoma cell line B16F10 transfected with the HSV-tk gene undergoes irreversible G2/M arrest and cytoskeleton reorganization when treated with ganciclovir (37). HdcDT produces very similar effects in toxin-sensitive tumor cell lines (24) and could potentially be used as anti-cancer therapy, if a selective delivery to tumor cells is provided.

It is also well established that bacterial toxins have been extremely valuable in dissecting several important aspects of cell biology, such as the role of small GTPases in the control of cytoskeleton rearrangement (reviewed in Ref. 38). The cross-talk between molecules regulating the cytoskeleton and those involved in control of cell cycle progression/arrest is complex and still poorly understood (reviewed in Refs. 39, 40), and elucidation of the HdcDT mode of action can be useful to further study this issue.

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