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M. Thelestam · T. Frisan

Cytolethal distending toxins

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Abstract The cytolethal distending toxins (CDTs) constitute the most recently discovered family of bacterial protein toxins. CDTs are unique among bacterial toxins as they have the ability to induce DNA double strand breaks (DSBs) in both proliferating and nonproliferating cells, thereby causing irreversible cell cycle arrest or death of the target cells. CDTs are encoded by three linked genes (*cdtA, cdtB* and *cdtC*) which have been identified among a variety of Gram-negative pathogenic bacteria. All three of these gene products are required to constitute the fully active holotoxin, and this is in agreement with the recently determined crystal structure of CDT. The CdtB component has functional homology with mammalian deoxyribonuclease I (DNase I). Mutation of the conserved sites necessary for this catalytic activity prevents the induction of DSBs as well as all subsequent intoxication responses of target cells. CDT is endocytosed via clathrin-coated pits and requires an intact Golgi complex to exert the cytotoxic activity. Several issues remain to be elucidated regarding CDT biology, such as the detailed function(s) of the CdtA and CdtC subunits, the identity of the cell surface receptor(s) for CDT, the final steps in the cellular internalization pathway, and a molecular understanding of how CDT interacts with DNA. Moreover, the role of CDTs in the pathogenesis of diseases still remains unclear.

A brief history of CDT

The first cytolethal distending toxin (CDT) was reported in 1987 as a novel type of toxin activity produced by pathogenic strains of *Escherichia coli*. The major hallmark of the observed cytotoxic effect was a remarkable cell distension, evident 3–5 days after addition of bacterial culture supernatants to cells growing in vitro, and resulting after a few more days in cell death (Johnson and Lior 1987a). The same authors later identified a similar activity in *Shigella* (Johnson and Lior 1987b) and *Campylobacter* spp (Johnson and Lior 1988a), and they designated this putative toxin as ‘cytolethal distending toxin’.

M. Thelestam (✉) · T. Frisan
Microbiology and Tumorbiology Center, Karolinska Institutet, Stockholm, Sweden
e-mail: Monica.Thelestam@mtc.ki.se · Tel.: +46-8-52487162 · Fax: +46-8-342651

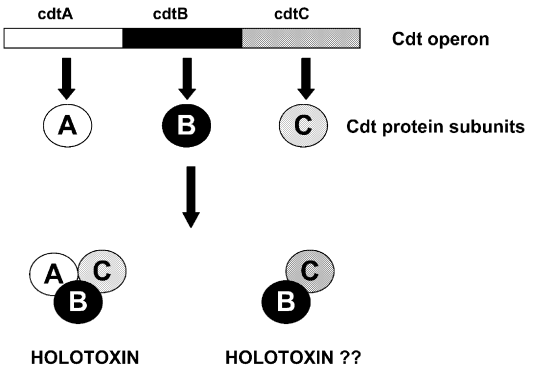

Fig. 1 Schematic representation of the three linked CDT genes and the respective protein subunits. Although the tripartite holotoxin (CdtA + CdtB + CdTC) has been found to be the most potent inducer of cellular intoxication, still some doubts exist whether this is the only possible subunit combination

CDTs from various *E. coli* strains were first cloned in 1994 by two American groups (Scott and Kaper 1994; Pickett et al. 1994) and in 1997 by French researchers (Peres et al. 1997). The CDTs turned out to be encoded by three linked genes, which were designated *cdtA*, *cdtB* and *cdtC*. Related CDT genes were identified in *Shigella spp* (Okuda et al. 1995), *Campylobacter jejuni* (Pickett et al. 1996), *Haemophilus ducreyi* (Cope et al. 1997), *Actinobacillus actinomycetemcomitans* (Mayer et al. 1999; Sugai et al. 1998), and several *Helicobacter* spp (Chien et al. 2000; Kostia et al. 2003; Taylor et al. 2003; Young et al. 2000a). The CDTs had no immediate resemblance to any other known proteins and thus, a new family of toxins rapidly emerged. So far no CDT has been identified from any Gram-positive bacterium. Since CDT variants are produced by many different bacterial species we previously proposed a CDT-nomenclature to avoid a confusing flora of different names in the future literature on CDTs (Cortes-Bratti et al. 2001a). A particular CDT is specified exactly by indicating the initials of the producing bacterium before CDT and, if necessary, the strain number or other common designation after CDT (e.g., HdCDT: *H. ducreyi* CDT).

The *cdtA, cdtB* and *cdtC* genes were found to encode proteins (CdtA, CdtB, CdtC) (Fig. 1) with predicted molecular masses of 23, 29 and 21 kDa and isoelectric points of 5.7, 8.3, 6.3, respectively, for the HdCDT. The purified HdCDT subunits show a normal migration pattern when subjected to SDS-PAGE. The CDT subunits were purified from some of the producing bacteria during the late 1990s and more recently in recombinant forms. The HdCDT was shown to enter sensitive target cells by receptor-mediated endocytosis (Cortes-Bratti et al. 2000) – a process likely to occur also with the other CDTs. In 2000 a deoxyribonuclease I (DNase I)-like enzymatic activity was identified in the CdtB component of CDTs from *E. coli* and *C. jejuni*, respectively (Elwell and Dreyfus 2000; Lara-Tejero and Galan 2000). Mutation of the catalytic residues abolished all cytotoxic effects. The proposed DNA damaging effect was consistent with our parallel observations on the cellular responses to HdCDT. They were identical to the cell cycle check point responses evoked by ionizing radiation (IR), a well known DNA damaging agent (Cortes-Bratti et al. 2001b). Finally, the HdCDT was reported in 2003 to induce DNA double strand breaks (DSBs) upon natural intoxication of mammalian cells (Frisan et al. 2003), thus giving direct proof that CDT attacks DNA. The crystal structure of the HdCDT holotoxin has been recently solved, and it reveals that indeed this toxin consists of an enzyme belonging to the DNase I family (CdtB), bound to two ricin-like lectin domains (CdtA and CdtC). HdCdtB shows the characteristic fold of the DNase I family: a central 12-stranded

β-sandwich packed between outer α-helices and loops on each side of the sandwich (Nesic et al. 2004).

This review deals mainly with the action of CDTs on mammalian cells. The possible roles of these toxins in pathogenesis, and their potential uses in the biomedical field will also be briefly discussed.

Actions of CDTs on mammalian cells

Morphological effect of CDT

The most conspicuous morphological effect of CDT on cells growing as an adherent monolayer is the cell distension leading within 72 h to a three- to fivefold increase of the cell size. Along with this slowly developing distension, the actin cytoskeleton is strongly promoted noted mainly as the appearance of prominent actin stress fibers (Fig. 2). This has been reported for *E. coli* CDT (EcCDT-II) in Chinese hamster ovary (CHO) cells (Aragon et al. 1997), for HdCDT in HEp-2/HeLa cells, in Don hamster lung fibroblasts and human foreskin fibroblasts (Cortes-Bratti et al. 1999; Frisan et al. 2003), and recently also in HeLa cells treated with CDT from *Helicobacter cinaedi* (Taylor et al. 2003). After several days of toxin exposure cells round up, show membrane blebbing in some cases, and then deteriorate completely. In contrast to fibroblasts and epithelial cells, T and B lymphocytes and dendritic cells do not distend but rather become rapidly apoptotic and fragmented after exposure to CDT (Cortes-Bratti et al. 2001b; Li et al. 2002; Shenker et al. 2004).

CDTs act on several cell types

As already evident from the morphological effect, CDTs affect various cell lines with differing outcomes depending on the cell type. Sensitive cells include HEp-2, HeLa, CHO, Vero and Don fibroblasts (reviewed in (Cortes-Bratti et al. 2001a; Pickett and Whitehouse 1999) as well as human embryonic intestinal epithelial cells (INT407) (Hickey et al. 2000), a human colon carcinoma cell line (Caco-2) and a human keratinocyte cell line (HaCat) (Cortes-Bratti et al. 1999). Human $CD4^+$ and $CD8^+$ T cells are highly sensitive to the *A. actinomycetemcomitans* CDT (AaCDT) (Shenker et al. 1999), which also inhibits proliferation of the murine B-cell hybridoma cell line HS-72 (Sato et al. 2002) as well as human periodontal ligament cells and gingival fibroblasts (Belibasakis et al. 2002). HdCDT affects normal human endothelial cells (HUVEC) (Svensson et al. 2002), foreskin keratinocytes and fibroblasts, embryonic lung fibroblasts, human B cells and dendritic cells (Figs. 3, 5) (Cortes-Bratti et al. 2001b; Li et al. 2002). Primary human fetal fibroblasts (IMR-90) (Hassane et al. 2003), Cos-7 cells (McSweeney and Dreyfus 2004), Jurkat and MOLT-4 cells (Ohara et al. 2004) are also sensitive to CDT intoxication.

In conclusion, most tested cells are sensitive to CDT. The only cells so far reported to resist CDTs are the Y-1 adrenal cells and 3T3 fibroblasts of mouse origin (Cope et al. 1997; Cortes-Bratti et al. 1999; Johnson and Lior 1988b). The reason for their resistance is not known but these cells might lack a cell surface receptor needed for binding of the toxin.

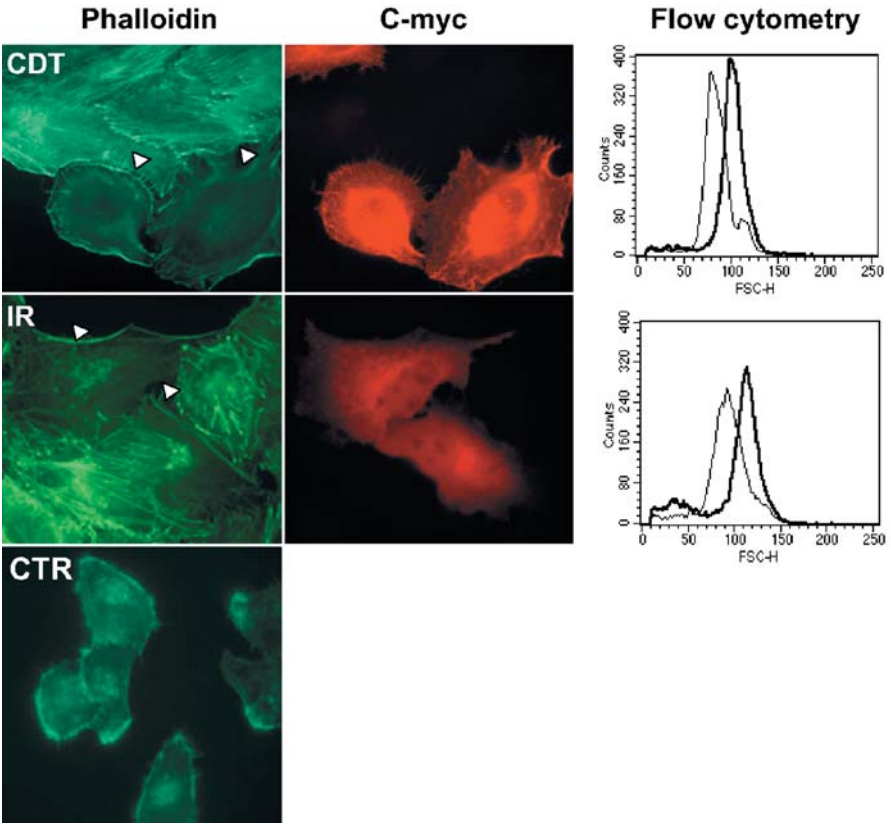

Fig. 2 CDT intoxication induces cell distension and promotion of actin stress fibers. HeLa cells were transfected with dominant negative RhoAN19, and 24 h after transfection cells were either left untreated (CTR) or exposed to HdCDT (2μg/ml) or irradiated (20 Gy) and incubated for additional 24 h in complete medium. Transfected cells were visualized by immunofluorescence using anti-Myc 9E10 antibody and F-actin was stained with FITC-phalloidin. *Arrows* indicate RhoAN19 transfected cells. The cell size of untreated (*thin line*) or treated (*thick line*) cells was analyzed by flow cytometry (FSC-H: forward scatter)

CdtB is a DNase

The CdtBs from several bacteria were shown to be able to cleave DNA in vitro. Indeed, all CdtBs on a close analysis turned out to have structural and functional homology to mammalian DNase I. The first paper describing position-specific homology between the CdtB subunit from EcCDT-II and mammalian DNase I was published by Elwell and Dreyfus (Elwell and Dreyfus 2000). The homology pattern was found at specific residues involved in enzyme catalysis (Glu86, His154, Asp229, His261), DNA binding (Arg123, Asn194) and metal ion binding (Glu62, Asp192, Asp260). EcCdtB-II also contained a pentapeptide sequence (aa 259–263: Ser-Asp-His-Tyr-Pro) found in all DNase I enzymes. Shortly after, similar conserved residues were described for the *C. jejuni* CdtB (CjCdtB) and in fact identified in all known CdtBs (Lara-Tejero and Galan 2000).

The position-specific homology is associated with functional activity, since crude Ec-CDT-II preparations have DNase activity as detected by in vitro digestion of the coiled

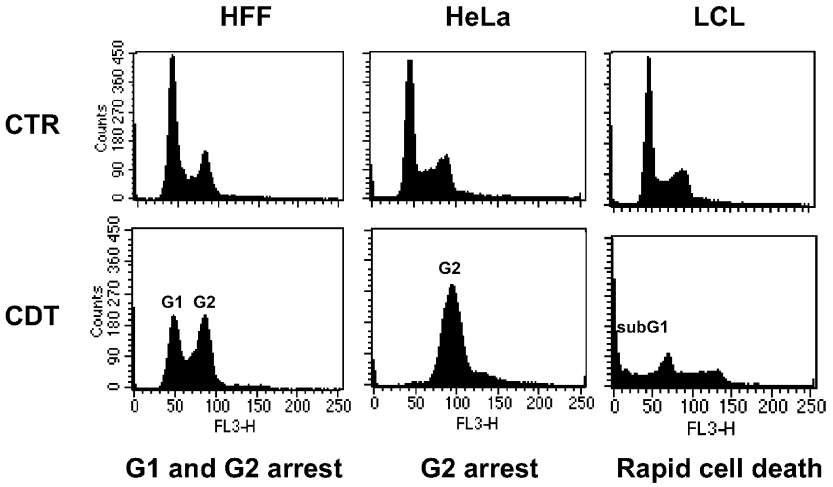

Fig. 3 Cell type-dependent effect of CDT. Human foreskin fibroblasts (*HFF*), HeLa cells and an EBV transformed lymphoblastoid cell line (*LCL*) show different patterns of cycle arrest or cell death upon CDT treatment. Cells were treated with HdCDT (2μg/ml) and cell cycle distribution was assessed by DNA staining with propidium iodide and flow cytometry analysis 24 h after treatment. The G_1 peak was arbitrarily set on the mean fluorescence intensity value of 50

pGEM-7zf plasmid (Elwell and Dreyfus 2000), and transfection of HeLa cells with CjCdtB was shown to induce a slowly appearing nuclear fragmentation and a marked chromatin disruption (Lara-Tejero and Galan 2000). Microinjected CjCdtB was also able to produce changes in the chromatin and enlargement of the nucleus. The DNase activity, as well as the cytotoxicity, was abolished by point mutations of conserved residues required for catalysis or for magnesium binding. Thus, the catalytic DNase activity appeared to be crucial for the cytotoxic activity of CDTs (Elwell and Dreyfus 2000; Lara-Tejero and Galan 2000).

CDT induces DNA double-strand breaks

We recently provided direct evidence that intoxication of HeLa cells with the HdCDT holotoxin induces DNA DSBs similarly to IR (Fig. 4) (Frisan et al. 2003). This first demonstration of CDT-induced DSBs in naturally intoxicated mammalian cells was made with a sensitive pulsed field gel electrophoresis (PFGE) method. It contradicts an earlier report showing that EcCDT did not induce DNA strand breaks, as measured with the alkaline single cell gel electrophoresis (Comet) assay (Sert et al. 1999). However, our findings agree with those of Hassane and co-workers who previously observed that yeast cells transfected with CjCdtB exhibited DSBs, also detectable by PFGE (Hassane et al. 2001). Furthermore, this effect on DNA is fully consistent with the DNase activity of CdtB and the widely reported observations that CDT-treated cells exhibit cell cycle arrest and/or undergo apoptosis (see below).

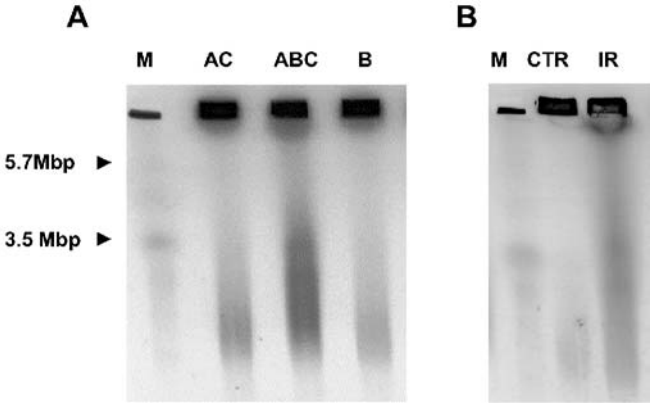

Fig. 4A, B CDT intoxication induces DNA DSBs. Twenty-five thousand HeLa cells per well were grown in 12-well plates in complete medium containing 4000 Bq [methyl-^{14}C]thymidine for 48 h. The cells were then washed three times in phosphate buffered saline and chased for 2 h in complete medium. **A** Cells were treated with (*AC*) CdtAC bacterial supernatant; (*ABC*) CdtAC bacterial supernatant incubated with purified *H. ducreyi* CdtB (20μg/ml); (*B*) purified *H. ducreyi* CdtB alone (20μg/ml) for 7 h, and processed for pulsed-field gel electrophoresis analysis. **B** HeLa cells were left untreated or irradiated (20 Gy, *IR*) and immediately processed for analysis by pulsed-field gel electrophoresis. *M*, molecular weight marker

Cellular responses to DNA damaging agents

It is well known that cells exposed to agents causing DNA damage activate checkpoint responses that arrest the cell cycle until the DNA damage has been repaired. These checkpoint responses can block cells in the G_1, S or G_2 phases of the cell cycle (Elledge 1996; Hartwell and Weinert 1989). The protein kinase 'Ataxia telangiectasia mutated' (ATM) and its homolog 'ATM and Rad3 related' (ATR) play a central role in sensing DNA damage. ATM is activated in response to DNA DSBs induced by IR, and can in turn trigger all the different checkpoints. ATR activation is mainly induced by other DNA damaging agents, such as UV irradiation (reviewed in Rotman and Shiloh 1999). Arrest in G_1 is mediated by the tumor suppressor gene p53, which is stabilized in an ATM dependent manner via phosphorylation on serine 20 by the chk2 protein kinase (Chehab et al. 2000; Siciliano et al. 1997). The G_2 arrest depends on inactivation of the cdc2 complex. Activation of cdc2 is achieved via dephosphorylation at Thr14 and Tyr15 by the Cdc25C phosphatase (Jackman and Pines 1997). The ATM-dependent protein kinases chk1 and chk2, activated in vivo in response to DNA damage, are both able to inactivate Cdc25C in vitro (Matsuoka et al. 1998; Sanchez et al. 1997), leading to accumulation of the inactive phosphorylated cdc2 and arrest of cells in the G_2 phase of the cell cycle.

Cellular checkpoint responses to CDTs

Cell cycle arrest

Already before the toxin was known to damage DNA, cells exposed to various CDTs had been reported to accumulate the phosphorylated (inactive) form of cdc2, implying G_2/M arrest (Comayras et al. 1997; Cortes-Bratti et al. 1999; Peres et al. 1997; Whitehouse et al.

1998). The inactive cdc2/cyclin B complex resulting after intoxication with EcCDT-III could be reactivated in vitro with recombinant Cdc25C (Sert et al. 1999). Furthermore, overexpression of Cdc25B or Cdc25C could override the G_2 arrest induced by this toxin, causing progression to a mitosis which, however, was abnormal (Escalas et al. 2000). Both results suggested that CDTs do not target specifically cdc2 but rather some upstream component, leading to inactivation of Cdc25C and consequent lack of cdc2 dephosphorylation as a secondary effect.

The cellular checkpoint responses induced by HdCDT were then shown to closely resemble those induced by IR. It became clear that the CDT-induced cell cycle arrest is not limited to the G_2/M phase, but that the checkpoint responses depend on the cell type (Cortes-Bratti et al. 2001b). A functional ATM protein was needed for rapid intoxication as demonstrated by the delayed responses in ATM deficient lymphoblastoid cell lines. In human fibroblasts, both IR and HdCDT treatment induced early activation of the p53 gene and the cyclin-dependent kinase inhibitor p21, which correlated with arrest in G_1 (Fig. 3). In epithelial cells both treatments induced chk2 kinase activation, accumulation of phosphorylated cdc2, and G_2 arrest. The checkpoint responses were detected already 4 h post-intoxication and therefore occurred much earlier than the morphological changes of intoxicated cells. Lymphoblastoid cell lines did not arrest in either G_1 or G_2 at the concentrations of HdCDT used, but rapidly underwent apoptosis (Cortes-Bratti et al. 2001b) (Fig. 3).

CDT-induced apoptosis

This aspect has been studied in some detail only in human T cells and T cell lines. Treatment of activated human T cells with the AaCDT (purified B subunit or recombinant holotoxin) was found to induce DNA fragmentation in 72–96 h, and FACS analysis showed reduction in cell size and increased nuclear condensation (Shenker et al. 2001). Mitochondrial changes were evident as a decrease in transmembrane potential and an elevation of reactive oxygen species, and the caspases 8, 9 and 3 were activated after the G_2 arrest. Overexpression of Bcl-2 decreased the CDT-induced apoptosis, but did not inhibit the CDT-induced G_2 arrest (Shenker et al. 2001). A more recent report on AaCDT-induced death of human peripheral T lymphocytes and the Jurkat and MOLT-4 cell lines, however, suggested that AaCDT has the ability to induce human T-cell apoptosis through activation of caspase-2 and caspase-7 (Ohara et al. 2004). In conclusion, the exact molecular responses on the way to apoptotic cell death have not been fully elucidated and can be expected to depend on the particular type of target cell.

CDTs activate sensors of DNA damage

We demonstrated that HdCDT induces ATM-dependent phosphorylation of the nuclear sensor of DNA damage, histone H2AX, as early as 1 h after intoxication. Moreover, the DNA repair complex Mre11 in the nuclei of HeLa cells was relocalized with kinetics similar to those observed upon IR (Li et al. 2002). Also the CjCDT was reported to induce both G_1 and G_2 arrest in primary human fetal fibroblasts (IMR-90 cells), and Rad50 foci were formed in the nuclei of the CjCDT-treated fibroblasts (Hassane et al. 2003). Thus, DNA damage-associated molecules are activated as an early response to the DNA DSBs induced by CDTs.

CDTs act on nonproliferating cells

It is noteworthy that CDTs also attack nonproliferating cells. The focus formation assays mentioned above enabled the discovery that human dendritic cells (DCs) (Li et al. 2002) as well as serum starved primary human fetal fibroblasts (Hassane et al. 2003) are sensitive to CDT. This contradicts previous notions that cells need to pass through the S phase in order to become intoxicated (Alby et al. 2001; Lara-Tejero and Galan 2000; Sert et al. 1999). The cell cycle analysis of HdCDT-treated human DCs shows that all control cells are in G_0 whereas the toxin-treated cells after 24 h either remain in G_0 or become apoptotic The short-term effect after 4 h toxin treatment of DCs is seen as a phosphorylation, i.e., activation, of H2AX (Fig. 5) (Li et al. 2002).

CDT-induced stress fiber promotion depends on ATM-mediated activation of RhoA

The reason that actin stress fibers are strongly promoted in certain CDT-treated cells was not understood in the early experiments (Aragon et al. 1997) although the possible involvement of small GTPases controlling the actin cytoskeleton was hypothesized (Cortes-Bratti et al. 1999). Later on it became feasible to investigate this aspect of CDT intoxication with new tools that allow biochemical assay of the activated small GTPases Rho, Rac and CDC42 (Benard et al. 1999; Ren et al. 1999). Thus, we could show that the HdCDT-activated stress fiber promotion in fibroblasts and HeLa cells depends on activation of RhoA, but not Rac or CDC42 (Frisan et al. 2003). This was observed both directly as activation of RhoA upon intoxication, and as an inhibition of the stress fiber formation in CDT-treated cells transfected with a dominant negative mutant of RhoA (see Fig. 2).

A cell distension and stress fiber promotion was observed also in irradiated cells. Interestingly, the increased size of CDT-treated or irradiated cells was not connected to the stress fiber formation, since dominant negative RhoA inhibited the formation of stress fibers without affecting the distension of the treated cells (Fig. 2). The distension phenomenon instead appears to depend on activation of PI3-kinase and its downstream effector mTOR, whereas this enzyme had no clear effect on induction of stress fibers (Frisan et al. 2003). The activation of RhoA upon intoxication or irradiation was ATM-dependent, thereby connecting the stress fiber promotion to the damaging action on DNA. Toxin-treated cells expressing a dominant negative form of RhoA detached and consequently died faster than cells expressing a functional RhoA (Frisan et al. 2003). Thus, the activation of RhoA was associated with prolonged cell survival, and it seems to represent an attempt of intoxicated cells to maintain cell adherence in order to stay alive while the DNA is supposed to be repaired. This ATM-dependent activation of RhoA constitutes a previously unknown type of cell survival response to induction of DNA DSBs, which seems to occur regardless of the agent inducing the DSBs.

Fig. 6 summarizes the intracellular responses activated by CDT intoxication.

Cellular internalization and nuclear localization of CdtB is required for cytotoxicity

Since CDT acts on DNA it is conceivable that this toxin, like most other intracellularly acting toxins, has to be internalized in target cells before it can act. Indeed, the HdCDT has been reported to enter HEp-2/HeLa cells by endocytosis via clathrin-coated pits

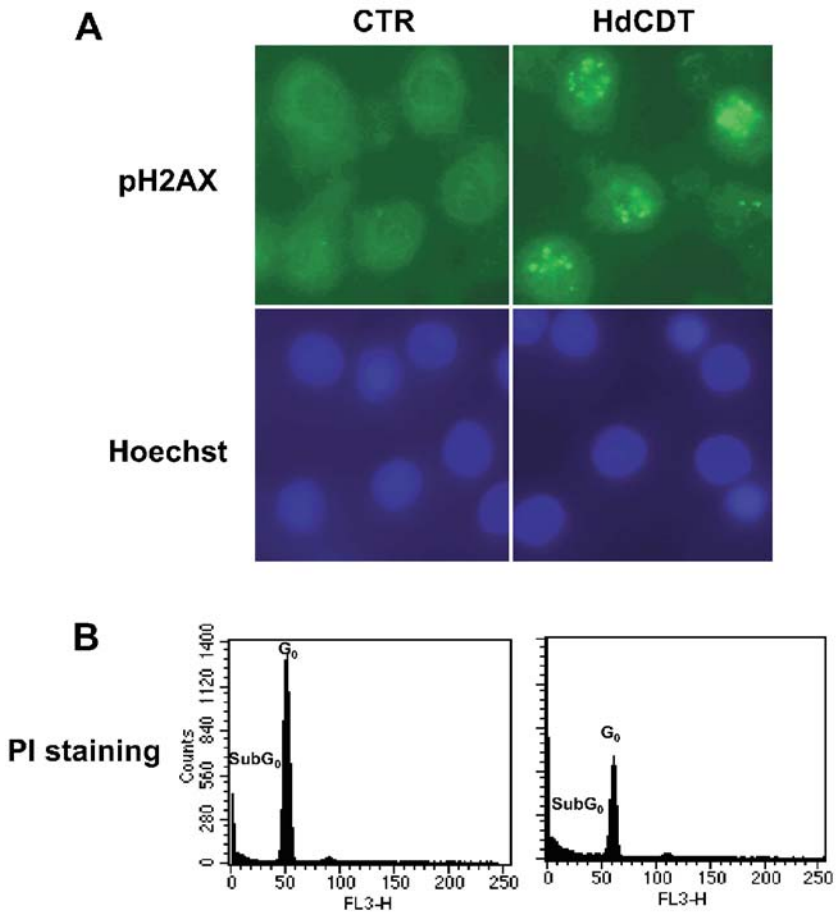

Fig. 5A, B CDT induces DNA damage and cell death in nonproliferating dendritic cells. **A** Five-day immature DCs were incubated with HdCDT (2μg/ml) for 5 h and stained with histone H2AX phospho-specific rabbit serum (*pH2AX*). Nuclei were counter-stained with Hoechst 33258. **B** Cell cycle distribution was assessed by DNA staining with propidium iodide (*PI*) and flow cytometry. The G_0 peak was arbitrarily set on the mean fluorescence intensity value of 50

(Cortes-Bratti et al. 2000). The cellular intoxication was completely inhibited at conditions that block the fusion of early endosomes with downstream compartments or after treatment of cells with agents that disrupt the Golgi complex. Apparently the toxin, after uptake via clathrin-coated pits, requires transport in vesicles at least to the Golgi complex before its activity can be expressed. From the Golgi it may be retrogradely transported to the endoplasmic reticulum (ER) and from there delivered either to the cytosol or directly to the nucleus (Cortes-Bratti et al. 2000).

CdtB contains no known conventional nuclear localization signal (NLS). However, microinjection experiments in HeLa cells showed that a 76-amino acid stretch (residues 48–124) in the AaCdtB constitutes an atypical NLS (Nishikubo et al. 2003). After microinjection, His-tagged CdtB-GFP entered the nucleus in 3–4 h. A lack of effect of leptomycin B on the speed of nuclear entry suggested that the relatively slow entry of the fusion protein

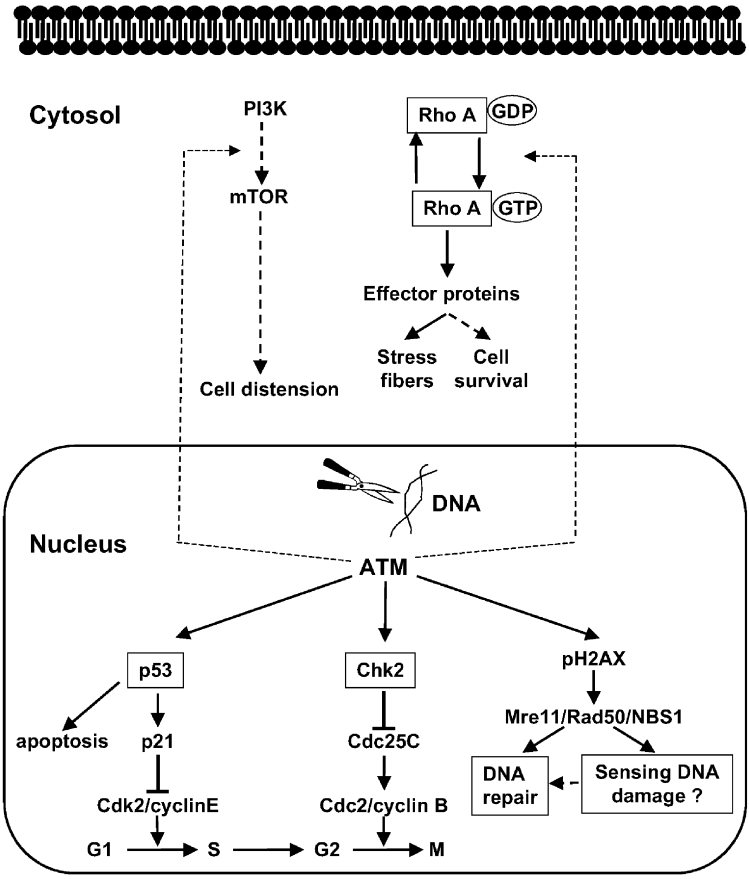

Fig. 6 Summary of the cellular responses activated by the CDT-induced DNA damage. *Dotted arrows* indicate pathways discovered using CDT as a tool for which intermediate effectors have not yet been completely elucidated

was not due to CRM1-dependent nuclear export of the protein. An in vitro transport assay demonstrated that the nuclear localization of CdtB was mediated via active transport requiring ATP and physiological temperature. Moreover, cells treated extracellularly with a holotoxin containing mutant CdtB, with an 11-amino acid truncation in the identified NLS, were unaffected. This observation suggested that the identified NLS may be functional for nuclear localization of the toxin also when mammalian cells are naturally intoxicated with the extracellularly added holotoxin (Nishikubo et al. 2003). Subsequently, two NLS sequences, designated NLS1 and NLS2, have been identified in the carboxy-terminal region of EcCdtB-II. Cell cycle arrest and nuclear localization were impaired in cells treated with CDT containing EcCdtB-II-ΔNLS mutants, while the in vitro DNase activity and the cell surface binding of the mutant holotoxins were not affected. Interestingly, fluorescence microscopy analysis showed a diffuse cytoplasmic distribution of EcCdtB-II-ΔNLS2 in cells intoxicated with the CDT holotoxin containing the EcCdtB-II-ΔNLS2 mu-

tant, while the EcCdtB-II-ΔNLS1 subunit localized preferentially in a perinuclear region of the intoxicated cells (McSweeney and Dreyfus 2004).

The studies cited above demonstrate beyond any doubt that at least the active CdtB subunit has to be endocytosed and undergo intracellular transport to the nucleus before it can damage cells. However, it is still not known whether in natural intoxication the nuclear entry of CdtB takes place from the cytosol or directly from the Golgi/ER. Nothing has yet been reported regarding the cellular localizations and fates of the other two toxin subunits.

Functions of the different CDT subunits

Early genetic studies demonstrated that all three genes need to be expressed in the producing bacterium for the generation of active (cytotoxic) CDT (Cope et al. 1997; Pickett et al. 1994, 1996; Sugai et al. 1998; Young et al. 2000a). However, some confusion was created by the difficulty to obtain highly purified subunits from bacteria, which possess the entire *cdt* operon and thus produce all three subunits. Although studies today are usually performed with recombinant subunits of CDT some discrepancies still remain as we shall see below. However, clarifying the roles of the three different subunits in cellular intoxication and other responses to CDT constitutes one of the most important frontiers in current CDT research.

CdtB. As stated already CdtB is definitely the most important active subunit showing DNase activity in vitro and in vivo. CdtB of various origins is cytotoxic after microinjection (Lara-Tejero and Galan 2000) or electroporation/facilitated entry into cells (Elwell et al. 2001; Mao and DiRienzo 2002). In contrast, CdtB added extracellularly alone is not able to bind to most target cells and consequently it is not cytotoxic by itself. T cells may be an exception as initial studies on the AaCDT suggested that its CdtB subunit alone was sufficient to cause G_2 arrest in phytohemagglutinin-activated human T cells (Shenker et al. 1999, 2000). However, the same authors demonstrated more recently that although their AaCdtB alone could bind to the Jurkat cell surface and was sufficient to induce G_2 arrest in human lymphocytes, the presence of both the CdtA and CdtC subunits was required to achieve maximum cell cycle arrest (Shenker et al. 2004). Indeed, a holotoxin comprised of CdtABC was >50,000-fold more toxic to Jurkat cells than toxins composed of either CdtAB or CdtBC. Today there is general consensus that all three subunits must be added together for extracellularly induced maximal intoxication (Lara-Tejero and Galan 2001; Lee et al. 2003).

CdtA. Shenker and coworkers (Shenker et al. 2004) report that immunoprecipitation of the CDT holotoxin from *A. actinomycetemcomitans* extracts, with a monoclonal antibody against CdtC, co-precipitated CdtA and CdtB. Interestingly, only a truncated form of CdtA (18 kDa) was immunoprecipitated with this holotoxin, suggesting that CdtA may undergo processing during the assembly of the holotoxin. The expression of two immunoreactive CdtA proteins corresponding to 25 kDa and 18 kDa has been observed also for the HdCDT (Frisk et al. 2001). CdtA seems to be able to bind to target cells, but alone it lacks cytotoxic activity (Lee et al. 2003; Mao and DiRienzo 2002). Also, the combination of only CdtA and CdtB was without cytotoxic effect on HeLa cells (Lara-Tejero and

Galan 2001; Lee et al. 2003). It is unclear whether the CdtA in these cases was in the truncated form or not.

CdtC. Besides the apparent maturation of CdtA in the presence of the two other subunits, also CdtC has been reported to be somehow processed in the holotoxin environment. The isoelectric point (pI) of the HdCdtC component was 1.5 pH units higher in recombinant strains expressing all three components than in recombinant strains expressing the CdtC protein alone (Frisk et al. 2001). A similar change of pI occurred after mixing the three individual recombinant components in vitro. Thus, it was suggested that HdCdtA/B may exert some kind of processing activity on HdCdtC, rendering it active (Deng et al. 2001; Frisk et al. 2001). Recently, however, the purified recombinant His-tagged AaCdtC alone, delivered to the cytosol with a lipid protein carrier, was found able to induce cell distension and eventually the death of CHO cells (Mao and DiRienzo 2002). The specific mechanism of this putative CdtC cytotoxicity has not been elucidated.

Cytotoxicity of combined CdtB and CdtC

Mao and DiRienzo (Mao and DiRienzo 2002) also found an additive cytotoxic effect exerted by the combination of AaCdtB and AaCdtC when delivered into the cytosol together. We previously observed cytotoxicity with an extracellularly added highly purified preparation of HdCDT in which we were not able to detect CdtA by Western blot analysis (Li et al. 2002). This is consistent with the observation that extracts from *H. ducreyi* producing CDT in which the *cdtA* gene was mutated still had some cytotoxic activity (Lewis et al. 2001). Also other workers found that the combination of purified HdCdtB with HdCdtC was slightly cytotoxic (Wising et al. 2002). Likewise, a mixture of recombinant AaCdtB and AaCdtC could induce G_2 arrest in HEp-2 cells, as measured by FACS analysis after a 72 h toxin exposure (Akifusa et al. 2001). Similarly, the extracellularly added combined recombinant subunits CjCdtB and CjCdtC were able to induce G2 arrest in HeLa cells (Lee et al. 2003). On a microgram basis, however, this combination was only about 25% as effective as the tripartite CjCDT holotoxin. It would be of interest to determine the toxin amount produced by the bacteria in vivo, in order to understand whether the CdtB/C combination could have any physiological relevance.

Cell surface binding of CDT subunits

Different CDTs have a documented ability to intoxicate cells even after exposure times as short as 2 to 15 min (Aragon et al. 1997; Cortes-Bratti et al. 1999) and HdCDT could be absorbed out from the medium by repeatedly passing the same toxin solution over fresh cultures of HEp-2 cells (Frisk et al. 2001). Such functional studies indirectly suggested that CDTs bind to cells rapidly and irreversibly, although no specificity of the binding could be demonstrated. It was earlier pointed out that a region in the CjCdtA, encompassing the amino acids 160 through 220, exhibits a lectin fold which is also present in the binding subunits of the plant toxins ricin and abrin (Lara-Tejero and Galan 2001). The recently determined crystal structure of HdCDT reinforced this observation and underscored the presence of a ricin-like lectin domain also in HdCdtC. However, no specific receptor for any CDT subunit has so far been identified.

When recombinant HdCdtA and HdCdtC were produced from two plasmids and expressed in the same *E. coli*, a noncovalent CdtA–CdtC complex was formed in the absence of CdtB (Deng and Hansen 2003). This CdtA–CdtC complex after association with HeLa cells (30 min, 4°C) was able to bind subsequently added CdtB, leading to cell killing within 72 h. Moreover, HeLa cells pretreated for 30 min at 37°C with the CdtA–CdtC complex became resistant to the cell killing induced by high concentrations of subsequently added HdCDT holotoxin (*H. ducreyi* culture supernatant). However, such treated cells were not resistant to the cell distension induced by lower amounts of the holotoxin (Deng and Hansen 2003), suggesting that cellular intoxication had indeed occurred. Thus, a more detailed analysis of the molecular markers of CDT intoxication should be performed.

Another recent piece of work considerably advanced our knowledge about binding of the CjCDT subunits to the HeLa cell surface (Lee et al. 2003). By competition experiments Lee and coworkers demonstrated that both the CdtA and CdtC subunits, but not CdtB, could bind with specificity to the HeLa cell surface. In unlabeled form these subunits were able to compete out binding of the corresponding biotinylated subunit. Interestingly, they could also compete with each other, suggesting that CdtA and CdtC were binding to the same receptor on the cell surface (Lee et al. 2003). Indeed, these two subunits of the CjCDT share about 40% sequence similarity with each other. McSweeney and Dreyfus recently noted the same binding competition between the CdtA and CdtC subunits of the EcCDT-II (McSweeney and Dreyfus 2004).

Lee and coworkers (Lee et al. 2003), in addition, produced in-frame deletion mutants of CdtA and CdtC from which 43 and 22 amino acids, respectively, were deleted. As the highest homologies within the CdtA and CdtC subunits from different bacteria are found in distinct regions, the deletions were made in one such region in CjCdtA (amino acids 126–168 deleted) and in the region of the highest homology within the CjCdtC subunit (amino acids 115–136 deleted). Both of these mutant subunits in biotinylated form were still able to bind HeLa cells as detected by an enzyme-linked immunosorbent assay on living cells. Moreover, each mutant subunit was able to compete out the binding of both the corresponding and the other wild-type subunit, as well as that of the holotoxin. The binding of the CDT holotoxin decreased nearly fivefold in the presence of the CdtA mutant subunit and almost threefold in the presence of the CdtC mutant subunit. These interesting findings then suggest that the deleted regions in each Cdt subunit do not play any significant role in cell surface recognition. On the other hand, the mutant subunits were unable to interact with CdtB and/or each other to reconstitute an active holotoxin, indicating that the deleted regions were instead critical for effective holotoxin formation (Lee et al. 2003).

In conclusion

The most reasonable interpretation of the combined current data on the CDT subunits is that CDT functions as an AB-toxin where the DNase CdtB constitutes the active subunit, and a CdtAC heterodimer constitutes the optimal binding subunit. This tripartite holotoxin is the subunit combination with maximal activity in all tested cell systems. However, the CdtC subunit alone can also function as a binding subunit, facilitating the cellular entry of CdtB, albeit with a much lower efficiency than the heterodimeric binding unit. That the AaCdtB alone can apparently act on Jurkat cells (Shenker et al. 2000, 2004) may have to

do with specific properties of T cells, which in contrast to epithelial cells or fibroblasts appear able to bind and take up CdtB.

Possible involvement of CDTs in bacterial infections

As CDTs are potent toxins produced by pathogenic bacteria they have to be regarded as potential virulence factors. CDTs are detected in an increasing number of clinical isolates but only few experiments with CDTs in animal model systems have been reported.

Occurrence of CDTs in clinical isolates from various geographic locations

Several studies on *E. coli* isolates have been performed, especially concerning the possible association of CDT with enteropathogenic (EPEC) serotypes. The general conclusion from these studies is that EcCDT is produced in some, but not all, EPEC serotypes (Ansaruzzaman et al. 2000; Ghilardi et al. 2001; Janka et al. 2003; Okeke et al. 2000; Pandey et al. 2003). For example, only 1 in 200 children in Brazil (0.5%) with acute diarrhea were infected with *cdt*-positive EPEC, while 1% of the nondiarrheic controls harbored *cdt*-positive isolates (Marques et al. 2003). CDT has also been identified in urosepsis *E. coli* isolates (Johnson and Stell 2000), in diarrhea isolates from dogs (Starcic et al. 2002), and from neonatal piglets (da Silva and da Silva Leite 2002), in extraintestinal human and animal urinary tract infection (UTI) and non-UTI strains (Toth et al. 2003), and in 83% of bovine isolates of necrotoxigenic *E. coli* (Mainil et al. 2003).

Pickett and coworkers recently investigated how the presence of *cdt* genes was related to a number of other virulence genes of *E. coli* (Pickett et al. 2004). Their study of a collection of 20 strains revealed that the CDT producers can be divided into three general groups with distinct differences in CDT type and in their complement of virulence-associated genes. Thus, *cdt* genes were found in a subset of uropathogenic *E. coli* strains, in additional extraintestinal isolates of uncertain clonal identity, and in certain strains with characteristics in common with Shiga-toxin producing *E. coli* and EPEC (Pickett et al. 2004). CDT and the *cdt* genes were also identified in 17 out of 340 non-O157 Shiga toxin-producing *E. coli* (STEC) of serotypes that were all *eae*-negative (Bielaszewska et al. 2004). Among these *eae*-negative STEC, *cdt* was proposed to be disease-associated as it was significantly more frequent in isolates from patients with hemolytic uremic syndrome (3 of 7) and in isolates from patients with diarrhea (14 of 138) than in isolates from asymptomatic carriers (0 of 65).

The production of CDT has been detected in few isolates of *Shigella dysenteriae*, *S. boydii* and *S. sonnei* (Okuda et al. 1995). In contrast, almost all investigated *C. jejuni* and *C. coli* strains have been reported to produce CDT or to possess the *cdt* genes. For instance, the *cdtB* gene was detected in all investigated isolates from chicken carcasses, i.e., the primary source of *C. jejuni* and *C. coli* in human infections (Eyigor et al. 1999a, 1999b). An in vitro assay showed that the *C. jejuni* isolates produced CDT at high toxin titers whereas *C. coli* produced little or no toxin. Likewise, 100/101 *C. jejuni* and 7 of 10 *C. coli* isolates from Danish broilers had the *cdt* genes. Again the *C. coli* strains produced lower amounts of the toxin (Bang et al. 2001). CDT production was also demonstrated from *C. fetus*, and PCR experiments suggested the presence of *cdtB* sequences in other species of *Campylobacter* (Pickett et al. 1996). A recent study on several virulence genes

including the *cdt* cluster in *C. jejuni* and *C. coli* isolates from Danish pigs and cattle demonstrated a high prevalence (83–95%) of *cdt* genes and CDT production (Bang et al. 2003).

In contrast to the situation in *Campylobacter* spp, the *cdt* genes and CDT activity is not present in all species of *Helicobacter*. The three genes have been identified in *H. hepaticus* (Young et al. 2000a), and the *cdtB* gene was detected in human clinical isolates of *H. pullorum* (Young et al. 2000b) and in canine isolates of *H. flexispira* (Kostia et al. 2003), but no CDT homology was found in *H. pylori* (Chien et al. 2000). Interestingly, CDT was recently reported as the first putative virulence factor present in *H. cinaedi*, which is the most commonly reported enterohepatic helicobacter in humans (Taylor et al. 2003). The *cdtB* gene detected by PCR, as well as CDT production detected by cytotoxicity, was present in all 11 investigated isolates, 10 of which were clinical isolates.

Out of 50 periodontitis strains of *A. actinomycetemcomitans*, 86% had the *cdt* genes and expressed cytotoxic activity (Ahmed et al. 2001). A majority (39/40) of clinical isolates from Brazil, Kenya, Japan and Sweden were also found to harbor the *cdt* genes and produce the AaCDT (Fabris et al. 2002). Although there was some variation in toxin production among the strains, no clear relationship between CDT activity and periodontal status could be found. In another study *A. actinomycetemcomitans* was detected in 106/146 subgingival plaque samples isolated from periodontitis patients, and among these only 13 sites were positive for the *cdt* genotype (Tan et al. 2002). However, 10 of these 13 positive sites were obtained from patients diagnosed with aggressive periodontitis, and the authors speculated that the cytotoxicity and immunosuppression by CDT may contribute to the pathogenesis of aggressive periodontitis (Tan et al. 2002). More recently CDT production and occurrence of the *cdtB* gene was studied in 73 strains of periodontopathogenic bacteria (Yamano et al. 2003) and CDT activity was found in 40/45 tested *A. actinomyctemcomitans* strains. The remaining 28 strains of other bacteria did not have CDT.

Finally, 89% of a group of isolates of *H. ducreyi* from different parts of the world showed CDT activity on HEp-2 cells (Purvén et al. 1995). A later study identified the *cdt* genes and CDT production in 83% of 29 isolates of *H. ducreyi* from chancroid (Ahmed et al. 2001). Likewise, 87% of 45 strains of *H. ducreyi* found in still more recent chancroid isolates from various parts of the world produced the toxin (Kulkarni et al. 2003).

In conclusion

A majority of strains of *C. jejuni, A. actinomyctemcomitans* and *H. ducreyi* appear to produce the CDT while the presence of the *cdt* genes in other bacteria is more variable. Table 1 summarizes the data reported in the previous paragraphs.

CDT-induced production of cytokines in vitro

CDT from *C. jejuni* has been shown to induce the release of the proinflammatory cytokine interleukin (IL)-8 from intestinal epithelial INT407 cells (Hickey et al. 2000). In contrast, a more recent study showed that while *C. jejuni* itself induced a number of proinflammatory cytokines, IL-8 among them, from the monocytic cell line THP-1, these responses did not depend on the presence of CDT (Jones et al. 2003). On the other hand, each of the purified recombinant subunits of AaCDT were found individually able to induce production

Table 1 Short summary of the CDT occurrence in clinical isolates

Toxin	Bacteria/disease	Species infected
E. coli CDTs	EPEC	Human
	Urosepsis *E. coli*	Human
	Diarrhea isolates	Dog
	Diarrhea isolates	Pig
	UTI strains	Human/animal
	Non-UTI strains	Human/animal
	Necrotoxigenic	Bovine
	STEC	Human
Shigella CDTs	Few isolates	
*Campylobacte*r CDTs	*C. jejuni*	Chicken/human
	C. coli	Chicken/human
	C. fetus	
Helicobacter CDTs	*H. pullorum*	Human
	H. flexispira	Human
	H. cinaedi	Human
	H. hepaticus	Human, mouse
AaCDT	Aggressive periodontitis	Human
HdCDT	Chancroid	Human

The references relative to the CDT distribution of clinical isolates are reported in the text

of the cytokines IL-1β, IL-6 and IL-8 but not tumor necrosis factor α, IL-12 or granulocyte-macrophage colony stimulating factor in human peripheral blood mononuclear cells (PBMC) (Akifusa et al. 2001). While CdtC was the most potent and CdtB had only minimal cytokine stimulating activity, the latter appeared to synergize with CdtA and CdtC to promote PBMC cytokine synthesis, and this synergy was most marked in inducing interferon (IFN)γ production (Akifusa et al. 2001).

Studies on CDTs in animal models

An *E. coli* strain expressing the *S. dysenteriae cdt* genes was able to induce watery diarrhea in the suckling mouse model, and the partially purified CDT had some effect also. In addition, the toxin caused a certain tissue damage in the descending colon of these mice (Okuda et al. 1997). When *cdtB* negative strains of *C. jejuni* were administered to severe combined immunodeficient mice no difference in enteric colonization was observed, but there was an impaired invasiveness of the bacteria into blood, spleen and liver tissues as compared to the wild-type bacteria (Purdy et al. 2000), implying that CDT might have a role in the pathogenesis (invasion) of *C. jejuni*. A more recent study of a *C. jejuni cdtB* mutant in wild-type and nuclear factor (NF)-κB deficient mice suggested that CDT may have pro-inflammatory activity in vivo, as well as a potential role in the ability of *C. jejuni* to escape immune surveillance (Fox et al. 2004). The *cdtB* mutant was less efficient than the wild-type *C. jejuni* in colonizing the wild-type (C57BL/129) but not NF-κB deficient mice. Despite 100% colonization of the NF-κB deficient 3X mice the *cdtB* mutant produced significantly less gastritis than the wild-type bacterium (Fox et al. 2004). The authors speculate that CDT may target the cells of the immune system in the lamina propria that influence the host's ability to clear bacterial pathogens. Young and coworkers recently provided further evidence for a potential pro-inflammatory activity of CDTs (Young et al. 2004). Strains of *H. hepaticus* expressing a functional CDT caused severe

colitis in a murine model of inflammatory bowel disease, using C57BL/6 IL-10 deficient mice. In contrast, the CDT-negative isogenic mutants were without effect (Young et al. 2004).

An isogenic *H. ducreyi cdtC* mutant was equally virulent as the parent strain when tested in the temperature-dependent rabbit model for experimental chancroid, despite the fact that it was not cytotoxic to HeLa cells and keratinocytes (Stevens et al. 1999). Isogenic *H. ducreyi cdtA* and *cdtB* mutants also proved to be as virulent as the wild-type strain with regard to lesion production in the same rabbit model (Lewis et al. 2001). In human volunteers, expression of CDT was also not required for pustule formation by *H. ducreyi*, although CDT might still be relevant for induction or persistence of the ulcer stage which is not testable in humans (Young et al. 2001). The purified HdCDT holotoxin induced dose-dependent pathologic skin reactions in rabbits (Wising et al. 2002). High levels of neutralizing antibodies against CDT were detected in only 22% and 2% of patients with chancroid and periodontitis, respectively. Moreover, a majority of healthy individuals also had HdCDT antibodies, and thus such antibodies may not be specific markers for chancroid infection (Mbwana et al. 2003).

In conclusion

The fact that CDTs are produced by diverse human pathogens suggests that these toxins might contribute to the development of different diseases. At the moment, however, there is no absolutely clear association between toxin and specific disease symptoms and one major problem is the lack of suitable animal models for this type of study. Conceivably, CDT could have a role in all instances where cell proliferation is required. Thus, CDTs produced by intestinal pathogens could possibly contribute to gastroenteritis by blocking the proliferation of crypt cells, although there are no studies yet on this particular aspect of the *E. coli*, *Campylobacter* or *Shigella* CDTs. The HdCDT could contribute to the very slow healing of chancroid ulcers. Furthermore, immunosuppression could be one important general effect of CDTs as suggested by the recent studies cited above and by the in vitro effect of CDT on DCs which have a central role for induction of both cellular and humoral-mediated immunoresponses (Li et al. 2002). Targeting DCs could represent a strategy developed by several pathogens in order to avoid or delay the onset of immunoresponses. In fact the AaCDT was originally isolated as the ‘immunosuppressive factor’ of *A. actinomycetemcomitans* and it might possibly play such a role in the pathogenesis of aggressive periodontitis and other infections caused by this bacterium (Shenker et al. 1999).

Future perspectives

In retrospect it is interesting to note that no toxicologist ‘jumped on’ the CDT directly after its discovery in the late 1980s, probably because it did not seem feasible to study something that requires several days to induce an effect on cells. However, during the 10 years after the CDT was first cloned this three-gene toxin has generated an increasing interest, and the development from ‘mysterious activity’ to well known toxin with defined mode of action, now even crystallized, has advanced rapidly. CDT turned out to be unique in that it is the only bacterial toxin known to target DNA as a primary action, subsequent-

ly activating a number of important cellular stress responses designed to save the attacked cell while it attempts to repair its DNA. Although—or perhaps because—we know today so much more than we did a decade ago there are many more questions waiting for their answers. In addition, there is reason to believe that CDT, like many other bacterial protein toxins, can be developed into a useful tool in cell biology and biomedicine.

Molecular mode of action of CDT

The receptor(s) for CDT has not yet been identified. This issue is complicated by the tripartite CDT structure with potentially two separate binding subunits, CdtA and CdtC, which might bind to separate receptor structures, or to the same as suggested by recent studies (Lee et al. 2003) and (McSweeney and Dreyfus 2004). The sequence homologies between the CdtA and CdtC subunits from different bacteria are much lower than between the different CdtB subunits. Thus, it is even possible that the receptor(s) for CDTs of different origin will turn out to differ slightly despite the fact that the active CdtB subunit apparently has the same DNase activity in all CDTs. That there are lectin-like regions in CdtA and CdtC (Nesic et al. 2004), similar to those found in the binding subunit of ricin, might point to a galactose-containing receptor, although Mao and DiRienzo (Mao and DiRienzo 2002) failed to inhibit the binding of AaCdtA to CHO cells with various galactosides and mannosides. Besides the binding to cell surface receptor(s) the three subunits must be able to interact with each other to form the fully active holotoxin. Obviously this interaction must depend on specific amino acid stretches, some of which have already been identified (Lee et al. 2003). It would also be of interest to combine subunits of CDTs from different bacteria to see whether they can fully complement each other. The binding of CdtB to T cells needs clarification; maybe there is a specific receptor for this subunit on these cells.

It is clear that CDT has to be internalized in cells so that the CdtB subunit can be transferred into the nucleus. Indirect functional studies suggested that HdCDT, or at least the active subunit, requires passage through an intact Golgi complex to intoxicate cells (Cortes-Bratti et al. 2000), and later observations in MDCK cells are fully consistent with these findings (T. Frisan and M. Thelestam, unpublished results). The CdtB subunit is able to enter the nucleus from the cytosol and this occurs via active transport (Nishikubo et al. 2003). However, the final steps in the transfer of the CdtB subunit, upon natural intoxication, from the vesicular uptake system to the nucleus are not clear. The cellular fates of CdtA and CdtC after fulfilling their putative roles to facilitate entry of CdtB have also not been clarified. Another unresolved issue is whether CdtC, if it really proves to be cytotoxic by itself, has an entirely different mode of action than CdtB.

CDTs as tools in cell biology

Bacterial toxins have been extremely useful in the study of different aspects of cell biology. So far, CDTs are the only bacterial protein toxins known to induce a subtle DNA damage. In the field of cell and tumor biology, regulation of the cell cycle is currently one of the major issues and CDT will probably be utilized as a tool in such studies. Toxins have also been very helpful in the study of endocytosis and intracellular transport in general. Studies with Shiga toxin showed for the first time that a molecule can be transported from

the cell surface to the Golgi apparatus and the ER (reviewed in Sandvig and Van Deurs 2000). Since the nucleus is its final destination CDT represents an interesting tool for studies of protein transport from the Golgi/ER to the nucleus. Finally, the study of CDT has already led to the discovery of a novel cellular signaling pathway transmitting survival signals from damaged DNA to the small GTPase RhoA. CDT will be useful in the continued efforts to clarify all the molecular details from DNA damage to RhoA activation, as well as from the latter event to the downstream components involved in protecting the cells from immediate death.

CDTs and disease

It will be interesting to see when CDTs will be more clearly demonstrated to cause, or at least to contribute to, the symptoms in infections caused by the bacteria producing them. Now that the mode of action of CDT is basically known this aspect will probably become more attractive for study even if the lack of really good animal models is still a problem. In any case CDTs are likely to affect the immune response as well as other situations in which cell proliferation is needed, such as the healing of chancroid or periodontic ulcers. Another potentially important aspect is that CDTs, being genotoxic, might possibly constitute a contributing factor in long term cancer development. Since CDT is produced by so many common pathogens this is a rather disturbing perspective, which has not yet been studied at all. On the other hand, the action of CDT on DNA makes it a potentially good candidate for an anti-tumor agent.

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References

Ahmed HJ, Svensson LA, Cope LD, Latimer JL, Hansen EJ, Ahlman K, Bayat-Turk J, Klamer D, Lagergard T (2001) Prevalence of cdtABC genes encoding cytolethal distending toxin among *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains. J Med Microbiol 50:860–864

Akifusa S, Poole S, Lewthwaite J, Henderson B, Nair SP (2001) Recombinant *Actinobacillus actinomycetemcomitans* cytolethal distending toxin proteins are required to interact to inhibit human cell cycle progression and to stimulate human leukocyte cytokine synthesis. Infect Immun 69:5925–5930

Alby F, Mazars R, de Rycke J, Guillou E, Baldin V, Darbon J-M, Ducommun B (2001) Study of the cytholethal distending toxin (CDT)-activated cell cycle checkpoint. Involvement of the CHK2 kinase. FEBS Letters 491:261–265

Ansaruzzaman M, Albert MJ, Nahar S, Byun R, Katouli M, Kuhn I, Mollby R (2000) Clonal groups of enteropathogenic *Escherichia coli* isolated in case-control studies of diarrhoea in Bangladesh. J Med Microbiol 49:177–185

Aragon V, Chao K, Dreyfus LA (1997) Effect of cytolethal distending toxin on F-actin assembly and cell division in chinese hamster ovary cells. Infection Immun 65:3774–3780

Bang DD, Nielsen EM, Scheutz F, Pedersen K, Handberg K, Madsen M (2003) PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and Campylobacter coli isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. J Appl Microbiol 94:1003–1014

Bang DD, Scheutz F, Ahrens P, Pedersen K, Blom J, Madsen M (2001) Prevalence of cytolethal distending toxin (cdt) genes and CDT production in *Campylobacter* spp. isolated from Danish broilers. J Med Microbiol 50:1087–1094

Belibasakis G, Johansson A, Wang Y, Claesson R, Chen C, Asikainen S, Kalfas S (2002) Inhibited proliferation of human periodontal ligament cells and gingival fibroblasts by *Actinobacillus actinomycetemcomitans*: involvement of the cytolethal distending toxin. Eur J Oral Sci 110:366–373

Benard V, Bohl BP, Bokoch GM (1999) Characterization of Rac and Cdc42 activation in chemoattractant-stimulated neutrophils using a novel assay for active GTPases. J Biol Chem 274:13198–13204

Bielaszewska M, Fell M, Greune L, Prager R, Fruth A, Tschape H, Schmidt MA, Karch H (2004) Characterization of cytolethal distending toxin genes and expression in shiga toxin-producing *Escherichia coli* strains of non-O157 serogroups. Infect Immun 72:1812–1816

Chehab NH, Malikzay A, Appel M, Halazonetis T (2000) Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53. Genes Dev 14:278–288

Chien CC, Taylor NS, Ge Z, Schauer DB, Young VB, Fox JG (2000) Identification of cdtB homologues and cytolethal distending toxin activity in enterohepatic *Helicobacter* spp. J Med Microbiol 49:525–534

Comayras C, Tasca C, Peres SY, Ducommun B, Oswald E, De Rycke J (1997) *Escherichia coli* cytolethal distending toxin blocks the HeLa cell cycle at the G2/M transition by preventing cdc2 protein kinase dephosphorylation and activation. Infect Immun 65:5088–5095

Cope LD, Lumbley S, Latimer JL, Stevens MK, Johnson LS, Purvén M, Munson RS, Lagergård T, Radolf J, Hansen EJ (1997) A diffusible cytotoxin of *Haemophilus ducreyi*. Proc Natl Acad Sci USA 94:4056–4061

Cortes-Bratti X, Chaves-Olarte E, Lagergård T, Thelestam M (1999) The cytolethal distending toxin from the chancroid bacterium *Haemophilus ducreyi* induces cell-cycle arrest in the G2 phase. J Clin Invest 103:107–115

Cortes-Bratti X, Chaves-Olarte E, Lagergård T, Thelestam M (2000) Cellular internalization of cytolethal distending toxin from *Haemophilus ducreyi*. Infect Immun 68:6903–6911

Cortes-Bratti X, Frisan T, Thelestam M (2001a) The cytolethal distending toxins induce DNA damage and cell cycle arrest. Toxicon 39:1729–1736

Cortes-Bratti X, Karlsson C, Lagergård T, Thelestam M, Frisan T (2001b) The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways. J Biol Chemy 276:5296–5302

da Silva AS, da Silva Leite D (2002) Investigation of putative CDT gene in *Escherichia coli* isolates from pigs with diarrhea. Vet Microbiol 89:195–199

Deng K, Hansen EJ (2003) A CdtA-CdtC complex can block killing of HeLa cells by *Haemophilus ducreyi* cytolethal distending toxin. Infect Immun 71:6633–6640

Deng K, Latimer JL, Lewis DA, Hansen EJ (2001) Investigation of the interaction among the components of the cytolethal distending toxin of *Haemophilus ducreyi*. Biochem Biophys Res Commun 285:609–615

Elledge SJ (1996) Cell cycle check points: preventing an identity crisis. Science 274:1664–1672

Elwell C, Chao K, Patel K, Dreyfus L (2001) *Escherichia coli* CdtB mediates cytolethal distending toxin cell cycle arrest. Infect Immun 69:3418–3422

Elwell CA, Dreyfus LA (2000) DNAase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. Mol Microbiol 37:952–963

Escalas N, Davezac N, De Rycke J, Baldin V, Mazars R, Ducommun B (2000) Study of the cytolethal distending toxin-induced cell cycle arrest in HeLa cells: involvement of the Cdc25 phosphatases. Exp Cell Res 257:206–212

Eyigor A, Dawson KA, Langlois BE, Pickett CL (1999a) Cytolethal distending toxin genes in *Campylobacter jejuni* and *Campylobacter coli* isolates: detection and analysis by PCR. J Clin Microbiol 37:1646–1650

Eyigor A, Dawson KA, Langlois BE, Pickett CL (1999b) Detection of cytolethal distending toxin activity and cdt genes in *Campylobacter* spp. isolated from chicken carcasses. Appl Environ Microbiol 65:1501–1505

Fabris AS, DiRienzo JM, Wikstrom M, Mayer MP (2002) Detection of cytolethal distending toxin activity and cdt genes in *Actinobacillus actinomycetemcomitans* isolates from geographically diverse populations. Oral Microbiol Immunol 17:231–238

Fox JG, Rogers AB, Whary MT, Ge Z, Taylor NS, Xu S, Horwitz BH, Erdman SE (2004) Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun 72:1116–1125

Frisan T, Cortes-Bratti X, Chaves-Olarte E, Stenerlöw B, Thelestam M (2003) The *Haemophilus ducreyi* cytolethal distending toxin induces DNA double strand breaks and promotes ATM-dependent activation of RhoA. Cell Microbiol 5:695–707

Frisk A, Lebens M, Johansson C, Ahmed H, Svensson L, Ahlman K, Lagergård T (2001) The role of different protein components from the *Haemophilus ducreyi* cytolethal distending toxin in the generation of cell toxicity. Microbial Pathogens 30:313–324

Ghilardi AC, Gomes TA, Trabulsi LR (2001) Production of cytolethal distending toxin and other virulence characteristics of *Escherichia coli* strains of serogroup O86. Mem Inst Oswaldo Cruz 96:703–708

Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. Science 246:629–634

Hassane DC, Lee RB, Mendenhall MD, Pickett CL (2001) Cytolethal distending toxin demonstrates genotoxic activity in a yeast model. Infect Immun 69:5752–5759

Hassane DC, Lee RB, Pickett CL (2003) *Campylobacter jejuni* cytolethal distending toxin promotes DNA repair responses in normal human cells. Infect Immun 71:541–545

Hickey TE, McVeigh AL, Scott DA, Michielutti RE, Bixby A, Carroll SA, Bourgeois AL, Guerry P (2000) *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun 68:6535–6541

Jackman MR, Pines JN (1997) Cyclins and the G2/M transition. Cancer Surv 29:47–73

Janka A, Bielaszewska M, Dobrindt U, Greune L, Schmidt MA, Karch H (2003) Cytolethal distending toxin gene cluster in enterohemorrhagic Escherichia coli O157:H- and O157:H7: characterization and evolutionary considerations. Infect Immun 71:3634–3638

Johnson JR, Stell AL (2000) Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis 181:261–272

Johnson WM, Lior H (1987a) Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. FEMS Microbiology Letters 43:19–23

Johnson WM, Lior H (1987b) Production of Shiga toxin and a cytolethal distending toxin (CLDT) by serogroups of *Shigella* spp. FEMS Microbiol Lett 48:235–238

Johnson WM, Lior H (1988a) A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. Microb Pathog 4:115–126

Johnson WM, Lior H (1988b) A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. Microb Pathog 4:103–113

Jones MA, Totemeyer S, Maskell DJ, Bryant CE, Barrow PA (2003) Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni.* Infect Immun 71:2626–2633

Kostia S, Veijalainen P, Hirvi U, Hanninen ML (2003) Cytolethal distending toxin B gene (cdtB) homologues in taxa 2, 3 and 8 and in six canine isolates of *Helicobacter* sp. flexispira. J Med Microbiol 52:103–108

Kulkarni K, Lewis DA, Ison CA (2003) Expression of the cytolethal distending toxin in a geographically diverse collection of *Haemophilus ducreyi* clinical isolates. Sex Transm Infect 79:294–297

Lara-Tejero M, Galan JE (2000) A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science 290:354–357

Lara-Tejero M, Galan JE (2001) CdtA, CdtB and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. Infection and Immunity 69:4358–4365

Lee RB, Hassane DC, Cottle DL, Pickett CL (2003) Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. Infect Immun 71:4883–4890

Lewis DA, Stevens MK, Latimer JL, Ward CK, Deng K, Blick R, Lumbley SR, Ison CA, Hansen EJ (2001) Characterization of *Haemophilus ducreyi* cdtA, cdtB, and cdtC mutants in in vitro and in vivo systems. Infect Immun 69:5626–5634

Li L, Sharipo A, Chaves-Olarte E, Masucci MG, Levitsky V, Thelestam M, Frisan T (2002) The *Haemophilus ducreyi* cytolethal distending toxin activates sensors of DNA damage and repair complexes in proliferating and non-proliferating cells. Cell Microbiol 4:87–99

Mainil JG, Jacquemin E, Oswald E (2003) Prevalence and identity of cdt-related sequences in necrotoxigenic *Escherichia coli*. Vet Microbiol 94:159–165

Mao X, DiRienzo JM (2002) Functional studies of the recombinant subunits of a cytolethal distending holotoxin. Cell Microbiol 4:245–255

Marques LR, Tavechio AT, Abe CM, Gomes TA (2003) Search for cytolethal distending toxin production among fecal *Escherichia coli* isolates from Brazilian children with diarrhea and without diarrhea. J Clin Microbiol 41:2206–2208

Matsuoka S, Huang M, Elledge SJ (1998) Linkage of ATM to cell cycle regulation by the chk2 protein kinase. Science 282:1893–1897

Mayer MP, Bueno L, Hansen EJ, DiRienzo JM (1999) Identification of a cytolethal distending toxin gene locus and features of a virulence-associated region in *Actinobacillus actinomycetemcomitans*. Infection and Immunity 67:1227–1237

Mbwana J, Ahmed HJ, Ahlman K, Sundaeus V, Dahlen G, Lyamuya E, Lagergard T (2003) Specificity of antibodies directed against the cytolethal distending toxin of *Haemophilus ducreyi* in patients with chancroid. Microb Pathogen 35:133–137

McSweeney LA, Dreyfus LA (2004) Nuclear localization of the *Escherichia coli* cytolethal distending toxin CdtB subunit. Cell Microbiol 6:447–458

Nesic D, Hsu Y, Stebbins CE (2004) Assembly and function of a bacterial genotoxin. Nature 429:429–433

Nishikubo S, Ohara M, Ueno Y, Ikura M, Kurihara H, Komatsuzawa H, Oswald E, Sugai M (2003) An N-terminal segment of the active component of the bacterial genotoxin cytolethal distending toxin B (CDTB) directs CDTB into the nucleus. J Biol Chem 278:50671–50681

Ohara M, Hayashi T, Kusunoki Y, Miyauchi M, Takata T, Sugai M (2004) Caspase-2 and caspase-7 are involved in cytolethal distending toxin-induced apoptosis in Jurkat and MOLT-4 T-cell lines. Infect Immun 72:871–879

Okeke IN, Lamikanra A, Steinruck H, Kaper JB (2000) Characterization of *Escherichia coli* strains from cases of childhood diarrhea in provincial southwestern Nigeria. J Clin Microbiol 38:7–12

Okuda J, Fukumoto M, Takeda Y, Nishibuchi M (1997) Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the *cdt* ABC genes of *Shigella dysenteriae*. Infection and Immunity 65:428–433

Okuda J, Kurazono H, Takeda Y (1995) Distribution of the cytolethal distending toxin A gene (*cdt*A) among species of *Shigella* and *Vibrio* and cloning and sequencing of the *cdt* gene from *Shigella dysenteriae*. Microbial Pathogen 18:167–172

Pandey M, Khan A, Das SC, Sarkar B, Kahali S, Chakraborty S, Chattopadhyay S, Yamasaki S, Takeda Y, Nair GB, Ramamurthy T (2003) Association of cytolethal distending toxin locus cdtB with enteropathogenic *Escherichia coli* isolated from patients with acute diarrhea in Calcutta, India. J Clin Microbiol 41:5277–5281

Peres SY, Marches O, Daigle F, Nougayrede JP, Herault F, Tasca C, De Rycke J, Oswald E (1997) A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. Mol Microbiol 24:1095–1107

Pickett CL, Cottle DL, Pesci EC, Bikah G (1994) Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. Infect Immun 62:1046–1051

Pickett CL, Lee RB, Eyigor A, Elitzur B, Fox EM, Strockbine NA (2004) Patterns of variations in *Escherichia coli* strains that produce cytolethal distending toxin. Infect Immun 72:684–690

Pickett CL, Pesci EC, Cottle DL, Russell G, Erdem AN, Zeytin H (1996) Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. cdtB gene. Infect Immun 64:2070–2078

Pickett CL, Whitehouse CA (1999) The cytolethal distending toxin family. Trends Microbiol 7:292–297

Purdy D, Buswell CM, Hodgson AE, McAlpine K, Henderson I, Leach SA (2000) Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. J Med Microbiol 49:473–479

Purvén M, Falsen E, Lagergård T (1995) Cytotoxin production in 100 strains of *Haemophilus ducreyi* from different geographic locations. FEMS Microbiol Lett 129:221–224

Ren XD, Kiosses WB, Schwartz MA (1999) Regulation of small GTP-binding protein rho by cell adhesion and the cytoskeleton. EMBO J 18:578–585

Rotman G, Shiloh Y (1999) ATM: a mediator of multiple responses to genotoxic stress. Oncogene 18:6135–6144

Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, Elledge SJ (1997) Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 277:1497–1501

Sandvig K, Van Deurs B (2000) Entry of Ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. EMBO J 19:5943–5950

Sato T, Koseki T, Yamato K, Saiki K, Konishi K, Yoshikawa M, Ishikawa I, Nishihara T (2002) p53-independent expression of p21(CIP1/WAF1) in plasmacytic cells during G(2) cell cycle arrest induced by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin. Infect Immun 70:528–534

Scott DA, Kaper JB (1994) Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxins. Infect Immun 62:244–251

Sert V, Cans C, Tasca C, Bret-Bennis L, Oswald E, Ducommun B, De Rycke J (1999) The bacterial cytolethal distending toxin (CDT) triggers a G2 cell cycle checkpoint in mammalian cells without preliminary induction of DNA strand breaks. Oncogene 18:6296–6304

Shenker BJ, Besack D, McKay T, Pankoski L, Zekavat A, Demuth DR (2004) *Actinobacillus actinomycetemcomitans* cytolethal distending toxin (Cdt): evidence that the holotoxin is composed of three subunits: CdtA, CdtB, and CdtC. J Immunol 172:410–417

Shenker BJ, Hoffmaster RH, McKay TL, Demuth DR (2000) Expression of the cytolethal distending toxin (Cdt) operon in *Actinobacillus actinomycetemcomitans*: evidence that CdtB protein is responsible for G2 arrest of the cell cycle in human T cells. J Immunol 165:2612–2618

Shenker BJ, Hoffmaster RH, Zekavat A, Yamaguchi N, Lally ET, Demuth DR (2001) Induction of apoptosis in human T cells by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin is a consequence of G2 arrest of the cell cycle. J Immunol 167:435–441

Shenker BJ, McKay T, Datar S, Miller M, Chowhan R, Demuth D (1999) *Actinobacillus actinomycetemcomitans* immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G2 arrest in human T cells. J Immunol 162:4773–4680

Siciliano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB (1997) DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev 11:3471–3481

Starcic M, Johnson JR, Stell AL, van der Goot J, Hendriks HG, van Vorstenbosch C, van Dijk L, Gaastra W (2002) Haemolytic *Escherichia coli* isolated from dogs with diarrhea have characteristics of both uropathogenic and necrotoxigenic strains. Vet Microbiol 85:361–377

Stevens MK, Latimer JL, Lumbley SR, Ward CH, Cope LD, Lagergård T, Hansen EJ (1999) Characterization of a *Haemophilus ducreyi* mutant deficient in the production of the cytolethal distending toxin. Infect Immun 67:3900–3908

Sugai M, Kawamoto T, Peres SY, Ueno Y, Komatsuzawa H, Fujiwara T, Kurihara H, Suginaka H, Oswald E (1998) The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. Infect Immun 66:5008–5019

Svensson LA, Henning P, Lagergard T (2002) The cytolethal distending toxin of *Haemophilus ducreyi* inhibits endothelial cell proliferation. Infect Immun 70:2665–2669

Tan KS, Song KP, Ong G (2002) Cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Occurrence and association with periodontal disease. J Periodontal Res 37:268–272

Taylor NS, Ge Z, Shen Z, Dewhirst FE, Fox JG (2003) Cytolethal distending toxin: a potential virulence factor for *Helicobacter cinaedi*. J Infect Dis 188:1892–1897

Toth I, Herault F, Beutin L, Oswald E (2003) Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV). J Clin Microbiol 41:4285–4291

Whitehouse CA, Balbo PB, Pesci EC, Cottle DL, Mirabito PM, Pickett CL (1998) *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infect Immun 66:1934–1940

Wising C, Svensson LA, Ahmed HJ, Sundaeus V, Ahlman K, Jonsson IM, Molne L, Lagergard T (2002) Toxicity and immunogenicity of purified *Haemophilus ducreyi* cytolethal distending toxin in a rabbit model. Microb Pathog 33:49–62

Yamano R, Ohara M, Nishikubo S, Fujiwara T, Kawamoto T, Ueno Y, Komatsuzawa H, Okuda K, Kurihara H, Suginaka H, Oswald E, Tanne K, Sugai M (2003) Prevalence of cytolethal distending toxin production in periodontopathogenic bacteria. J Clin Microbiol 41:1391–1398

Young RS, Fortney KR, Gelfanova V, Phillips CL, Katz BP, Hood AF, Latimer JL, Munson JR RS, Hansen EJ, Spinola SM (2001) Expression of cytolethal distending toxin and hemolysin is not required for postule formation by *Haemophilus ducreyi* in human volunteers. Infection and Immunity 69:1938–1942

Young VB, Knox KA, Schauer DB (2000a) Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus*. Infection and Immunity 68:184–191

Young VB, Chien CC, Knox KA, Taylor NS, Schauer DB, Fox JG (2000b) Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*. J Infect Dis 182:620–623

Young VB, Knox KA, Pratt JS, Cortez JS, Mansfield LS, Rogers AB, Fox JG, Schauer DB (2004) In vitro and in vivo characterization of *Helicobacter hepaticus* cytolethal distending toxin mutants. Infect Immun 72:2521–2527