Intoxication of mammalian cells by the cytolethal distending toxin of *Haemophilus ducreyi*: a novel mechanism of action

by

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SUMMARY

The cytolethal distending toxins (CDTs) are a newly described family of bacterial protein toxins with a novel mechanism of action: DNA damage. *Haemophilus ducreyi* produces a cytotoxin that belongs to this family. This bacterium causes chancroid, a sexually transmitted disease, characterised by mucocutaneous, slowly healing genital ulcers. As all genital ulcerative diseases, chancroid is a predisposing factor in the transmission of HIV. Since the pathogenesis of the disease is not known the *Haemophilus ducreyi* cytolethal distending toxin (HdCDT) represents a putative virulence factor.

At the beginning of our studies with HdCDT little was known about CDTs. To understand the mode of action of this toxin, we studied how it intoxicates mammalian cells. The morphological effect induced by the toxin was studied in epithelial-like cells and hamster fibroblasts. The intoxication was irreversible and appeared as a gradual cell distention, followed by cell death. A promotion of actin stress fibers was observed concomitantly with the cell enlargement. As shown for other CDTs, we found that HdCDT-intoxicated HEp-2 cells were arrested in the G2 phase of the cell cycle due to an accumulation of the tyrosine phosphorylated (inactive) form of the cyclin dependent kinase cdc2.

To characterize better the mode of action of HdCDT we tested a broad panel of human cell lines. We could demonstrate that the HdCDT effect is cell type specific and not exclusively related to G2 arrest. B cell lines underwent apoptosis, epithelial cells and keratinocytes arrested exclusively in G2 whereas normal fibroblasts arrested both in G1 and G2. Moreover, we showed that the response induced by HdCDT is similar to the checkpoint response activated by ionizing radiation (IR). Both responses were characterized by an early induction of the p53 gene and the cyclin dependent kinase inhibitor p21 in human fibroblasts and activation of chk2 kinase in HeLa cells. Our work also suggested that ATM, a key molecule in sensing DNA damage, is needed for the early response to HdCDT. However, in the absence of functional ATM the checkpoint was activated after a delay, probably by a homologue such as ATR. We also demonstrated that the promotion of actin stress fibers induced by HdCDT is dependent on Rho activation. Our observations made a link between Rho activation and DNA damage.

Finally, we demonstrated that these effects can occur only after cellular internalization of the toxin, and we have clarified some steps of the intracellular pathway followed by HdCDT. The toxin was found to enter HEp-2 cells via clathrin coated pits and to need an intact Golgi complex in order to induce intoxication.

In conclusion, this work, using HdCDT as a model, has improved our understanding of the mode of action of CDTs.
This thesis is based on the following papers, which will be referred to in the text by their Roman numbers:


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ABBREVIATIONS

ACSK  actin cytoskeleton
ADP  adenosine diphosphate
ATM  protein kinase “Ataxia telangiectasia mutated”
ATR  protein kinase “ATM and Rad3 related”
BafA1  bafilomycin A1
BFA  brefeldin A
BrdU  bromodeoxyuridine
Cdc2 (Cdk1)  cell division cycle 2 kinase
CDT  cytolethal distending toxin
Cdk  cyclin dependent kinase
CNF  cytotoxic necrotizing factor
DNase I  deoxyribonuclease I
ER  endoplasmic reticulum
GDP  guanosine diphosphate
GTP  guanosine triphosphate
HdCDT  Haemophilus ducreyi cytolethal distending toxin
HFS  human foreskin fibroblast
HLF  human lung fibroblasts
HU  hydroxyurea
IR  ionizing radiation
LCL  lymphoblastoid cell line
LCT  large clostridial cytotoxin
TcdA  Clostridium difficile toxin A
TcdB  Clostridium difficile toxin B
TcsL  Clostridium sordelli lethal toxin
UDP  Uridine diphosphate
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1. INTRODUCTION

*Haemophilus ducreyi* is a Gram-negative bacterium that causes chancroid (soft chancre), a sexually transmitted disease. The disease develops ulcers that are painful and show a pronounced retardation of healing; 50% of the patients also present inguinal lymphadenopathy. The lesion begins as a tender papule that becomes pustular, erodes and ulcerates. Chancroid is endemic in many developing countries and renewed attention has been focused on *H. ducreyi* since the transmission of HIV has been associated with genital ulceration (98, 114). Despite its significance, the pathogenesis of the disease remains poorly understood. Different bacterial components have been proposed as potential virulence factors, including: pili, lipooligosaccharide, hemolysin, iron regulated proteins, heat shock proteins and outer membrane proteins like DsrA (14, 114). *H. ducreyi* also produces a potent toxin that belongs to the family of cytolethal distending toxins (CDTs). This toxin may represent an important virulence factor since it is produced by the majority of strains from clinical samples and neutralizing antibodies have been detected at significantly higher levels in sera from patients compared to healthy donors (56, 82). This thesis deals with the intoxication of mammalian cells by the *H. ducreyi* CDT (HdCDT). The understanding of the mode of action of this toxin in cell models might help to clarify the pathophysiology of the disease.

1.1 The family of cytolethal distending toxins

The cytolethal distending toxins (CDTs) are a new family of bacterial protein toxins with the unique ability to interfere with the cell cycle, causing cell cycle arrest and eventually
death of the target cells. The mode of action and cellular targets of CDTs only now begin to be elucidated.

The first CDT activity was described in 1987 as a new toxin occurring in clinical isolates of *Escherichia coli*. Its effect was differentiated from the morphological changes of cells treated with the *E.coli* heat-labile enterotoxin, heat-stable toxin, verotoxin and hemolysin. The new cytotoxic effect was characterized by a remarkable cell distention, evident 96-120 h after addition of bacterial culture supernates, and resulting in cell death (52). The new toxin was designated “cytolethal distending toxin”, reflecting the characteristic distention observed in intoxicated cells. In the following year, a similar toxic activity was described in *Shigella* spp and *Campylobacter* spp (50, 51) and more recently several other Gram-negative bacteria have been shown to produce CDT.

1.1.1 Nomenclature

Since CDTs are produced by many different bacterial species, we have proposed a nomenclature to avoid confusions with different names in the future literature on CDTs (21). This nomenclature specifies a particular CDT by indicating the initials of the producing bacterium before CDT and the strain number or other common designation after CDT. Thus, the only CDT identified so far from *Haemophilus ducreyi* will be designated as HdCDT whereas the three presently known *E.coli* CDTs will be denoted as EcCDT-I, EcCDT-II and EcCDT-III, respectively (Table I).

1.1.2 CDT genes and protein products

The CDT activity is encoded by three linked genes (figure 1). The toxin genes in *E. coli* strain 6468/62 (086:H34) were cloned and sequenced by Scott and Kaper and designated
as *cdtA*, *cdtB* and *cdtC*, encoding proteins with predicted molecular masses of 25.5, 29.8 and 20.3 kDa respectively (99). Later, CDT genes from *E. coli* strain 9142-88 (0128:H-)(78) and *E. coli* strain S5 (015:H21) (76) were found to encode similar but not identical proteins. The respective toxins were denoted as CDT-I (strain 6468/62), CDT-II (strain 9142-88) and CDT-III (strain S5). The CDT-I and CDT-II genes are located on the chromosome whereas the CDT-III genes were found in a large virulence plasmid (76).

Recently CDT genes have been found in a diverse group of gram-negative pathogens, including *Campylobacter jejuni* (78), *Salmonella dysenteriae* (74), *Haemophilus ducreyi* (19), *Actinobacillus actinomycetemcomitans* (66, 109), and *Helicobacter hepaticus* (120). The presence of *cdtB* has been demonstrated or suggested for additional *Campylobacter* spp (31, 78), and homologues of *cdtB* have been identified in enterohepatic *Helicobacter* species (17, 119). The *cdtA* gene has been detected in *Shigella sonnei* (75) (Table 1).

Lagergård and Purven detected a soluble activity from *H. ducreyi* that was shown to have a cytotoxic effect on human cell lines of epithelial origin (56, 82, 83). In 1997, Cope and coworkers established that this soluble cytotoxic activity was encoded by a cluster of three genes. The proteins encoded by these genes had calculated molecular masses of 25, 30 and 20 kDa and had 38%, 51% and 24% identity to the products of the *E. coli cdtABC* genes encoding CDT-I. Therefore, the *H. ducreyi* cytotoxic activity was characterised as a cytolethal distending toxin (19).
<table>
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<tr>
<th>Species</th>
<th>Gene(s) detected</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em> (strain 6468/62, CDT I)</td>
<td>cdtABC</td>
<td>+</td>
<td>EcCDT I</td>
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<tr>
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<td>+</td>
<td>EcCDT II</td>
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<td>+</td>
<td>EcCDT III</td>
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<td>+</td>
<td>SdCDT</td>
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<tr>
<td><em>S. boydii</em></td>
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<td>+</td>
<td>ShCDT</td>
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<td>SsCdtA</td>
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<td>+</td>
<td>HpCdtB</td>
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<td>+</td>
<td>HbCdtB</td>
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<td>+</td>
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<td>HspCdtB-98-6070</td>
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<td>+</td>
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</tr>
<tr>
<td><em>C. &quot;upsaliensis&quot;</em></td>
<td>cdtB*</td>
<td>+</td>
<td>CuCdtB</td>
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**Table 1. Distribution of CDT genes in bacterial species**

nr : not reported
* : probable presence of cdtB sequences
The Cdt proteins from different species present diverse degrees of similarity. Even in *E. coli* there are variations, the genes encoding CDT-II being more related to the genes of CDT-III than to those of CDT-I. The closest relative of HdCDT is the *A. actinomycetemcomitans* CDT (AaCDT). The identities between these two CDTs are 91%, 97% and 94% for the A, B and C subunits respectively. When the amino acid identities from CDTs are compared, the *cdtB* products are the most conserved, with 47% identity even between the most distantly related CDTs. The *cdtA* and *cdtC* products present more variability, 21 to 39% identity between the different CDTs (79). Exceptions where this percentage is higher are the closest relatives mentioned above, i.e. the *E. coli* CDT-II and CDT III as well as the HdCDT and AaCDT.

1.1.3 *Properties of the CDT proteins*

Genetic studies have indicated that expression of all the three genes is required for the production of an active CDT (19, 78, 109, 120). Recent studies have shown that when applied individually recombinant CdtA, CdtB or CdtC are non-toxic but toxicity can be reconstituted by combining the three components (29, 36, 58). The combination of only two components was not sufficient for toxicity. Frisk and coworkers found that the CdtC protein purified from a toxic preparation of HdCDT (strains expressing *cdtABC*) has an isoelectric point (pI) value 1.5 units higher than a non-toxic CdtC from strains expressing *cdtC* alone (36). Furthermore, the combination *in vitro* of the three non-toxic components (produced individually as recombinant proteins) also resulted in a similarly changed pI value of CdtC. This suggests that CdtA and/or CdtB exerts some kind of modifying effect on CdtC that is needed for its activation (36). The CdtC component indeed plays a crucial role for the cytotoxic activity of HdCDT, as demonstrated by the ability of an anti-CdtC monoclonal antibody to neutralize the activity of this toxin (19). Moreover, toxin
preparations from a *H. ducreyi cdtC* mutant had no effect on Jurkat T cell growth. The toxic activity of this mutant was restored by complementation in *trans* (37). Another recent study with HdCDT supports the notion that a modification is needed for the formation of a fully active CDT. Deng and coworkers reported that the presence of functional CdtA was required for the formation of a noncovalent CdtB-CdtC complex and the production of a fully active CDT (24). It has also been showed that supernatants from isogenic *H. ducreyi cdtA, cdtB* and *cdtC* mutants failed to kill HeLa cells until four days after intoxication, in contrast to the wild-type parent strain that caused extensive killing. The *cdtA* mutant induced cell distention but not cell death after four days. Lewis and coworkers proposed that this would be consistent with CdtA being essential for the formation of a fully active toxin (60).

The CdtBs from *E.coli, C.jejuni* and *H. ducreyi* were recently demonstrated to have structural and functional homology to DNase I. CdtB preparations from *E. coli* and *H. ducreyi* were shown to have DNase activity as detected by *in vitro* digestion of a DNA plasmid substrate (29, 36). For *C. jejuni* it was reported that transfection of HeLa cells with the CdtB gene induced a slowly appearing (48h) nuclear fragmentation and a marked chromatin disruption (57). Microinjected CdtB protein was also able to induce an enlargement of the nucleus and changes in the chromatin. The DNase activity, as well as the induction of cell cycle arrest, was abolished by point mutations of conserved residues required for catalysis or for magnesium binding, indicating that the cytotoxic effects are related to this enzymatic activity (29, 57). CdtB alone added extracellularly did not induce cell cycle arrest. However, *C. jejuni* CdtB microinjected (57) and *E. coli* CdtB electroporated (28) into HeLa cells were able to produce the same effects as the
holotoxin, suggesting that CdtB alone, when present intracellularly can induce cell cycle arrest.

Recent work with *C. jejuni* CDT, showed that a domain in CdtA shares similarity to a lectin fold present in the B-chain of ricin and abrin, which are two AB toxins (42). The B subunit of A/B toxins is responsible for binding to toxin receptors on the cell surface and for mediating translocation across the cell membrane (33). The observed similarity between CdtA and these toxins may suggest a role for CdtA in the internalization of CdtB. The work of Lara-Tejero and colleagues, also showed that the Cdt proteins can interact with each other and form a tripartite complex that was isolatable by gel filtration chromatography. Therefore, these authors proposed that CDT is a tripartite toxin with CdtB as the enzymatically active subunit and CdtA and CdtC as the heterodimeric B subunit required for the delivery of CdtB into the cell (58).

![Figure 1: Genes encoding CDTs.](image)
1.2 Roles of CDTs in pathogenesis

1.2.1 Occurrence of CDTs in clinical isolates

*E. coli* CDT has been found in isolates of different serotypes. A possible association with enteropathogenic (EPEC) serotypes has been suggested. However, different studies showed that CDT is produced in some but not all EPEC serotypes (5, 73). In a study on urosepsis isolates of *E. coli*, some strains positive for *cdtB* were found, and the authors suggested that CDT in the future should be also considered as a possible extraintestinal virulence factor (49). At the moment, however, there is no clear association between *E. coli* CDTs and some specific disease or serotype. The production of CDT has been detected in isolates of *S. dysenteriae*, *S. boydii* and *S. sonnei* but the numbers are too low to allow any conclusion about the possible role of CDT in *Shigella* infections.

*Campylobacter* infections are common but very little is known about the pathogenesis. Different toxins have been described but only CDT is well characterized (40, 78, 117). CDT activity or *cdt* genes have been found in almost all the investigated strains of *C. jejuni* and *C. coli*. In one study the *cdtB* gene was detected in all the isolates from chicken carcasses, i.e., the primary source of *C. jejuni* and *C. coli* in human infections (32). *C. jejuni* isolates produced CDT at high toxin titers whereas *C. coli* produced little or no toxin as detected by an *in vitro* assay. CDT production has also been demonstrated from *C. fetus*, and PCR experiments suggest the presence of *cdtB* sequences in other species of *Campylobacter* as well (78). Recently, CDT activity has also been detected in *C. upsaliensis*, which is an emerging human enteropathogen (67).
CDT activity is not so common in *Helicobacter*. The three genes have been identified in *H. hepaticus* (120), and *cdtB* homologues have been reported for enterohepatic helicobacters. The *cdtB* gene was detected in human clinical isolates of *H. pullorum* (119) but no CDT homologue was found in *H. pylori* (17).

1.2.2 Effects of CDTs in experimental animals

An *E.coli* strain expressing the *S.dysenteriae cdt* genes was able to induce watery diarrhea in the suckling mouse model, and also the partially purified CDT had some effect. Moreover, the toxin caused a certain tissue damage in the descending colon of these mice (74). A recent study showed that CDT from *C. jejuni* induced the release of interleukin-8 from human embryo intestinal epithelial cells (44). Furthermore, a reduced toxicity in HeLa cells was observed with *cdtB* negative strains of *C. jejuni* (although one of the strains retained a low level of toxic activity). When these *cdtB* mutants were administered to severe combined immunodeficient mice no difference in enteric colonization was registered as compared to the wild type. However, there was an impaired invasiveness of the bacteria into blood, spleen and liver tissues (81), implying that CDT might have a role in the pathogenesis of *C. jejuni*.

1.2.3 Actinobacillus actinomycetemcomitans and Haemophilus ducreyi

*Actinobacillus actinomycetemcomitans* produces several human diseases, including endocarditis, meningitis, and periodontal disease. Different studies suggested that impaired host defense mechanisms may contribute to infectious diseases associated to this bacterium. It has been reported that the *A. actinomycetemcomitans* CdtB is able to block the proliferation of T lymphocytes (101, 103). Akifusa and coworkers recently reported that Cdt proteins, either alone or in combination, were able to induce the
production of interleukin-1β (IL-1β), IL-6, IL-8 and IFN-γ. CdtA was the most potent and CdtB the least potent cytokine inducer, and a synergistic effect was observed when the proteins were combined (1). The inhibition of cell proliferation and the induction of cytokines are activities that might facilitate the colonization by this bacterium.

The presence of the cdt genes has not been studied in many H. ducreyi strains, but 89% of a group of 100 isolates from different parts of the world showed CDT activity on HEp-2 cells (82). The high prevalence of the toxin in clinical isolates supports the notion of its role as a virulence factor of this bacterium.

A human model of H. ducreyi infection has been established to study the pathogenesis of this bacterium. Volunteers are inoculated into the skin of the arm and the infection is allowed to proceed until the stage of pustule (3). A cdtC mutant with no cytotoxic effect on keratinocytes, fibroblasts, HeLa cells or T cells was tested in this model. Inoculation of the mutant or the parent strain caused pustules at similar rates. The same result was found with a CDT and hemolysin double mutant; therefore expression of neither CDT nor hemolysin was required for the formation of pustules (118). Other putative virulence factors of H. ducreyi like the outer membrane protein DsrA (14), facilitated pustule formation. One major limitation of this human model is that the disease can not be studied beyond the pustular stage, and therefore it is not possible to evaluate the role of CDT at the ulcer stage. Conceivably, other factors are involved in the first pustular stage of the disease, whereas the CDT could be associated with formation of the ulcer and, not least, the retardation of healing characteristic in this disease. The role at a later stage of the disease is supported by the ability of HdCDT to affect human T cells and B cells (22,
The toxin might exert an immunosuppressive activity in the human host, which could contribute to the delayed healing of the chancroidal ulcers.

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, the information available is not sufficient to establish a clear association between toxin and disease.

1.3 The cell cycle and checkpoint responses

CDTs are the first protein toxins shown to interfere with the cell cycle. Progression of the cell cycle through the different phases, i.e. G1, S, G2 and M, is controlled by the activation and deactivation of various cyclin-dependent kinases (Cdks). The Cdk complexes are formed by a protein kinase catalytic subunit (cdk) and a cyclin which is the activating subunit. Different Cdk complexes are required for the transitions between the various phases of the cell cycle (figure 2). Most of the time, the Cdk catalytic subunits are inactive and the cellular levels of Cdks tend to be constant during the cell cycle. In order to become activated they require association with a cyclin subunit and phosphorylation of a conserved threonine by Cdk-activating kinases. This complex can be reversibly inactivated by phosphorylation of a conserved threonine-tyrosine pair or by binding to Cdk- inhibitory subunits (CKIs) (69). Finally, after the cell cycle transition is complete the cdk-cyclin complex is irreversibly inactivated by degradation of the cyclin subunit (64).
Cell cycle checkpoints are regulatory pathways that control the cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity (27). Cells exposed to genotoxic stress activate checkpoint responses that prevent cell cycle progression until the DNA damage has been repaired. Cell cycle arrest can occur at different stages of the cycle: the G1/S transition (G1 checkpoint), S phase progression, and the G2/M transition (G2/M checkpoint) (27). The protein kinase “Ataxia telangiectasia mutated” (ATM) and its homologue “ATM and Rad3 related” (ATR) play a central role in the DNA damage response, and they can act in concert or separately. Irradiation-induced DNA double strand breaks have been found to activate ATM which in turn can trigger all the different checkpoints. ATR activation is also associated to other types of DNA damage, as that induced by UV irradiation, and to replication arrest (89, 104). The G1 arrest induced upon DNA damage requires the tumor suppressor protein p53. The p53 protein is stabilized in an ATM dependent manner via

Figure 2: Phases of the eukaryotic cell cycle
G: gap, S: synthesis, M: mitosis
phosphorylation on serine 20 by the chk2 protein kinase (16, 105). The transition of cells from G2 into mitosis requires activation of the cyclin-dependent kinase cdc2 /cyclin B complex. The final step in the activation of this complex is a dephosphorylation of Thr-14 and Tyr-15 by the phosphatase Cdc25C (47) (figure 3). The G2 arrest depends on an inactive cdc2 complex. The protein kinases chk1 and chk2 are activated in vivo in response to DNA damage and both are able to inactivate Cdc25C in vitro (65, 90) giving rise to the lack of cdc2 activation at the end of the G2 phase.

**Figure 3:** Activation of the cdc2/cyclinB complex for the transition from G2 to M phase

P: phosphorylation
1.4 The actin cytoskeleton and its regulators

In some cell types, CDTs have been shown to promote a rearrangement of the actin cytoskeleton (ACSK), characterized by the formation of prominent stress fibers (6, 20). The ACSK mediates essential biological functions in all eukaryotic cells, including maintenance of the cell shape and polarity and providing the driving force for cells to move and to divide (38). Monomeric actin (G actin) is polymerized to form actin filaments (F actin). The polymerization and depolymerization of filaments is a highly dynamic process that depends on cellular needs and is regulated by a variety of actin-binding proteins. Actin filaments can be organized in different structures; stress fibers are large bundles of actin filaments linked to the plasma membrane at focal adhesions. Filopodia are formed by parallel bundles of actin protruding from the membrane, and membrane ruffles are formed when the polymerization of actin occurs beneath the plasma membrane (38).

The Rho family of small GTPases are key regulators of the actin cytoskeleton (38). Rho is involved in the formation of stress fibers and focal adhesions (88). Cdc42 induces the formation of filopodia (55) and Rac stimulates the production of membrane ruffles (87). Furthermore, Rho proteins are involved in several other cellular processes like membrane trafficking, transcriptional regulation, cell proliferation, transformation and apoptosis (9, 10). Small GTPases regulate all these molecular events by cycling between two conformations: an inactive form (GDP-bound) and an active form (GTP-bound). In the GTP form, the proteins are able to interact with effector molecules and exert their function; after hydrolysis of the GTP the proteins change to the GDP conformation and are inactivated. The nucleotide exchange is facilitated by nucleotide exchange factors.
(GEFs) and the intrinsic GTPase activity can be promoted by GTPase activating proteins (GAPs) (111).

Several bacterial toxins are known to affect the ACSK either indirectly by targeting small GTPases or directly by affecting actin (113). For example the Clostridium botulinum C2 toxin ADP-ribosylates G-actin (115). Rho proteins can be either inactivated (53, 54) or activated (34, 46, 97) by bacterial toxins. Rho-inactivating toxins include the C. botulinum exoenzyme C3, some C3-like ADP-ribosyltransferases and the “large clostridial toxins” (LCTs), such as toxins A and B from C. difficile. All LCTs have glucosyltransferase activity and the glucosylation of a variety of small GTPases causes a collapse of the ACSK (113). Rho-activating toxins include the cytotoxic necrotizing factors (CNF1 and CNF2) from E.coli and the dermonecrotic toxin produced by various Bordetella species (2). CNF activates Rho by deamidation of glutamine 63 (34, 97), and also exerts its activity on Cdc42 and Rac. The CNF-mediated Rho-subfamily activation induces the promotion of stress fibers, membrane ruffles, microspikes and lamellipodia and finally results in the formation of multinucleated cells (2).

1.5 Cellular internalization of toxins

Bacterial toxins can act on the cell surface or intracellularly. Toxins with intracellular targets enter cells by first binding to cell surface receptors, then they are endocytosed and finally they become translocated into the cytosol from an intracellular compartment. The receptor provides the binding site at the cell surface and has several other functions, for example if it is internalized efficiently, the uptake of the toxin is rapid. Receptors can
target the toxin to the relevant compartment before it enters the cytosol or may play a
direct role in toxin penetration across the membrane (33).

Endocytosis is known to take place via clathrin-coated pits or clathrin-independent
mechanisms, for example via caveolae (93). Endocytic vesicles fuse and form endosomes
that mature by additional fusion processes. After this, endocytosed macromolecules may
be delivered either to lysosomes for degradation or to the Golgi complex for processing.
Mechanisms exist for retrograde transport from the Golgi compartment to the ER of
macromolecules containing appropriate signaling sequences.

Most protein toxins are endocytosed, although by differing mechanisms. Some toxins, for
instance the plant toxin ricin, can use both clathrin-dependent and independent
mechanisms for entry into the cell. Presumably this toxin is internalized by all the
endocytic mechanisms available in the cell. In contrast, Shiga toxin is endocytosed
preferentially via the clathrin-coated pathway (93).

Two major group of toxins have been defined based on the compartment they are
translocated from: i) toxins able to enter the cytosol directly from endosomes like the
diphtheria and anthrax toxins, and ii) toxins that need to be transported to the Golgi
complex and the ER before membrane translocation (33). This second pathway is used by
the majority of toxins studied to date: Shiga and Shiga-like toxins (48, 91), pertussis toxin
(26, 43), cholera toxin (71), the E. coli heat labile enterotoxin (59), the Pseudomonas
exotoxin A (121) and ricin (84, 107). The retrograde transport of toxins from the Golgi
complex to the ER may take place by more than one mechanism. There are KDEL
receptors which can retain newly synthesized proteins with the sequence KDEL in the
ER, and they can also retrieve such proteins from the Golgi apparatus to the ER in a retrograde manner (61). Some toxins (cholera toxin, *Pseudomonas* exotoxin A) have a KDEL sequence that might bind to these receptors thereby facilitating their retrograde transport. Other toxins do not have this sequence (ricin, Shiga) and may use a different pathway(s) to reach the ER.

The mechanism of the ER-to-cytosol translocation is still not completely clear but these toxins may take advantage of the translocation machinery present in the ER membrane, such as the Sec61 translocon (62, 80). It has been suggested that toxins can disguise themselves as misfolded proteins in order to be transported across the ER membrane (62).
2. AIMS

The long term aim of this work is to understand the mode of action of the *Haemophilus ducreyi* cytolethal distending toxin in mammalian cells. More specific aims for this thesis were:

(i) To clarify the general characteristics of cellular intoxication by HdCDT (Paper I)

(ii) To achieve a more detailed understanding of the HdCDT-induced cell cycle arrest in normal human cell types that may be toxin targets *in vivo* (Paper II).

(iii) To determine if cellular internalization of HdCDT is required for its cytotoxic action and if so clarify the major events in the internalization pathway (Paper III).

(iv) To elucidate the role of small GTPases in the HdCDT-induced rearrangement of the actin cytoskeleton (Paper IV).
3. RESULTS AND DISCUSSION

3.1 Effects of HdCDT on mammalian cells (papers I and II)

3.1.1 General early observations

At the beginning of this study, little was known about CDTs. Therefore we studied some general features of the cellular intoxication of HdCDT in eukaryotic cells. It had been previously reported that HdCDT was able to intoxicate human cell lines of epithelial origin (83). We began our study with the intoxication of a human keratinocyte cell line HaCat, a human epithelial tumor cell line HEp-2 and also a hamster lung Don fibroblasts which turn out to be toxin sensitive. The observed morphological change was characterized by a remarkable cell distention. After 24 hours, HEp-2 cells were three times, and after 72 hours five times, larger than the average control cell (Paper I, fig.2). The same distending effect was observed in Don fibroblasts, which were maximally enlarged after 24 hours. In both cell lines, the distention was accompanied by a stress fiber promotion and followed by cell death. Addition of HdCDT to mouse 3T3 fibroblasts, however, did not cause any change of their morphology.

Among the bacterial toxins which act intracellularly, two enzymatic activities are common: ADP-ribosylation and glucosylation. At the beginning of this study when the mode of action of CDTs was not known, we asked whether HdCDT might have one of these activities. Fresh HEp-2 cell lysates were mixed with the toxin and the radiolabeled cofactor needed for the reaction. To detect ADP-ribosyltranferase activity, lysates were treated with the toxin in the presence of $[^{32}\text{P}]$ NAD and using \textit{C. botulinum} exoenzyme

25
C3 as positive control. No cellular proteins became ADP-ribosylated. We also did not detect any glucosyltransferase activity as shown by a similar assay, but using UDP-[14C] glucose as the cofactor and *C. difficile* TcdB as the positive control.

Another result of HdCDT intoxication was the inhibition of cell proliferation. The synthesis of DNA in toxin-treated cells was not affected up to 18 hours after treatment, but the incorporation of both BrdU and [3H]-thymidine was decreased 24-36 hours after toxin treatment (Paper I, fig.6). It was concluded that an inhibition of DNA synthesis was not the primary cause of the toxin-induced antiproliferative effect but that a subtle DNA damage could not be excluded.

### 3.2 HdCDT induces cell cycle arrest and checkpoint responses

#### 3.2.1 *HdCDT* induces *G2* arrest in *HEp-2* cells

CDTs had been shown to block cells in the G2 phase of the cell cycle. Studying the cell cycle distribution after intoxication with HdCDT by flow cytometric analysis, we also demonstrated a block in the G2/M phase of HdCDT-treated HEp-2 cells. This cell cycle arrest was associated with accumulation of the inactive hyperphosphorylated form of the cyclin dependent kinase cdc2, as previously shown for other CDTs (18, 117). To confirm the inactivation of cdc2, the protein was immunoprecipitated and its kinase activity towards histone H1 measured in the presence of [γ-32P]. As positive control we used nocodazole to synchronize cells into the G2/M phase, in which the cdc2 complex is active. As expected, the nocodazole-treated cells showed a high kinase activity while in toxin treated cells the kinase activity was much lower and the tyrosine phosphorylated (inactive) form of cdc2 was detected (Paper I, fig.8).
In HEp-2 cells, the distention was detected 12-15h after HdCDT-intoxication. We asked whether the accumulation of inactive cdc2 was an event preceding the distention. Time kinetic experiments showed that the inactive tyrosine-phosphorylated form of cdc2 appeared already six hours after addition of the toxin (Paper I, fig.9). For other CDTs, this effect had been determined only 24 hours after toxin exposure (18, 117). Thus, our work pinpointed cdc2-inactivation as the earliest biochemical sign of HdCDT-intoxication, which was present hours before the cell distention and ACSK promotion were detectable. The interaction of CDTs with the cell cycle regulatory machinery suggested that the promotion of the ACSK was secondary to the molecular events leading to cell cycle arrest.

As mentioned before, intoxication with different CDTs induces accumulation of the tyrosine-phosphorylated (inactive) form of cdc2 (18, 76, 109, 117). After intoxication with an *E. coli* CDT, the inactive cdc2/cyclin B complex could be reactivated *in vitro* with recombinant Cdc25C (100). Furthermore, overexpression of Cdc25B or Cdc25C was able to override the G2 arrest induced by this toxin, and allowed the cells to enter an abnormal mitosis (30). Both results suggested that CDTs do not target specifically cdc2 but rather some upstream component, leading to inactivation of Cdc25C with subsequent lack of cdc2 dephosphorylation as a secondary effect.

3.2.2 *The HdCDT-induced cell cycle arrest is cell type dependent*

To better characterize the mode of action of HdCDT, we studied its effects on a broad panel of human cell lines (paper II), including normal keratinocytes and fibroblasts, which are conceivable toxin targets *in vivo*. We now tested HdCDT in two epithelial cell
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Table 2: HdCDT induced-cell cycle arrest is cell type dependent

<sup>a</sup> Detection of the sub-G1 population by propidium iodine staining 24 h after treatment
<sup>b</sup> Burkitt’s lymphoma-derived cell line
<sup>c</sup> Epstein-Barr virus-transformed B lymphocytes derived from healthy donors
<sup>d</sup> Normal foreskin keratinocytes
<sup>e</sup> Normal foreskin (HFS) and lung (HF) fibroblasts
lines, three keratinocyte lines, two normal fibroblast lines and three lines of B cell origin (Table 2). After intoxication with HdCDT, the epithelial tumor cells and keratinocytes were arrested in G2. Intoxicated normal fibroblasts, however, accumulated in G2 only partially. Even 48 hours post-intoxication the cells were arrested also in S and G1 (Paper II, fig.1A). The B cell lines showed a 50% decrease of the G1 peak, and this was associated with a 2-to 4-fold increase of the apoptotic sub-G1 population 24h after intoxication. Thereby we had demonstrated that the effect of HdCDT is cell type dependent and not exclusively related to a G2 block (Table 2). The CDT effect previously had been associated only with G2 arrest because of the accumulation of the inactive form of cdc2. The induction of apoptosis in human T cells by the A. actinomycetemcomitans CDT has been shown to be a consequence of G2 arrest (102). However, in the case of HdCDT-intoxicated B cells a slight increase of the G2 phase could be detected only in some experiments.

3.2.3 HdCDT activates checkpoint responses

DNA damage is known to activate checkpoint responses which prevent progression through the cell cycle until the damage is repaired (27). Our observation that HdCDT can induce arrest at different phases of the cell cycle, suggested that the toxin may activate DNA damage checkpoint responses. Therefore we decided to compare the effects of HdCDT and ionizing radiation (IR) on HEp-2 cells and human lung fibroblasts (HLF). Both treatments were found to induce similar responses. In toxin treated fibroblasts, accumulation of the inactive form of cdc2 was detected only at early time points (4 and 6 hours) and the same was observed after exposure to IR. The tumor suppressor protein p53 was stabilised and phosphorylated on serine 15 with the same kinetics in intoxicated as in irradiated cells. This was associated with an upregulation of the p53-regulated cyclin-
dependent kinase inhibitor p21 and to a lesser extent of p27 (Paper II, fig.2C), suggesting that the G1-checkpoint response in normal fibroblasts is p53-dependent. In HEp-2 cells a different pattern was detected, consistent with the fact that the two cell lines respond different to HdCDT intoxication. Phosphorylation of cdc2 was detected 4 hours after intoxication and was maintained up to 24 hours. Increased expression of p53 and p21 was detected much later than the accumulation of inactive cdc2, suggesting that p53 is not involved in the G2 arrest observed in these cells. Furthermore, the HeLa and HaCaT cell lines, which carry a nonfunctional p53, still arrested in G2 and did not up-regulate p21, supporting the involvement of a p53-independent pathway. The chk2 kinase is known to play a central role in activation of the G2 checkpoint response upon exposure to IR (65). In HEp-2 cells, we found that after both treatments, IR and HdCDT, the chk2 protein was phosphorylated, as detected by a shift of its electrophoretic mobility. Involvement of the chk2 kinase in CDT-intoxication was also confirmed by Alby and coworkers, who demonstrated accumulation of phosphorylated chk2 in EcCDT-treated HeLa cells (4).

The protein kinase “Ataxia telangiectasia mutated” (ATM) and its homologue “ATM and Rad3 related” (ATR) play a central role in sensing DNA damage. ATM is activated exclusively by DNA double strand breaks and in turn can trigger all the different checkpoints. Therefore we tested the involvement of ATM in the HdCDT-induced response. ATM wild type lymphoblastoid cell lines (LCLs) and ATM-deficient cell lines were intoxicated. After 24 hours, wild type cells showed a 2- to 4-fold increase of the apoptotic sub-G1 population and a strong decrease of the G1 peak, whereas no major changes were observed in the S or G2/M phases. The ATM-deficient cells were more resistant to the toxin, only minor changes being observed in the sub-G1 and G1
populations after 24 hours of treatment (Paper II, fig.4). Furthermore in ATM-deficient cells the increase in p53 was also delayed (Paper II, fig.5).

Caffeine is known to override in mammalian cells the G2/M block induced by DNA damage by inhibiting the activation of the ATM kinase and therefore blocking the activation of chk2 (13, 122). The involvement of ATM was supported by the ability of caffeine to partially override the cell cycle arrest induced by HdCDT. Caffeine treatment prevented the accumulation of tyrosine-phosphorylated cdc2 and this was associated with a partial release of the G2 arrest (Paper II, fig.3).

The delayed intoxication observed in ATM-deficient cells suggested that the early molecular response to HdCDT is ATM dependent. However, checkpoint responses can still be activated in the absence of functional ATM, probably by homologous molecules such as ATR. Our findings then suggested that the toxin targets DNA or some molecule upstream ATM and directly involved in activation of checkpoint responses (figure 4).

Sert and coworkers did not detect any DNA strand breaks in cells intoxicated with E. coli CDT, using the single cell gel electrophoresis assay (“comet assay”) (100). Therefore, the authors suggested a pathway independent of DNA damage. In contrast to this, CdtB later was found to have structural and functional similarity to DNAse I (29, 36, 57). Moreover, intracellular CdtB from C. jejuni and E. coli were able to induce the same effects as the holotoxin. It is well established that the Mre11 complex associates with damaged DNA and forms discrete nuclear foci after induction of double strand breaks (DSBs) following irradiation (77). Recent data demonstrate indeed that HdCDT induces re-localization of the DNA repair complex Mre11 (Frisan, unpublished results).
Further evidence of CdtB DNase I-like activity has been provided in a yeast model system, used to analyze *C. jejuni* Cdt subunits. Hassane and coworkers found that expression of CdtB alone was sufficient to cause a G2 block in *Saccharomyces cerevisiae* (41). The CdtB toxicity was not avoided in mutant yeasts altered to lack their DNA damage checkpoint control or to constitutively promote cell cycle progression via mutant Cdk1 (cdc2). This work also demonstrated the activation of RNR2, a gene known to be upregulated in response to direct DNA damage. Moreover, chromosomal degradation was associated with CdtB expression. In conclusion, all these data strongly support the idea that DNA is the toxin target. CDTs may induce a subtle DNA damage, not detectable by the “comet assay”, but leading to the activation of cell cycle checkpoint responses, in agreement with our data presented in paper II.
Figure 4: Cellular responses induced by HdCDT

P: phosphorylation
3.3 Cellular internalization of HdCDT (paper III)

3.3.1 *HdCDT* is endocytosed via clathrin-coated pits

At the beginning of this thesis work it was not known if cellular internalization was required for the cytotoxic action of HdCDT. Thus, we undertook internalization studies with HEp-2 cells (paper III). The observation that cell surface-bound HdCDT was not accessible to proteases or antibodies was suggestive of a rapid internalization. Moreover, the accumulation of tyrosine-phosphorylated cdc2 was reduced in cells treated with ammonium chloride, methylamine or monensin (Paper III, fig.1), agents that inhibit endosomal acidification. The partial protection observed with these drugs was an indication that HdCDT requires passage via an intracellular low-pH compartment, suggesting that the toxin was internalized by endocytosis.

Endocytosis can take place via clathrin coated-pits. To determine if this was the case with HdCDT, three different experimental approaches were used. i) Removal of clathrin coats by K⁺ depletion (70). The HdCDT effect was reduced 40-80% (Paper III, fig.3). Diphtheria toxin (DT) was used as a positive control since it had been reported to enter cells via clathrin coated pits (70, 106). The reduction of the toxic effect of DT in potassium-depleted cells, was quantitatively similar to the reduction of the HdCDT-induced effect. ii) Treatment with drugs known to inhibit receptor clustering in coated pits. HEp-2 cells were exposed to the cationic amphiphilic drug chlorpromazine (108). The intoxication in such treated cells was decreased by 80% (Paper III, fig. 3). iii) The third approach was to use a cell line genetically manipulated to fail in its endocytosis via clathrin coated pits. The importance of dynamin in clathrin-mediated endocytosis is well established (23, 96). It is needed for the constriction of coated pits and subsequent
budding of the coated vesicles. Overexpression of dominant negative mutant dynamin was shown to specifically block endocytic clathrin-coated vesicle formation although the fluid phase uptake continues in these cells (23). Therefore, to corroborate the clathrin dependent uptake of HdCDT we used HeLa cells stably transfected with dominant negative dynamin (dyn<sup>K44A</sup>). Cells cultivated in medium with tetracycline, allowing expression only of endogenous dynamin, were fully sensitive to the toxin as shown by the tyrosine phosphorylation of cdc2 (Paper III, fig.4). In the absence of tetracycline these cells overexpress the dyn<sup>K44A</sup>, the clathrin-dependent pathway is inhibited (23) and the toxic effect of HdCDT was prevented (Paper III, fig.4).

3.3.2 <i>HdCDT requires transport from early endosomes to downstream vesicular compartments</i>

The next step was to clarify whether the toxin can be translocated to the cytosol directly from early endosomes or needs to be transported to some downstream compartment(s) before being able to act. Three different treatments were used: i) Bafilomycin A1 (BafA1), which is a specific inhibitor of vacuolar proton ATPases, and is known to raise the vesicular pH and block protein transport from early to late endosomes (11). ii) The microtubule-disrupting agent nocodazole which is known to block the fusion of endosomal carrier vesicles with downstream compartments, such as late endosomes, lysosomes or the Golgi complex (11). iii) Incubation of cells at 18°C which is another treatment known to block the transport of proteins from endosomes to the lysosomes and/or the Golgi complex (92). In all these conditions, the cellular intoxication by HdCDT was completely inhibited (Paper III, fig.5).
3.3.3 An intact Golgi complex is required for the intoxication

Many bacterial toxins need to be transported to the Golgi complex and ER before translocation to the cytosol. To determine if this is the case for HdCDT, we used brefeldin A (BFA). This drug disrupts the Golgi complex with redistribution of its proteins to the ER as well as inhibition of vesicular transport from the ER to the Golgi (15). It is well established that the cytotoxicity of toxins internalized via the Golgi complex is inhibitable by BFA. When HEp-2 cells were treated with BFA before and after toxin exposure, the intoxication was completely inhibited as scored by flow cytometric analysis after 6, 8 and 12 hours (Paper III fig.6A-C) and as a lack of cdc2 tyrosine phosphorylation. Since BFA has been reported to interfere also with the cell cycle (68) an additional experiment was performed to ensure that the inhibition by this drug was actually due to disruption of the Golgi complex. BFA was not added until 1 hour after a 15 min toxin exposure and in this case it did not interfere with the intoxication (Paper III fig.6D). This implies that internalisation was the step prevented by BFA as shown in fig.6A-C. Our observations with BFA were corroborated with Ilimaquinone, another drug causing fragmentation of Golgi membranes and their dispersion throughout the cytoplasm (72, 112). The actions of BFA and Ilimaquinone differ in that the latter does not induce a retrograde transport of Golgi enzymes to the ER (112). Thus, two drugs that disrupt the Golgi complex in differing ways inhibited the HdCDT-induced intoxication.

In conclusion, all these results support the notion that HdCDT undergoes clathrin-dependent endocytosis and vesicular transport at least to the Golgi complex before it can induce cell cycle arrest. So far, this is the only study on cellular internalization of a CDT but the pathway described is likely to be common for all CDTs.
All toxins previously found to enter via the Golgi complex have been either implied to or actually demonstrated to be transported also to the ER before being translocated to the cytosol. Therefore, it is conceivable that HdCDT follows the same pathway and undergoes retrograde transport to the ER. Moreover, since DNA is the target of CDTs, they must reach the nucleus. This presents two possibilities: (i) the toxin is released from the ER to the cytosol and transported to the nucleus, or ii) the toxin is directly delivered from the ER to the nucleus. In either case, it will represent a completely new mechanism of protein toxin internalization.

Microinjected or electroporated CdtB from various bacteria is cytotoxic, indicating that at least this component of the toxin might not require any chemical processing during cellular internalization, and apparently it can be transferred from the cytosol to the nucleus. However, it is not yet known which route into the nucleus is taken when the holotoxin is added extracellularly and follows the described pathway to the Golgi.

3.4 HdCDT-induced cytoskeletal effects (papers I and IV)

3.4.1 *HdCDT promotes actin stress fibers formation*

Cell intoxication was associated with actin rearrangements in HEp-2 and Don cells (Paper I, fig.3). This effect was characterized by the promotion of stress fibers. A similar pattern was observed for hamster ovary (CHO) cells intoxicated by *E. coli* CDT-II (6). To study small GTPases we used two glucosyltransferase toxins, TcdB and TcsL, produced by *C. difficile* and *C. sordelli*, respectively. TcdB targets are Rho, Rac and Cdc42 and TcsL attacks Ras, Rap, Ral and Rac (25, 45). In lysates from HdCDT-treated cells, TcdB and TcsL were able to fully glucosylate all their substrates and no changes were detected.
in electrophoretic mobility or in the extent of GTPase glucosylation (Paper I, fig.4). These observations suggested that no covalent modification had taken place and seemed to exclude a direct effect of HdCDT on these proteins.

3.4.2 

HdCDT intoxication activates Rho

The reason for the promotion of the ACSK in HEp-2 cells and hamster lung Don fibroblasts by HdCDT was still not clear. It was not known how this promotion of stress fibers was regulated or related to the DNA damage induced by CDTs. Therefore our study of small GTPases was continued in more detail. HeLa cells were transfected with dominant negative and dominant positive mutants of Rho, Rac and Cdc42 and the HdCDT-induced actin cytoskeleton rearrangements were observed. In HeLa cells transfected with mutants of Rac and Cdc42, no evident change of response to HdCDT was detected. However, in cells transfected with dominant negative Rho, the toxin-induced promotion of actin stress fibers was clearly prevented (Paper IV, fig.1 Panel A).

To further elucidate the role of Rho, Rac and Cdc42 we used a recently developed affinity binding assay to detect the active form of the protein in lysates from intoxicated cells. The assay is based on the ability of the GTP-bound, but not the GDP-bound form to bind to a specific domain. GTP-Rac and GTP-Cdc42 bind to the p21-binding domain (PBD) of p21-activated kinase (PAK) and GTP-Rho binds to the Rho-binding domain (RBD) of the effector protein Rhotekin (12, 86). We used as positive control CNF, a toxin known to activate GTPases of the Rho family (34, 96). HdCDT did not induce any increases of GTP-Rac or GTP-Cdc42. In contrast RhoA was activated in HdCDT-treated HeLa cells already two hours after addition of the toxin. At 24 hours post-intoxication, there was a small decrease in this activation (Paper IV, fig. 2B). Rho activation was also
detected in human foreskin (HFS) fibroblasts but not in the SN-B1 lymphoblastoid cell line (LCL) (Paper IV, fig3A,C). This result suggested that Rho activation is a cell type dependent event and not a general effect of the toxin directly on Rho.

3.4.3 Activation of Rho is related to the DNA damage induced by the toxin

To highlight the association between DNA damage and Rho activation, we studied the effect of ionizing radiation (IR) on the ACSK. Irradiated cells showed a promotion of actin stress fibers as well as a cell distention comparable to that seen in HdCDT-treated cells (Paper IV, fig.4A). We could detect an early activation of RhoA (2h) followed by a decrease (Paper IV, fig.4B), which is a time course similar to the one observed in toxin-treated cells. Moreover, transfection of HeLa cells with dominant negative RhoA prevented the stress fiber promotion in irradiated cells (Paper IV, fig.5 a,c). This result is consistent with our previous findings that HdCDT induces a cellular response resembling the one induced by IR (Paper II).

Likewise, the promotion of actin stress fibers induced by hydroxyurea (HU) treatment apparently requires Rho, since it was also inhibited in HeLa cells transfected with dominant negative Rho (Paper IV, fig.5 b,d). HU blocks DNA synthesis by reducing the intracellular levels of deoxynucleoside triphosphates. This agent has been shown to arrest HeLa cells exclusively in the G1/S phase (63), thus evoking a checkpoint response differing from that induced by HdCDT and IR.

An interesting observation was that cell distention occurred also in the absence of stress fiber-promotion, as can be clearly observed in fig.1 (Paper IV). Moreover, the stress fiber promotion which occurred after transfection with the dominant positive Rho was not
associated with cell distention in the controls whereas distention occurred as usual in the HdCDT-treated cells (Paper IV fig1: Panel B). Thus, the distention phenomenon induced after DNA damage is separable from the Rho-activated stress fiber promotion and the reason for the distention is still not understood.

Our data demonstrated that the toxin-induced promotion of the actin cytoskeleton, requires the small GTPase RhoA but not Rac or Cdc42. This study also suggests that RhoA activation is related to the DNA damage induced by the toxin. It remains to be clarified whether this general activation of Rho is part of the cell cycle checkpoint responses. The fact that RhoA is activated in two cell lines which respond to HdCDT with arrest at different phases of the cell cycle (HeLa cells and HFS fibroblasts), suggests that a component upstream the specific checkpoint responses may be involved. Alternatively, Rho activation could be evoked along a completely separate signalling pathway, activated directly at the level of DNA in parallel with the activation of checkpoint responses. This hypothesis is supported by our observation that in human foreskin fibroblasts activation of p53 was not detectable before the GTP-RhoA, as well as by the early appearance of the RhoA activation. Both these results suggest a separate pathway activated by the induction of DNA damage.
4. CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 Mode of action of CDTs

The understanding of the novel toxin family of CDTs is just in its beginnings, although a significant development in the field has been made during the past two years. Our studies with HdCDT have provided new information on different aspects of this toxin, therefore making an important contribution to the field. Based on our results and the recent finding that CdtB has DNAse-I like activity (29, 36, 57) (Frisan, unpublished results) we can establish a model for the mode of action of HdCDT (figure 5).

HdCDT binds to an unknown receptor, is internalized via clathrin-coated pits and is transported to the nucleus via the Golgi complex. Once in the nucleus, the toxin causes a subtle DNA damage as detected by the re-localization of the DNA repair complex Mre11 (Frisan, unpublished results). Subsequently, the activation of DNA damage checkpoint responses leads to: i) cell cycle arrest and apoptosis and ii) activation of RhoA with promotion of the ACSK. ATM is involved in the early response after intoxication, however the checkpoint response could be activated in absence of a functional ATM. At the moment it is not known which component(s) might transmit the signal from DNA damage to Rho activation.

Several questions related to the mode of action of CDTs remain to be answered, for instance, the composition of the holotoxin. Evidence suggests that a modification of CdtC is required for intoxication, and that CdtA is also needed to have an active holotoxin. One possibility is that CdtC is the binding component which is modified by CdtA. However this remains to be clarified. Though we described some steps of the internalization of HdCDT, the route of entrance of CDTs is not completely clear. Some toxins use more
than one pathway to enter the cells. The possibility that HdCDT uses also a clathrin-independent pathway could not be excluded. The identity of the receptor is unknown. The compartment from which the toxin is translocated or how it reach the nucleus is unknown as well. The ability of intracellular CdtB to induce cell cycle arrest, suggests that no activation is required when the toxin is internalized. However, it can not be excluded that the holotoxin has to be processed by proteolysis as is the case with several other bacterial toxins. We described the activation of DNA damage checkpoint responses. An interesting point that remains to be clarified is the signalling pathway from DNA damage to Rho activation.
Figure 5: Proposed model for HdCDT intoxication

Endocytosis (clathrin dependent)

Holotoxin ?

Early endosome

Late endosome

Golgi complex

ER

Nucleus

CdtB-mediated DNA damage

Cell cycle arrest  RhoA activation  Re-localization of the DNA repair complex Mre11

Apoptosis
4.2 CDTs as tools in cell biology

Bacterial toxins have contributed to the study of different aspects of cell biology, sometimes leading to the discovery of important new pathways in cellular signalling. As an example, various toxins that affect small GTPases have been used for the functional characterization of these proteins (113). Similarly, tetanus toxin is an endopeptidase which acts specifically on a membrane protein localized on synaptic vesicles and secretory granules. This finding was essential in the definition of the molecular mechanisms of regulated secretion (95).

So far, CDTs are the only bacterial protein toxins known to induce a subtle DNA damage, thereby interfering with the cell cycle. In the field of cell and tumor biology, regulation of the cell cycle is currently one of the major issues and the use of cytotoxic prodrugs has been considered for cancer gene therapy. Attention has been focused on the Herpes Simplex virus-thymidine kinase (HSV-tk) gene, which induces single strand breaks in synthesised DNA in the presence of the nucleoside analogue ganciclovir (35, 85). The murine melanoma cell line B16F10 transfected with the HSV-tk gene undergoes irreversible G2/M arrest and cytoskeleton reorganisation when treated with ganciclovir (39). HdCDT produces very similar effects in toxin sensitive tumour cell lines and could potentially be used as anti-cancer therapy. Understanding the detailed molecular mode of action of the CDTs will also help to create a new useful tool for studying the cell cycle control mechanisms.

Studies with Shiga toxin indicated for the first time that a molecule can be transported from the cell surface to the Golgi apparatus and the ER (94). The mechanisms working in
this retrograde route are still not fully characterized (116). Moreover, the communication routes between the endocytic and the biosynthetic pathways, are still poorly understood (48). CDTs might be useful in clarifying these trafficking pathways. Even more interesting is the fact that the nucleus is the final destination of CDT. Once the internalization pathway of HdCDT is well characterized, it may represent a new tool for studies of protein transport from the ER to the nucleus.

Because of the participation of small GTPases in multiple intracellular pathways at the membrane/cytosolic and nuclear levels, the details of the regulation of some of these pathways are still not clear. Indeed, the crosstalk between molecules that control the cytoskeleton and those that control cell cycle progression/arrest is not well understood (7, 8). HdCDT may become a useful tool to study the relation between small GTPases and molecules involved in the control of the cell cycle and DNA damage responses.
1. Modification of the cytoskeleton
2. Inhibition of membrane fusion
   Vesicular trafficking
3. Membrane permeabilization
   Modification of membrane components
4. Interfering with signal transduction

CELLULAR PROCESSES TARGETED BY BACTERIAL TOXINS
Adapted from Schiavo and Van der Goot Nature Reviews, 2001
Las toxinas citoletas distensivas (cytolethal distending toxins, CDTs) forman una familia de toxinas bacterianas que presentan un mecanismo de acción nuevo: daño al ADN. A esta familia pertenece una citotoxina producida por Haemophilus ducreyi. Esta bacteria es el agente causal del chancro blando, una enfermedad de transmisión sexual caracterizada por la presencia de úlceras mucocutaneas genitales de lenta curación. Como todas las enfermedades que presentan úlceras genitales, el chancro es un factor predisponente para la transmisión del virus de inmunodeficiencia adquirida (VIH). La patofisiología de la enfermedad es poco conocida y la citotoxina de Haemophilus ducreyi (HdCDT) representa un posible factor de virulencia en el desarrollo de la enfermedad.

Al principio de nuestros estudios con HdCDT, poco se conocía sobre esta familia de toxinas. Para comprender el mecanismo de acción de esta toxina, se comenzó un estudio sobre la intoxicación en células mamíferas. El efecto morfológico inducido por esta toxina se estudió en células de origen epitelial y en fibroblastos de hamster. La intoxicación es irreversible y aparece como una distensión gradual de la célula, seguida por muerte celular. Al mismo tiempo que la distensión, se observa una promoción de fibras de actina (actin stress fibers). Como se había observado en otras CDTs, se demostró que las células HEP-2 intoxicadas con HdCDT son bloqueadas en la fase G2 del ciclo celular debido a una acumulación de la forma fosforilada (inactiva) de la quinasa ciclina dependiente cdc2.

Para obtener una mejor caracterización del modo de acción de la HdCDT se probaron varias líneas celulares de origen humano. Se pudo demostrar que el efecto de esta toxina es específico para cada tipo de célula y no exclusivamente relacionado a bloqueo en la fase G2 del ciclo celular. Las células B entran en apoptosis, las células epiteliales y los queratinocitos se bloquean exclusivamente en la fase G2 mientras que los fibroblastos normales se bloquean en las fases G1 y G2. Además, se demostró que la respuesta inducida por la HdCDT es similar a la respuesta de punto de control (checkpoint) activada por radiación ionizante. Ambas respuestas se caracterizan por una inducción temprana del gen p53 y del inhibidor de quinasas ciclina dependientes p21 en fibroblastos humanos y por la activación de la quinasa chk2 en células HeLa. Nuestro trabajo también sugiere que ATM, una molécula clave en la detección de daño celular, es necesaria para la respuesta temprana inducida por HdCDT. Sin embargo, en ausencia de ATM funcional, el punto de control puede ser activado más tarde, probablemente por un homólogo como ATR. Se demostró también que la promoción de fibras de actina inducida por HdCDT es dependiente de la activación de Rho. Nuestros observaciones señalan una relación entre la activación de Rho y el daño al ADN.

Finalmente se demostró que todos estos efectos ocurren después de la internalización celular de la toxina y se clarificaron algunos de los pasos en el recorrido intracelular de HdCDT. Se demostró que la toxina ingresa en las células por medio de vesículas cubiertas de clatrina y que se necesita de un complejo de Golgi intacto para poder lograr una intoxicación celular.

En conclusión, los datos recopilados en este trabajo han contribuido a entender el modo de acción de esta familia de toxinas.
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