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MiniReview

Cytolethal distending toxin (CDT): a bacterial weapon to control host cell proliferation?

Jean De Rycke *, Eric Oswald

UMR 960 de Microbiologie Moléculaire, Institut National de la Recherche Agronomique et Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles, 31076 Toulouse, France

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Abstract

Cytolethal distending toxins (CDT) constitute a family of genetically related bacterial protein toxins able to stop the proliferation of numerous cell lines. This effect is due to their ability to trigger in target cells a signaling pathway that normally prevents the transition between the G2 and the M phase of the cell cycle. Produced by several unrelated Gram-negative mucosa-associated bacterial species, CDTs are determined by a cluster of three adjacent genes (*cdtA, cdtB, cdtC*) encoding proteins whose respective role is not yet fully elucidated. The CDT-B protein presents sequence homology to several mammalian and bacterial phosphodiesterases, such as DNase I. The putative nuclease activity of CDT-B, together with the activation by CDT of a G2 cell cycle checkpoint, strongly suggests that CDT induces an as yet uncharacterized DNA alteration. However, the effective entry of CDT into cells and subsequent translocation into the nucleus have not yet been demonstrated by direct methods. The relationship between the potential DNA-damaging properties of this original family of toxins and their role as putative virulence factors is discussed.

Keywords: Toxin; Phosphodiesterase; Cell cycle; DNA damage; G2 checkpoint

1. Introduction: initial characterization of CDT activity in cell cultures

An original distending and lethal cytopathic activity in cultured CHO, Vero, HeLa and Hep-2 epithelial cell lines was demonstrated in the late 1980s in culture supernatants of clinical isolates of *Escherichia coli* and various *Campylobacter* spp. [1]. Later on, the cytopathic effect of cytolethal distending toxin (CDT) was described according to criteria used in cellular biology. It was found that cultured epithelial cells exposed to CDT are irreversibly blocked at stage G2 of the cell cycle, i.e. before entry into mitosis [2,3] (Fig. 1). In fibroblasts and keratinocytes a block in both G2 and G1 has been observed [4]. In this non-proliferative state, surviving cells enlarge for several days and acquire the morphology of giant mononucleated cells. In some epithelial cell lines, such as CHO and Hep-2, CDT also trigger a rearrangement of the actin cytoskeleton [5,6]. In epithelial cell lines exposed to CDT, lethality starts to be significant only after 2 days of incubation, a feature that clearly distinguishes CDT from true cytotoxins, such as Shiga-like toxins that kill cells sooner after exposure. In HeLa epithelial cells exposed to CDT, lethality results from abortive alleviation of the G2 block leading to mitotic death, and the formation of non-viable micronucleated cells [7]. In lymphocytes, the effect of CDT is somewhat different in that no significant increase in size is observed and that a proportion of cells die from apoptosis [4,8].

2. Molecular heterogeneity and dispersion of the CDT family

CDTs are present in a large spectrum of Gram-negative bacterial species, all known as potential mucosal pathogens in man and animals, including *E. coli* [2,3,9,10], *Shigella dysenteriae* [11], *Campylobacter* species such as *C. jejuni*, *C. fetus*, *C. coli* [12–15], *Helicobacter* species such as *H. hepaticus* [16,17] and *H. pullorum* [18], *Actinobacillus actinomycetemcomitans* [19–21], and *Haemophilus ducreyi*

* Corresponding author. Tel.: +33 (5) 61 19 38 85; Fax: +33 (5) 61 19 39 75.
E-mail address: j.derycke@envt.fr (J. De Rycke).

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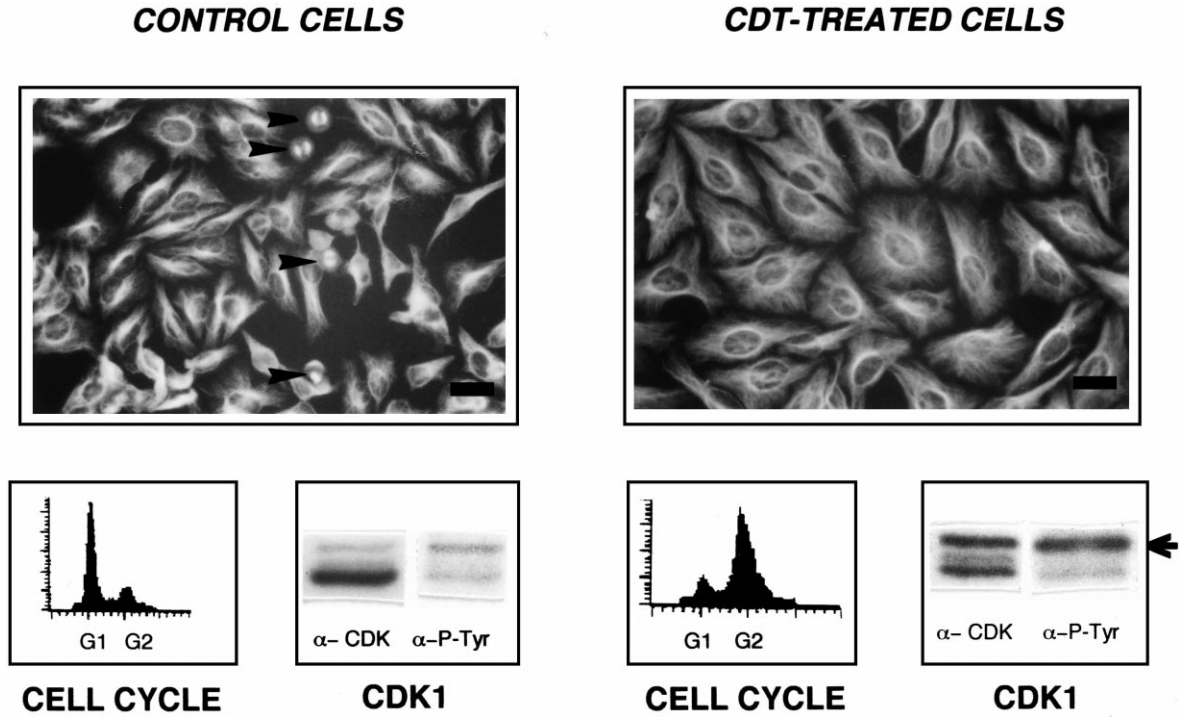


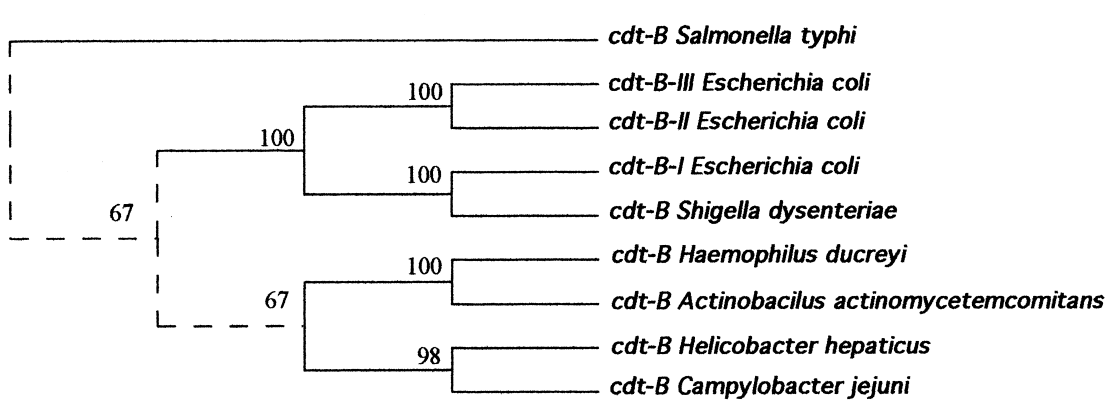
Fig. 1. Cytopathic and cell cycle effect in HeLa cells exposed to an *E. coli* CDT-I crude preparation. Upper panel: Fluorescence microscopy of control (not exposed) and CDT-exposed cells (24 h) after indirect immunofluorescence staining of α-tubulin. Note the absence of mitotic figures and the enlarged size in CDT-treated cells. Mitotic figures are shown in control cells. Bar = 10 μm. Lower panel: Cell cycle distribution by flow cytometry analysis (left) and relative concentration of CDK1 phosphorylated isoforms as shown in SDS–PAGE immunoblotting (right) in control and CDT-exposed cells (after 24 h). After 24 h of exposure a majority of CDT-treated cells are arrested in the G2 stage of the cell cycle (4n chromosomes). The inactive slowly migrating, tyrosine-phosphorylated form of CDK1 is concentrated in CDT-treated cells as compared to control cells.

[22,23]. In *E. coli*, three different types of CDT have even been described so far [2,9,10], one of them, CDT-III, being encoded by a large plasmid [2].

The genes encoding CDT have been cloned and sequenced in several bacterial species. CDT activity is encoded by three closely linked genes termed *cdtA*, *cdtB* and *cdtC*. Transcript analysis carried out for the *cdt* genes of *H. ducreyi* suggests that *cdtA*, *cdtB* and *cdtC* are organized into a single operon [22]. The expression of all three genes is required for CDT activity and the three genes encode proteins with a consensus leader sequence for secretion [9,10]. It is worth noting, however, that recently released sequencing data indicate that the chromosome of *Salmonella typhi* and that of *S. paratyphi* A contain a *cdtB* gene without the expected *cdtA* and *cdtC* partners (Genome sequencing center of Washington University and University of Wisconsin-Madison Genome Project). This gene is downstream of *mgsA*, which encodes a protein involved in the survival of *S. typhimurium* in macrophages [24]. The presence in certain CDT proteins, such as *E. coli* CDT-IIIc [2], of a conserved consensus sequence recognized by signal peptidase II suggests also that some of these proteins could be typical membrane lipoproteins. The predicted molecular masses of the mature secreted proteins are 22–27 for CDT-A, 27–29 for CDT-B and 18–19 kDa for CDT-C. The homologies between different CDT-B proteins are always higher than the homologies between the CDT-A and CDT-C proteins. The phylogenetic relationships between *cdt-B* alleles are illustrated in Fig. 2. There clearly exist at least four different lineages of *cdt-B* alleles. The presence of *cdt* genes in different bacterial species and the analysis of the DNA in the vicinity of the *cdt* genes suggest that the toxin has been acquired from heterologous species by horizontal transfer. Indeed, near the plasmid-encoded *E. coli cdt-III* genes we found sequences that appear to be phage and insertion sequence remnants [2]. Similarly, sequences near *cdt* genes in *A. actinomycetemcomitans* are related to a bacteriophage *att* site, and to sequences previously identified in different plasmids [19,21]. Transposase genes have also been found near the *H. ducreyi cdt* genes [22]. However, such sequences are not always present near the *cdt* locus, for instance in *C. jejuni*, the *cdt* locus is between a gene encoding a lactate permease and a gene encoding a cytochrome oxidase [25].

Since CDT-B is the most conserved protein, it has been postulated that it could be the catalytic unit of the toxin, and its possible homologies with known enzymes were then examined. Comparison of the CDT-B amino acid sequence with known three-dimensional structures using the hybrid fold recognition method (D. Fischer, Hybrid fold recognition: Combining sequence derived properties with evolutionary information. Pacific Symp. Biocomputing, Hawaii, January 2000, pp. 119–130) shows that CDT-B has the best compatibility score with human DNase I,

(A) Phylogenetic tree of *cdt-B*



(B) Percentage of amino acid similarities in the different CdtB

	CDT-B-St	CDT-B-Ec-III	CDT-B-Ec-II	CDT-B-Ec-I	CDT-B-Sd	CDT-B-Hd	CDT-B-Aa	CDT-B-Hh	CDT-B-Cj
CDT-B-St	100%	58%	58%	59%	59%	66%	66%	61%	60%
CDT-B-Ec-III		100%	95%	71%	71%	63%	63%	62%	64%
CDT-B-Ec-II			100%	70%	70%	63%	63%	61%	62%
CDT-B-Ec-I				100%	100%	66%	64%	62%	66%
CDT-B-Sd					100%	66%	64%	62%	66%
CDT-B-Hd						100%	97%	67%	66%
CDT-B-Aa							100%	67%	67%
CDT-B-Hh								100%	72%
CDT-B-Cj									100%

Fig. 2. A: Relationships between the *cdt-B* alleles present in different pathogenic bacterial species. The bacterial origin of these alleles is noted on the right. The evolutionary relationships inferred from the *cdt-B* structure are represented by the dendrogram, which is derived from the nucleotide differences between *cdt-B* alleles. The neighbor joining method was used and the nucleotide distances were calculated with the method of Tamura-Nei. The consensus bootstrap (10 000 reps) tree is shown. The *cdt-B* alleles can be grouped in four different clusters but the low bootstrap values (below 70) did not allow us to clearly link these clusters together in the phylogenetic tree. B: The percentage of amino acid similarities between the different mature CDT-Bs was calculated from a pairwise alignment using the blosum matrix. Although no clear phylogenetic link could be demonstrated at the nucleotide level the CDT-B of *S. typhi* (like the CDT-B of *S. paratyphi* A, not shown) seems more closely related at the protein level to the CDT-B of *H. ducreyi* and *A. actinomycetemcomitans* than to the CDT-B of *E. coli* or *S. dysenteriae*. These results may suggest that *cdt-B* of *Salmonella* is part of a particular genetic lineage in which the products of the *cdt-A* and *cdt-C* genes may not be needed for the activity on eukaryotic cells.

human DNA repair endonuclease Hap1, exonuclease III from *E. coli* and also with certain bacterial sphingomyelinases [26] and inositol phosphatases [27]. Alignment of the different CDT-Bs shows the conservation of functionally important residues, involved in the catalytic, metal ion binding or substrate binding of several diphosphodiesterases such as the human DNase I and the sphingomyelinase (SMase) from *Bacillus cereus* (Fig. 3).

Mutation of some of these putative critical residues in CDT-B abrogated the cytological effect of the toxin, which shows unambiguously that they participate in the enzymatic activity of the toxin [28,29]. However, the residual

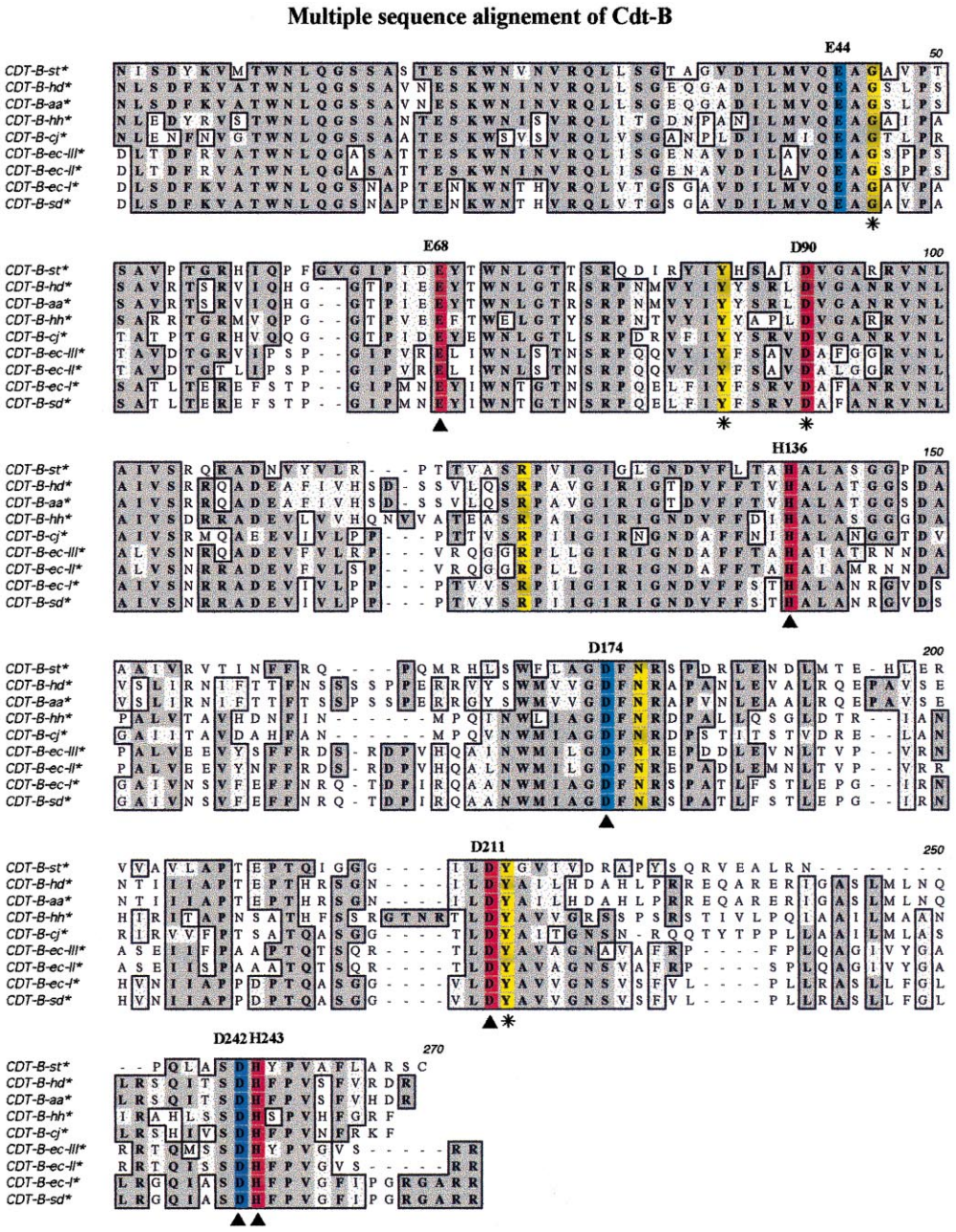


Fig. 3. Sequence alignment of CDT-Bs from several bacterial species. The predicted three-dimensional structure of CDT is highly compatible with the structure of bacterial SMases, DNase I from different mammalian species, and apurine/apyrimidine base excision repair endonucleases such as *E. coli* exonuclease III. Despite the lack of overall sequence identity (less than 15%), most of the catalytic and metal ion binding residues are conserved between these different phosphodiesterases. The same residues are also important for the activity of the inositol polyphosphate 5-phosphatases, another family of phosphodiesterases [27]. In this multiple sequence alignment with the different mature CDT-Bs, residues common to these different phosphodiesterases are highlighted. The putative catalytic residues are in red. The putative residues important for metal ion binding are in blue. The putative substrate contact residues are in yellow. The asterisks indicate residues that are not always conserved in the other phosphodiesterases. Arrows indicate residues that were mutated and shown to completely or partially abolish the cell cycle arrest activity of CDT [28,29]. In conclusion, CDT-B and the different classes of phosphosdiesterases share a common mechanism for catalysis that may have evolved from a common ancestor employing the same residues for catalysis. Based on this homology, a suggested mechanism for CDT-B activity is that H136 and H243 provide general acid and general base catalysis, respectively. Two magnesium ions (coordinated to E44 and D174) neutralize the charges on the non-bridging oxygen atoms of the scissile phosphate and provide electrophilic catalysis. st*, *S. typhi*; hd*, *H. ducreyi*; aa*, *A. actinomycetemcomitans*; hh, *H. hepaticus;* cj, *C. jejuni*; ec*, *E. coli*; sd*, *S. dysenteriae*.

activity observed in the E68 residue mutant [28] would suggest that this residue is not as critical for CDT as it is for mammalian DNase I homologues and that the catalytic residue that bonds to H136 is not E68 to form the catalytic domain but is more likely the asparagine D90, as is the case for *S. aureus* and *B. cereus* SMases [26]. Although the exact nature of CDT-B enzymatic activity and the range of its potential substrates are not yet fully

elucidated, purified CDT-B was shown to exhibit a DNA-nicking activity on supercoiled plasmid DNA [30]. Complete digestion of DNA was also previously reported in unpurified preparations of the whole toxin or of CDT-B [28]. In the light of this new result, this lytic effect may be interpreted as a possible potentiation by CDT-B of a endonuclease activity naturally present in unpurified extracts.

3. Induction by CDT of a G2 cell cycle control pathway

The ability of CDT to induce a G2 block in exposed cells is reminiscent of the mode of action of DNA-damaging agents that activate a cycle checkpoint in G2 called the DNA damage cascade. Cell cycle checkpoints are complex regulatory cascades, highly conserved in eukaryotic cells, that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. Several G2 checkpoint cascades have been described which all end up in the maintenance of the cellular cyclin-dependent kinase CDK1, a key effector in the physiological transition from G2 to mitosis, in an inactive tyrosine-phosphorylated form [31]. CDK1 is inactivated by tyrosine phosphorylation in epithelial cell lines exposed to the CDTs produced by *E. coli* [3], *C. jejuni* [32], and *H. ducreyi* [6], and in human peripheral T cells exposed to CDT produced by *A. actinomycetemcomitans* [20] (Fig. 1). CDK1 is not, however, directly inhibited by CDT since it can be fully reactivated in vitro [33] and in cellulo [34] by the CDC25 phosphatase, an effector immediately upstream of CDK1 in the G2 control cascade (Fig. 4). Inhibition of CDK1 activation by CDT is probably the ultimate event of a complex pathway involving specific effectors of the DNA damage cascade. One postulated effector has been identified: CHK2 kinase [4,35]. When activated, CHK2 phosphorylates the CDC25C phosphatase on serine 216, which in turn causes CDC25C to be sequestered in the cytoplasm, thus unable to activate CDK1 (Fig. 4).

Activation of a G2 cascade in target cells upon exposure to CDT suggests that CDT is able to induce some form of genomic alteration. This hypothesis appears consistent with the fact that CDT-B contains an enzymatic motif with distant homology to phosphodiesterase enzymes such as DNase I, as reported above [28,29] (Fig. 3). In vitro, the existence of a DNase activity has indeed been reported in crude supernatants of a laboratory *E. coli* strain expressing CDT [28]. In exposed cells, no early DNA alterations could be detected using single gel cell electrophoresis, also called the comet assay [33], an observation which does not support the hypothesis of an early in vivo nuclease activity. In the same way, CDT, in contrast to most DNA-damaging agents, does not induce a detectable delay in S phase progression [3,33]. This observation would also argue against the induction by CDT of early DNA strand breaks. In contrast, when microinjected into cells at high concentration, CDT-B alone was able to cause chromatin disruption [29]. It is not easy to reconcile these apparently contradictory observations, but one can speculate that CDT-B nuclease activity is mild and not easily detectable by conventional methods, or that it is restricted to a certain conformation of the replicative DNA, as suggested by the fact that G2 checkpoint activation by CDT is strictly dependent upon cell transit through the S phase [3,33].

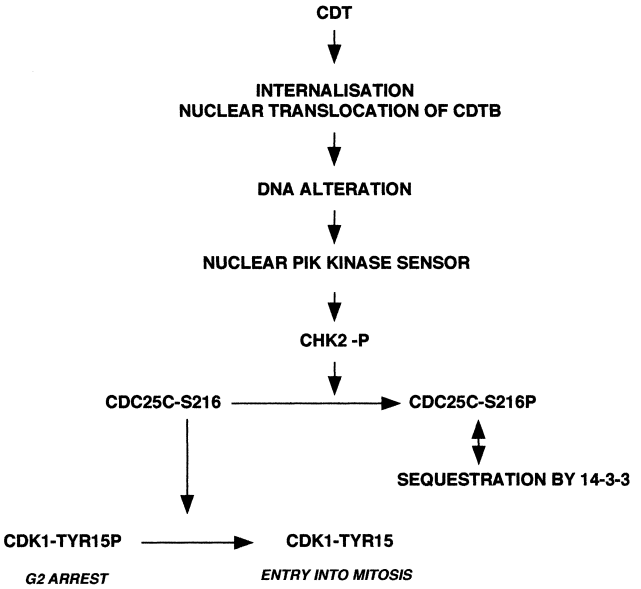


Fig. 4. Activation of a G2 checkpoint control by CDT in epithelial cells: a tentative model. CDT would be internalized by a clathrin-dependent pathway and processed through the Golgi [4], then CDT-B might be translocated to the nucleus, bind to the DNA and induce a specific DNA alteration. This putative damage activates the kinase CHK2, through as yet unidentified upstream effectors, and consequently the cytoplasmic sequestration of CDC25C phosphatase by 14-3-3 protein. The nuclear unavailability of CDC25C prevents the activation of CDK1 by tyrosine dephosphorylation of serine 216. Cells are then eventually prevented to enter mitosis and remain blocked in the G2 stage of the cell cycle.

If CDT-B does exert a direct effect on nuclear DNA, then it should be translocated into the nucleus. This property is suggested by the fact that COS-1 cells transfected with a plasmid encoding tagged CDT-B exhibited exclusive nuclear localization [29]. To our knowledge, there is currently no published example of other bacterial toxic proteins naturally translocated into the nucleus of target cells. The modalities of CDT entry into the cytoplasm have been approached in a study using inhibitors of defined stages of internalization [36]. This study shows that CDT activity is dependent upon a functional clathrin-coated pits pathway and on the integrity of the Golgi, which suggests, but does not prove, that the toxin uses these pathways. There is as yet no published direct demonstration of the effective binding or trafficking of the

toxin in target cells, nor any data on the role of CDT-A and CDT-C in these processes. The role of CDT-A and CDT-C is possibly restricted to binding and internalization and would be dispensable in specific situations where other internalization mechanisms are present in the bacteria, as suggested by the existence of orphan *cdtB* genes in *S. typhi* and of *S. paratyphi* (Section 2 and Fig. 2).

4. CDT as a potential virulence factor

The powerful antiproliferative effect of CDT, observed in vitro in various epithelial cell lines and immune primary cells, could affect in priority epithelia, which are characterized by a rapid renewal from crypt stem cells and by the presence of enormous numbers of intraepithelial lymphocytes. If effective in situ, inhibition of epithelial renewal could prolong local colonization of bacteria, which are normally shed along with epithelial cells to which they are bound. Likewise, the profound cell alteration could lead to significant changes in the distribution of cell surface receptors, possibly enhancing the adhesion and invasiveness of bacterial organisms. Eventually, the lack of epithelium renewal would produce epithelial lesions altering irreversibly the functional capacity of the tissues or facilitating bacterial invasion of underlying tissues. Inhibition of the activation of local lymphocytes would also logically impede the capacity of the host to control bacterial colonization or invasion. However, to our knowledge, there is currently no published information indicating that the antiproliferative effect of CDT has ever been examined in vivo. There is notwithstanding clear evidence that *A. actinomycetemcomitans* CDT is able to inhibit the function of peripheral human T and B cells isolated from blood of healthy donors [20]. Moreover, CDT generates a subset of T cells which abnormally expresses both the CD4 and CD8 surface markers [20].

Suggestions that CDT may be a virulence factor have been gathered essentially in murine models. In a suckling mouse model, CDT from *S. dysenteriae* administered orally was shown to induce diarrhea [37]. Although the mechanism leading to diarrhea has not yet been elucidated, the short lag period between toxin administration and the first signs of diarrhea (about 3 h) and the nature of the lesions (necrosis and reparative hyperplasia) do not suggest an antiproliferative effect of CDT in vivo. In another study, the effects of oral administration of a *C. jejuni* strain and of isogenic *cdtB* mutants to adult severe combined immunodeficiency (SCID) mice were compared [38]. Mutant strains were unaffected in enteric colonization ability but displayed an impaired invasiveness into blood, spleen and liver tissues. This study could suggest that CDT may contribute to the invasiveness of the challenge strain but the relation between such an in vivo property and the effect of CDT on the cell cycle is not clear. In contrast, CDT does not contribute to the ability of *H. ducreyi* to cause experimental chancroid in the rabbit skin [39].

5. Conclusions and outlook

Evidence is accumulating that several bacterial species are able to control the eukaryotic cell cycle through specific effectors and mechanisms. This constitutes an original field of research in cellular microbiology, the discipline that studies how microorganisms are able to target and control fundamental mechanisms of eukaryotic cell biology. If CDT appears as only one among various bacterial effectors able to inhibit cell proliferation, its originality resides in its ability to trigger, in various primary cells and cell lines, a physiological cell cycle checkpoint similar to that induced by DNA-damaging agents. The specificity and universality of this activity in cultured cells leads us to think that it bears some relevance in vivo, in relation to either the virulence of the producing organisms or their adaptation to their ecological niche. However, one could also imagine that the cell cycle block induced by CDT is only a secondary effect of the toxin, bearing little relevance per se to microbial pathogenicity. To address more relevantly the role of CDT in microbial pathogenicity, we need to know in particular the precise nature of the enzymatic activity exerted by CDT-B on its substrate(s) in vivo.

Research on CDT is just beginning and several broad areas of research deserving priority can be identified: (1) the role of *cdtA*, *cdtB* and *cdtC* products in the biogenesis, assembly, structure, host recognition and enzymatic activity of CDT; (2) the complete molecular determinism of the CDT G2 blocking activity, including the determination of its primary target, its mode of internalization and possible nuclear translocation, and the identification of the cellular effectors of the G2 checkpoint cascade; (3) the in vivo relevance of the G2 blocking activity of CDT in specified animal models, and more specifically its effect on epithelial cell proliferation and the local immune response. Besides its relevance to bacterial pathogenicity, and possibly carcinogenesis, research on the mode of action of CDT is of interest for cell biologists if, as anticipated, it brings original insights into the mechanisms of cell cycle control, an area of intensive research.

Acknowledgements

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