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Chapter 6

Effects of the Cytolethal Distending Toxin from Gram-Negative Bacteria on Mammalian Cells

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Abstract

The cytolethal distending toxin (CDT) is produced by many pathogenic Gram-negative bacteria (*Escherichia coli, Helicobacter hepaticus, Haemophilus ducreyi* and others) colonizing the oral cavity, the intestinal tract, the liver, etc. Depending on the affected organs, these bacteria are responsible for different pathologies. However, as a virulence factor, CDT has been shown to allow bacteria to persistently colonize the body, evade the immune system, induce inflammation and trigger genetic instability. Moreover, *in vivo*, the production of CDT by *Helicobacter hepaticus* induces the development of dysplastic liver nodules, thus defining CDT as a potential carcinogen. Given the important role of the toxin in the physiopathology of these bacteria, it is essential to accurately identify its mode of action.

In human cells, CDT exposure leads to a singular cytotoxicity associated with characteristic cell distension, and induces cell cycle arrest at the G2/M transition that is dependent on the DNA damage response. Thus, CDT has been classified as a cyclomodulin and a genotoxin, as this toxin induces DNA double-strand breaks. It is therefore important to characterize the outcome of CDT-induced DNA damage and the consequences for the intoxicated cells and organisms. Indeed, the presence of unrepaired damage can lead to cell death, whereas effective repair will allow cells to resume cell cycle. However, improper repair of DNA damage can induce genetic instability and lead to cancer.

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After years of study, the CDT mode of action begins to reveal itself, however, many aspects remain to be identified. In this chapter, we briefly present the bacteria producing CDT as well as their cellular niche. We will discuss CDT trafficking within eukaryotic cells, in order to understand cell entry and nuclear targeting of the toxin. We will then consider host cell behavior in response to CDT intoxication, by focusing on morphological changes, cell cycle modulation and cell death response. Finally, we will examine the biochemistry of CDT, specifically regarding its catalytic activities and relate these aspects to the overall physiopathology of the toxin.

Keywords: Gram-negative bacteria; cytolethal distending toxin; virulence factor; pathogenicity; retrograde traffic; DNA damage response; double-strand breaks; cell cycle checkpoints; apoptosis

Introduction

Human beings are constantly in contact with a huge amount of bacterial agents. Some, non-pathogenic commensal bacteria, are essential for our body, whilst pathogenic bacteria are the source of various diseases. For example, pathogenic bacteria are responsible for a lot of food-borne contaminations. Among the most common bacteria, *Salmonella enterica* induces gastroenteritis, whilst *Salmonella enterica* serovar Typhi is the agent responsible for typhoid fever. *Campylobacter jejuni* causes diarrhea and acute intestinal diseases, and some *Escherichia coli* strains are responsible for gastrointestinal infections, etc. The type of induced disease depends on the bacterial strains capacity to induce a disease (pathogenicity of the strain), and on the location of the bacterial niche. However, infection by a pathogenic bacteria strain does not necessarily imply the development of a disease in infected individuals. In fact, the bacteria pathogenicity depends on several factors: the genetic background of the affected individual, the presence of other bacterial strains in the niche that may act synergistically or antagonistically, and finally the virulence of the bacterial strain itself. Indeed, the virulence of bacteria is the ability to induce damage, directly or indirectly, at the cellular level (DNA damage and cell death) and at the tissular or organ level (inflammation, fibrosis, necrosis...), particularly through the production of virulence factors (Casadevall and Pirofski, 1999).

The production of virulence factors allows bacteria to evade or compromise host defenses, in order to survive and/or exit the host and be transmitted to another. Among them can be distinguished exotoxins and endotoxins. Endotoxins are found within the outer membrane of Gram-negative bacteria and enter the systemic circulation of infected host once the bacteria are killed, whereas exotoxins are usually secreted into the surrounding environment of bacteria. This class of toxins varies considerably in specificity, as some act only on certain cell types while others affect a wide range of cells or tissues. Pathogens usually produce these toxins in an inactive form, activated only after having left the bacteria. Roughly, three types of exotoxins can be considered: cytotoxins that kill cells, neurotoxins interfering with the transmission of the neurological signal and enterotoxins, affecting the digestive epithelium. Many exotoxins consist of A and B moieties. The B moiety is involved in the binding of a receptor found on specific target host cells whereas the A moiety is associated with the interference of host cell metabolism. Moreover, a specific bacterial

pathogen may produce one or more exotoxins. *Helicobacter pylori*, for example, produces different factors, including CagA, VacA, DupA and OipA, and the production of these toxins has an important impact on the related physiopathology. In fact, some strains expressing CagA and VacA are involved in the development of gastric diseases, whilst production of DupA induces duodenal ulcer cancers (Yamaoka, 2011). This demonstrates the key role of virulence factors produced by bacteria and the importance of characterizing and understanding their mode of action.

Cytoletal Distending Toxin Producing Bacteria

Reciprocally, if bacteria can produce several virulence factors, different bacteria can produce a particular virulence factor. One of these virulence factors, inducing the distension and the death of eukaryotic cells, was discovered by Johnson and Lior in 1987. Because of its action, this factor was named cytolethal distending toxin (CDT). In the two years following its discovery, several bacterial strains, obtained from clinical isolates, were characterized as producing CDT, including *Escherichia coli* (*Ec*CDT) (Johnson and Lior, 1987) and *Campylobacter jejuni* (*Cj*CDT) (Johnson and Lior, 1988). In 1994, the genes encoding CDT, produced by *Escherichia coli*, were cloned (Scott and Kaper, 1994; Pickett et al., 1994), which led to the discovery of novel Gram-negative pathogenic bacterial strains producing CDT, through systematic gene detection. Among most characterized are *Helicobacter hepaticus (HhCDT)* (Chien et al., 2000), *Haemophilus ducreyi (Hd*CDT*)* (Cope et al., 1997), *Aggregatibacter actinomycetemcomitans* (*Aa*CDT) (Sugai et al., 1998) and *Salmonella enterica* serovar Typhi (*St*CDT) (Haghjoo and Galán, 2004), although CDT toxin production appears atypical. To date, no Gram-positive bacteria have been characterized to produce CDT.

Implication of the Cytoletal Distending Toxin in the Pathogenicity

Our body constantly coexists with microorganisms. These relationships can be mutualistic or commensal. There is therefore a balance established between the microbiota and the host, particularly at the involved epithelium and regarding the immune system. A change in the presence or amount of a bacterial strain in the microbiota can modify a mutualistic or commensal situation to a host-pathogen relationship (Sansonetti, 2011). To persist and colonize host organisms, pathogenic bacteria must adapt to an ever-changing microenvironment, renewal of the epithelium, competition with other bacterial strains, etc. To do so, bacteria have developed adherence, invasion and survival strategies in response to environmental signals of the host. In addition, pathogens not only have to colonize the host body to induce disease, but also have to evade the immune system, and finally, damage the host, either at the cellular, tissue or organ level. Whatever the case, the pathogenicity of bacteria is dependent on the production of exotoxins, i.e. virulence factors (Finlay and Falkow, 1997).

Virulence factors may be involved in the adhesion of bacteria to eukaryotic cells, invasion, damage of host cells or in the exhaustion of the immune system (Finlay and Falkow, 1997). Indeed, the interaction of a pathogen with eukaryotic cells almost always leads to the activation of host signaling pathways and to an immune response (Sansonetti, 2011). Different lines of defense are implemented by host organisms to deal with pathogens. First, a physical barrier formed by the epithelial cells, and sometimes mucous, prevents the penetration and invasion of bacteria into host tissues. In case of damages and passage of bacteria through the epithelial barrier, actors of innate immunity (macrophages, neutrophils, mast cells) recognize the bacterial antigen (lipopolysaccharide (LPS) for example) and limit the infection through the production of pro-inflammatory cytokines, chemokines, which will attract other immune effectors. Amongst the immune effectors recruited, T- and B-lymphocytes are key actors of the adaptive immune response. This response will among other things, produce specific antibodies against the bacteria or against the virulence factors produced (McGhee and Fujihashi, 2012; Hayashi et al., 2011). In most cases, activation of the immune system will control and stop the infection. Therefore, pathogens must develop mechanisms, and virulence factors, to escape the immune system in order to persist in the body and induce diseases. Among them, the CDT virulence factor has been shown to be expressed in many pathogenic Gram-negative bacteria. Several examples of CDT involvement in the pathogenicity have been demonstrated. Importantly, depending on the producing bacteria and the induced disease, the involvement of CDT in the pathogenicity may be more or less important. However, for all CDT producing strains, CDT production leads to damage and necrosis of the mucosa through destabilization of the epithelial barrier (Damek-Poprawa et al., 2011; Okuda et al., 1997; Pokkunuri et al., 2012; Wising et al., 2005), allowing bacteria to infect the tissue and bypass the first defense system of the host organism. In addition, numerous studies have shown that CDT is highly cytotoxic to cells of the immune system (Gelfanova et al., 1999; Shenker et al., 2006; Shenker et al., 1999). These results suggest that CDT could induce localized immunodepletion during the bacterial infection. Herein are presented examples of bacteria for which the CDT toxin has been found to be involved in disease development.

Escherichia coli

Escherichia coli (*E. coli*) bacterial strains form the major components of the intestinal flora (Tenaillon et al., 2010). These bacteria, commensal most of the time, in some cases may be involved in the induction of diseases (gastroenteritis, urinary tract infections, meningitis). Some studies have investigated the involvement of *Ec*CDT production in these diseases (Van Bost et al., 2003). The involvement and the importance of *Ec*CDT in the pathogenicity of *E. coli* have not been clearly demonstrated. Moreover, CDT is not expressed in all strains of pathogenic *E. coli* (Johnson et al., 2002; Kim et al., 2009; Pandey et al., 2003). Indeed, many studies have investigated the prevalence of CDT encoding genes in *E. coli* strains involved in diarrhea (1.6-2.7% prevalence) (Kim et al., 2009), meningitis (46%) (Johnson et al., 2002), etc.

These studies suggest that although the production of CDT by *E. coli* is involved in its pathogenicity, CDT is not the only virulence factor produced by *E. coli* responsible for disease induction.

To date, five different CDTs have been characterized in *E. coli* strains (Smith and Bayles, 2006). *Ec*CDT-I and *Ec*CDT-II were characterized in 1994 in strains of serotype 086:H34 (Scott and Kaper, 1994) and 0128:H- (Pickett et al., 1994), respectively. *Ec*CDT-III was found in strains of *E. coli* producing the cytotoxic necrotizing factor-2 (CNF2) (Pérès et al., 1997). *Ec*CDT-IV was found in many strains colonizing humans and some animals (Toth et al., 2003) and, finally, *Ec*CDT-V was discovered in shiga toxin producing *E. coli* (STEC) O157:H7 (Janka et al., 2003). Genes encoding these five CDTs exhibit 54 to 89% sequence similarity (Toth et al., 2003) and are not found in the same DNA structures, suggesting that they were acquired from different means. The genes encoding *Ec*CDT-I are localized into a lambdoid prophage (Asakura et al., 2007), suggesting that they have been acquired following a lysogenic conversion, after a prophage infection. In contrast, genes encoding *Ec*CDT-II and *Ec*CDT-V are found in the bacterial chromosomal DNA. As these later ones are flanked by P2-phage and λ homologous sequences (Janka et al., 2003), this suggests that they were acquired after phage infection and integration of viral material into the bacterial genome. Finally, the genes encoding *Ec*CDT-III are located in the pVir plasmid, carrying the CNF2 virulence genes (Pérès et al., 1997).

Shigella dysenteriae

Among the bacteria of the *Shigella* genus, *Shigella dysenteriae* (*S. dysenteriae*) is the most virulent specie. Interestingly, *S. dysenteriae* is also the only specie where CDT encoding genes were found, in 22% of cases (Okuda et al., 1995). This characterization was made by PCR, and as these genes may present some sequence variability, it is possible that this quantification has been underestimated. *S. dysenteriae* colonizes the colon and is responsible for shigellosis, in some cases characterized by diarrhea and/or dysentery. Okuda et al. have studied the involvement and the importance of *Sd*CDT production in the pathogenicity of *S. dysenteriae*. In a mouse model, the administration of purified *Sd*CDT into the stomach induced diarrhea in the majority of individuals, as well as necrosis of the colon mucosa (Okuda et al., 1997). These results suggest the involvement of CDT in the induction or in the severity of symptoms developed after infection with *S. dysenteriae*.

Campylobacter jejuni

Bacteria of the *Campylobacter* genus colonize the intestine and are the cause of many food contaminations. The specie *Campylobacter jejuni* (*C. jejuni*) is responsible for gastroenteritis characterized by fever and diarrhea of variable severity. Almost all *C. jejuni* strains possess the genes encoding CDT (Bang et al., 2001; Jain et al., 2008). Therefore, PCR tests directed against *Cj*CDT are available to diagnose gastro-enteritis caused by *C. jejuni* (Kabir et al., 2011).

Experiments on human Hela cells showed that *Cj*CDT increases the adhesion and invasion capacity of *C. jejuni* (Jain et al., 2008). In a rat model of post-infectious irritable bowel syndrome (PI-IBS), *Cj*CDT does not increase the percentage of colonization by *C. jejuni* (Pokkunuri et al., 2012). However, CDT seems to increase the duration of *C. jejuni* colonization, suggesting that CDT is involved in the persistence of *C. jejuni* at the intestinal

epithelium, and not in the initial colonization. The *in vivo* study of CDT involvement in the pathogenicity of *C. jejuni* was performed in a mouse model (Jain et al., 2008). Intra-gastric treatment of mice with the supernatant of a CDT producing *C. jejuni* culture, showed diarrhea and severe inflammation of the entire gastro-intestinal tract (for 6 out of 10 mice), whereas treatment with the catalytically inactive *Cj*CDT supernatant induces no diarrhea and only a slight colon inflammation (Jain et al., 2008). Moreover, Pokkunuri et al. demonstrated in a PI-IBS rat model that the *Cj*CDT production alters stool forms, and, at a histological level, damages the epithelial barrier and induces an inflammation (Pokkunuri et al., 2012). Altogether, these results demonstrate the importance of *Cj*CDT in the pathogenicity of the bacteria *C. jejuni*.

Haemophilus ducreyi

Haemophilus ducreyi (*H. ducreyi*) is the bacteria responsible for a sexually transmissible disease named chancroid. The niche of *H. ducreyi* is in the genital mucosa (Spinola et al., 2002; Janowicz et al., 2011). This disease develops in different phases: first, the development of erythema within a few hours, before appearance of pustules within 2-3 days. Several weeks after infection, and in the absence of treatment, ulcers and in some cases suppurative lymphadenopathy appear. A temperature-dependent rabbit model for chancroid has been developed and used in many *in vivo* studies (Spinola et al., 2002; Stevens et al., 1999; Lewis et al., 2001; Wising et al., 2005). In this model, *Hd*CDT is not required for the initiation of the disease and the development of early symptoms, such as erythema and pustules (Lewis et al., 2001; Stevens et al., 1999). However, these studies examine the symptoms only after a few days of treatment, therefore CDT involvement in disease progression (such as ulcer development), or remission, has not been studied.

Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*) is part of the human oral flora and is involved in periodontal disease, but also in cardiac and neurological diseases. The prevalence of *Aa*CDT production in individuals with periodontitis is between 27% and 79% (Bandhaya et al., 2012; Jentsch et al., 2012; Wahasugui et al., 2013) and the presence of the toxin is associated with an increase in gingival bone loss. *Ex vivo* studies on gingival explant models showed that the purified *Aa*CDT induces damages and destabilization of the epithelial barrier (Damek-Poprawa et al., 2011). However, *in vivo*, in a rat model, CDT production by *A. actinomycetemcomitans* does not seem to cause any periodontal disease, nor bone loss (Schreiner et al., 2013).

Salmonella Typhi

Salmonella enterica serovar Typhi (*S.* Typhi) is the pathogen responsible for typhoid fever, which causes more than 200,000 deaths per year worldwide. For a long time, factors

involved in the induction of typhoid fever were unknown. Recently, the virulence factor produced by *S.* Typhi has been characterized (Song et al., 2013; Spanò et al., 2008). This factor, named the typhoid toxin, is an atypical A_2B_5 toxin consisting of three different subunits, one of the catalytic A moieties being identical to a CDT constituent (see after) (Song et al., 2013).

A recent *in vivo* study, in a mouse model, showed that systemic administration of purified typhoid toxin induces huge weight loss, a decrease in the rate of circulating immune cells and, after few days, the death of individuals. In contrast, injection of a catalytic mutant of the toxin (mutant of the CDT catalytic moiety) induces none of these typhoid fever symptoms (Song et al., 2013). In conclusion, in the case of *S.* Typhi, the catalytic CDT subunit is clearly involved, and even essential, to the pathogenicity.

Helicobacter hepaticus

Helicobacter hepaticus (*H. hepaticus*) is found in the gastrointestinal mucosa and, in some cases, can colonize the hepato-biliary channels and be found in the liver. For a long time, *H. hepaticus* has been characterized as a mouse pathogen, although this bacteria has also been found in the human body (Murakami et al., 2011). *H. hepaticus* induces an acute hepatitis, inflammation and even liver cancers in a mouse model (Ge et al., 2007). In this immunocompetent mouse model, *Hh*CDT is not involved in the induction of chronic hepatitis or in the colonization of the mucosa by *H. hepaticus*. However, only mice infected with a strain of *H. hepaticus* producing a functional CDT exhibited increased levels of pro-inflammatory mediators and developed hepatic dysplastic nodules (Ge et al., 2007). This study clearly shows CDT involvement in the *H. hepaticus* pathogenicity. In addition, it is also the first study to demonstrate the carcinogenic potential of CDT.

CDT Biogenesis and Secretion by Bacteria

CDT Is a Tripartite AB Toxin

The three genes encoding CDT were characterized in 1994 (Pickett et al., 1994; Scott and Kaper, 1994). The *CdtA*, *CdtB* and *CdtC* genes, organized in one operon, encode the three subunits constituting the CDT holotoxin (Figure 1). Among all bacterial strains producing CDT, the CdtB protein is the most conserved, with at least 45% sequence identity (Hu et al., 2006). On the other hand, CdtA and CdtC exhibit up to 19% sequence identity. All three subunits are required to trigger maximal cytotoxicity, albeit in some cases CDT lacking CdtA retains some toxic activity (Gargi et al., 2012).

The crystal structures of the *Aa*CDT natural form and of a recombinant *Hd*CDT, reconstituted *in vitro*, have been determined (Nesić et al., 2004; Yamada et al., 2006). As with many other exotoxins, CDT adopts an AB functional structure. Depending on the structural properties of the A and B subunits (i.e. mode of assembly, monomer subunits, homo- or hetero-polymer subunits, etc.), many classes of these toxins can be differenciated (Henkel et

al., 2010). CDT assembles into a heterotrimeric holotoxin, where CdtB is the catalytic A-subunit.

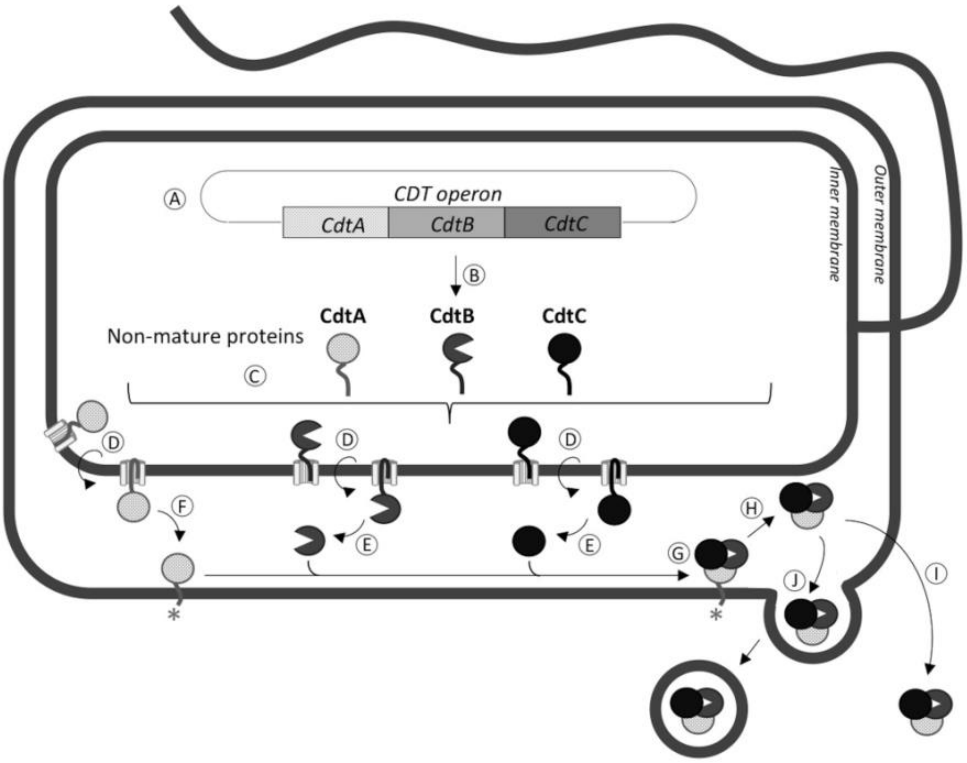

Figure 1. *CDT biogenesis and secretion*. A. CDT operon structure. B. Expression of the three sub-units. C. Localisation of the three sub-units to the inner membrane. D. Periplasm localisation may be via the general secretory pathway. E. Cleavage of the signal peptide leading to the release of CdtB and CdtC into the periplasm. F. Cleavage of the signal peptide and lipid modification of CdtA, leading to his relocalization at the outer membrane. G. Folding of the holotoxin. H. Processing of CdtA, which is N-terminally truncated. Depending on the bacterial strain: I. secretion of the holotoxin in the supernatant or J. secretion of the holotoxin into outer membrane vesicles (OMVs).

The B-moiety, essential for the holotoxin binding to the host cell membrane, is composed of the CdtA and CdtC subunits. Thus, CDT can be classified as an AB_2 exotoxin.

One exception is the CDT-related toxin produced by *S.* Typhi, called the typhoid toxin. Although *S.* Typhi has the *CdtB* gene, it does not possess the *CdtA* and *CdtC* genes. Interestingly, *CdtB* is expressed within the same pathogenicity islet as *PltA* and *PltB* (Spanò et al., 2008), encoding the pertussis-like toxin A (homologous to the pertussis toxin ADP-ribosyltransferase subunit) and the pertussis-like toxin B (homologous to one of the pertussis B subunits) (Kaslow and Burns, 1992) respectively. The structure of this toxin has been recently described (Song et al., 2013), demonstrating that the typhoid toxin is an A_2B_5 toxin. The regulatory subunit (B_5) is composed of a pentameric PltB structure, whereas the catalytic subunit (A_2) is composed of the *St*CdtB/PltA proteins, covalently linked by a disulfide bond.

CDT Biogenesis and Secretion

In an initial report studying CDT biogenesis, using an *E. coli* strain carrying the *AaCDT* genes, it was shown that CdtA is a lipoprotein associated to the outer membrane (Ueno et al., 2006). However, in the periplasm or in the culture supernatant, the CDT holotoxin is found as a complex where CdtA is N-terminally truncated and associated with CdtB and CdtC (Ueno et al., 2006). This suggests that CdtA undergoes lipid modification during the export process from the inner membrane to the outer membrane and, for a second time, a N-terminal processing (Figure 1).

Finally, as CdtA, CdtB and CdtC present a signal sequence, they can be directed to the general secretory pathway leading to CDT genotoxin secretion into the culture supernatant (Zijnge et al., 2012) (Figure 1). However, different studies have showed that certain bacteria release CDT *via* outer membrane vesicles (OMVs). OMVs are constitutively released from some Gram-negative bacteria and have been shown to contain a great variety of virulence factors (Amano et al., 2010; Deatherage and Cookson, 2012). In extra-intestinal pathogenic *E. coli*, the vast majority of secreted CDT was associated to OMVs (Berlanda Scorza et al., 2008). Lindmark et al. studied *C. jejuni* CDT production and release, by OMVs electronic microscopy and bacteria subcellular fractionation: the three CDT subunits have been detected in the periplasmic compartment, and, in the bacterial culture supernatant, extracellular CDT was found tightly associated to OMVs (Lindmark et al., 2009). Isolated OMVs induced the cell distending effects typical of CDT on human intestinal cells, indicating that CDT is present at OMVs under a biologically active form and suggesting that the release of OMVs is a delivery route for *Cj*CDT. In addition, *A. actinomycetemcomitans* OMVs have also been identified as a vehicle for CDT delivery into human cells (Rompikuntal et al., 2012). Concerning the OMVs interaction with the host plasma membrane, the OMVs proteins were found to internalize, in both HeLa cells and human gingival fibroblasts (HGF), *via* the OMVs fusion with lipid rafts (specialized membrane microdomains, enriched in specific lipids, such as cholesterol). In contrast to OMVs isolated from a CDT defective strain, OMVs isolated from a wild type CDT strain induced a characteristic cytolethal distending effect on HeLa and HGF cells, indicating that the OMV-associated CDT was biologically active (Rompikuntal et al., 2012). Association of CDT with OMVs was observed in *A. actinomycetemcomitans* isolates belonging to a, b and c serotypes, indicating that OMV-mediated release of CDT may be conserved in all *A. actinomycetemcomitans* genus. To conclude, whereas CDT may be freely secreted into the extracellular environment (Figure 2A), it is clearly apparent that CDT is also associated to OMVs (Figure 2B). The detailed mechanisms of CDT secretion still remain to be elucidated.

In contrast to these secretory pathways, the *S.* Typhi toxin is once again an exception (Spanò et al., 2008). Infection studies revealed that bacterial uptake into host cells triggers CdtB/PltA/PltB expression, leading to the formation of an intracellular multipartite toxin. Interestingly, following its production, the typhoid toxin is secreted into the extracellular medium. Only after this step, the toxin can interact in an autocrine and paracrine way with the eukaryotic plasma membrane and be internalized to exert its cytotoxic activity (Figure 2C). Inhibition of typhoid toxin export into the extracellular medium inhibits its effects, demonstrating that this step is limiting for toxin activity (Spanò et al., 2008).

All the Same but Different: CDT Host-Cell Binding and Trafficking

Even if all CDT toxins induce the same characteristic effects on eukaryotic cells, they are produced by bacterial strains located within different niches.

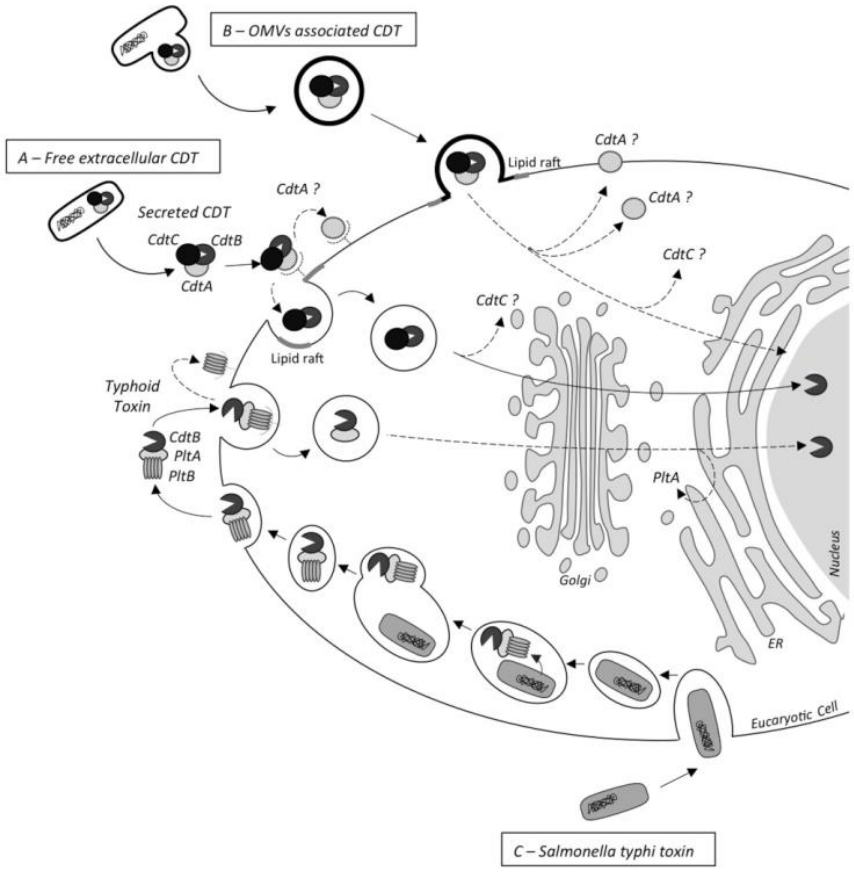

Figure 2. *CDT binding and trafficking in host cell*. Global representation of the CDT binding and trafficking steps. A. Free extracellular CDT. Mainly CdtA, and in a lesser extent CdtC, are involved in the CDT binding to the host cell surface, probably through the interaction with a cargo/receptor. Rafts microdomains may also be important for the binding to the membrane. The CdtA subunit may stay, or be rapidly recycled, at the cell surface, whereas CdtC is found both on the cell surface and in the cytosol and may participate in the CdtB trafficking. CdtB follows a retrograde endosome-Golgi trafficking to translocate at the ER and, later, at the nucleus. However, it has to be noted that different CDT toxins may use different cargoes and traffic through different routes (see text for details). B. Outer Membrane Vesicles (OMVs) associated CDT. After production by bacteria, OMVs are fusing with the host cell membrane. Lipid raft microdomains are important for this fusion step. The CDT toxin is released in the host-cell cytoplasm and CdtB is relocalized to the nucleus of intoxicated cells, apparently independently of the retrograde transport. The CdtA and CdtC outcomes are not clearly understood. C. *Salmonella* Typhi toxin. After its intracellular uptake, *S.* Typhi expresses the CdtB, PltA and PltB subunits, forming the multipartite typhoid toxin. Following its production, the typhoid toxin is secreted into the extracellular medium. The typhoid toxin can bind, in an autocrine and paracrine way, host cells membrane, leading to endocytosis and retrograde trafficking of PltA and CdtB to the ER where they split apart.

This implies that all CDT toxins are not secreted in the same microenvironment (mucus or not, different cell types, highly proliferating host cells or not) and raises different questions regarding the specificity of the CDT holotoxins (Gargi et al., 2012). Are all CDTs the same? Are they acting the same way in all these diverse biological niches? As explained above, there is large sequence variability between CdtC and CdtA homologs. Some authors have hypothesized that this variability may allow CDT to interact specifically with the different cell types residing in the different bacterial niches. Although CdtA and CdtC are clearly involved in the binding of CDT to host cells, a number of questions remain unanswered. Firstly, it is still not clear how CdtA and CdtC contribute to the binding of CDT on the host cell membrane, and furthermore the nature of the CDT receptor remains unknown. Secondly, the internalization and the trafficking of the toxin are still obscure. Hereafter, we present an overview surrounding the current knowledge regarding these different points.

CDT Binding at the Host Membrane

CdtA and CdtC Bind to the Host Plasma Membrane

It is known that CdtA, CdtB and CdtC interact with one another to form an active tripartite holotoxin (Lara-Tejero and Galàn, 2001). CdtA and CdtC are essential for CDT binding to the host cell surface, as *Cj*CdtA or *Cj*CdtC subunits can specifically bind to HeLa cell surface whereas *Cj*CdtB cannot (Lee et al., 2003). Interestingly, mutants containing deletions of regions important for the CdtA/CdtC interaction are still able to bind to the HeLa cell surface (Lee et al., 2003).

The study of the CDT 3D structure by X ray crystallography brought new information on the potential role of CdtC and CdtA in cell surface binding. Indeed, these studies have revealed the presence of an aromatic patch in the structure of CdtA and a groove formed at the CdtA and CdtC interface. The aromatic patch and the groove were shown as key elements of the CDT holotoxin, as mutations in these structures impair host cell intoxication, with or without impairing toxin binding to host cells or toxin stability (Nesić et al., 2004; Nesic and Stebbins, 2005). This key role in the toxin function was recently confirmed by mutational analysis of the CdtA W115 residue. Located in the aromatic patch, this residue exhibits a critical role for host cell binding, without interfering with the holotoxin assembly (Li et al., 2013).

Intriguingly, another point mutation (Y181A), outside of the aromatic patch, has been shown to impair the CDT binding (Li et al., 2013), suggesting that other domain(s) may be involved in host cell binding. Finally, *Ec*CdtA-II and *Ec*CdtC-II subunits may form a binding platform for *Ec*CdtB-II, as *Ec*CdtB-II binds to the HeLa cell surface in a *Ec*CdtA-II and *Ec*CdtC-II dependent manner, as shown by cell cytometry (Mcsweeney and Dreyfus, 2005). Altogether, these results lead to the consensus that CdtA and CdtC form a heterodimeric complex allowing for CDT attachment to the host cell membrane (Figure 2A). This is supported by the fact that *Aa*CdtA and *Aa*CdtC are localized to the plasma membrane (Damek-Poprawa et al., 2012).

Binding to Sugar and to Cholesterol

Interestingly, CdtA and CdtC both display a structure resembling that of lectins (Nesić et al., 2004), characterized as carbohydrate-binding proteins displaying specificity to varying sugars. More precisely, CdtA and CdtC structures are homologous to the lectin-like B-chain repeats of the ricin toxin, important for ricin toxin cellular uptake. It has been shown that CdtA and CdtC structures are conserved between all CDT subtypes, suggesting that they are essential and have key functions in the cell surface binding and holotoxin internalization (Nesic and Stebbins, 2005). In addition, CdtA and CdtC seem to compete for a similar receptor (or binding protein) on the HeLa cell surface, as preincubating cells with specific lectins blocks CdtA and CdtC binding (Mcsweeney and Dreyfus, 2005). This is confirmed by the fact that CdtA or CdtC preincubation with specific glycoproteins prevents their binding to host cells. Moreover, removal of N-linked, but not O-linked, sugars at the host cell surface impedes CDT intoxication (Mcsweeney and Dreyfus, 2005). At the same time, another study showed that CDT could bind to specific glycolipids (Mise et al., 2005). Particularly, gangliosides (ceramide-based oligosaccharide with sialic acids linked on the sugar chain) may be involved, as the pre-incubation of CDT with GM3 (monosialodihexosyl ganglioside)-enriched liposomes reduced CDT toxicity on host cells (Mise et al., 2005). Moreover, *Aa*CDT effects also depend on cholesterol, as cholesterol-depletion reduces CDT binding and cholesterol-repletion restores the binding potential of CDT (Boesze-Battaglia et al., 2009). Further analyses indicate that the CdtC subunit contains a cholesterol recognition/interaction amino acid consensus (CRAC) site, mutation of which results in decreased CDT binding (Boesze-Battaglia et al., 2009). Rafts microdomains are enriched in cholesterol and sphingolipids, explaining why rafts microdomains are required for binding and intoxication by CDT (Boesze-Battaglia et al., 2006; Guerra et al., 2005). Finally, cell intoxication by *Cj*CDT is also achieved through CDT association to cholesterol-rich microdomains (Lin et al., 2011), as a mutation in the *Cj*CdtC CRAC-like region impacts the cholesterol-binding and leads to an impaired CDT intoxication (Lai et al., 2013).

CDTs from *H. ducreyi*, *A. actinomycetemcomitans*, *E. coli* and *C. jejuni* may differ in their abilities to intoxicate host cells, leading to a variable cell target tropism. Enriching the cholesterol-content in host cells enhances the effects of all CDTs tested except *Cj*CDT, whilst the absence of cell surface sialic acid sensitized CHO cells to all CDTs with the exception of *Cj*CDT (Eshraghi et al., 2010). In contrast, it has to be noted that fucosylated N-linked glycans and glycolipids were not required for intoxication by any of the CDTs tested in this study. As stated by the authors, *Aa*CDT and *Hd*CDT behave similarly, whereas *Ec*CDT-III and *Cj*CDT display distinct host cell preferences. This is consistent with the homology presented by these four CDTs, and also illustrates that CDTs produced by different pathogens may possess unique features to intoxicate host cells and may do so through different host receptors (Eshraghi et al., 2010). The CDT tropism may thus correlate to the amino-acid sequence of the CDT-binding subunits. Thus, although *A. actinomycetemcomitans* and *H. ducreyi* have different niches, their CdtA and CdtC subunits display high sequence homologies and respond similarly to host factors alterations (such as glycans, cholesterol content, etc.).

In addition, these niches (oral cavity and genital mucosa respectively) are however characterized by a similar epithelial organization (stratified epithelia), underlining again the necessity of a similar mode of action. Reciprocally, *E. coli* and *C. jejuni* have similar niches

but their binding subunits are divergent and display different target cell preferences (Eshraghi et al., 2010). The CDT receptor(s) identification for these different CDT toxins may ultimately provide insights regarding cellular tropism and shed light on host CDT-producing bacteria interactions.

A Specific Membrane Receptor for CDT?

As stated before, the divergent results on factors that mediate CDT binding to the host cell surface may depend on the receptor specificity inherent to each CDT. To underline this specificity, it is important to note that CDT toxins may be specific to host species, as *Hd*CDT cannot intoxicate rodent cells (Cortes-Bratti et al., 1999; Guerra et al., 2005). Recently, two genetic screens conducted on the KBM7 chronic myeloid leukemia cell line identified several genes important for CDT intoxication (Carette et al., 2009; Carette et al., 2011). These host genes have not been found with other toxin screens (i.e. diphtheria toxin, ricin, etc.) and may therefore be considered specific to the CDT intoxication. Different CDT toxins share some common host factors, such as the SGMS1 gene, encoding the sphingomyelin synthase 1, implied in the host-cell sensitivity for all CDTs tested (*E. coli*, *C. jejuni*, *A. actinomycetemcomitans* and *H. ducreyi*).

However, CDTs also exploit specific host proteins, which may help in defining a characteristic profile for each CDT. *Ec*CDT-I specifically requires TMEM181, a putative G protein-coupled receptor, expressed at the cell surface, which has been shown to co-immunoprecipitate with CDT (Carette et al., 2009).

GLG1 (Golgi glycoprotein 1) and ATP6V0A2 (the vacuolar ATPase subunit 2) were also found as important genes for host cell sensitivity to *Ec*CDT-I (Carette et al., 2011), although their cellular localization does not make them good receptor candidates. *Aa*CDT shows a gene pattern similar to the *Ec*CDT-I, except that it does not require TMEM181 but, instead, relies on the SYNGR2 (synaptogyrin 2), an ubiquitous integral membrane protein. *Cj*CDT depends on different host factors: besides proteins involved in vesicular transport, three specific plasma membrane proteins were identified as essential: TMEM127, GPR107 and TM9SF4. Because the SGMS1 depletion reduces levels of sphingomyelin, a key component of lipid rafts, this confirms and underlines the role of these microdomains in the CDT intoxication process.

In addition, these data suggest that the TMEM181 protein may be the *bona fide Ec*CDT-I receptor, however it could not be ruled out that TMEM181 plays an indirect role, yet to be identified, in the receptor-toxin interaction and/or trafficking.

CDT Trafficking into the Mammalian Cells and CdtB Nuclear Localization

Recently, through live cell microscopy analysis, *Aa*CdtA has been found at the cell surface, whereas *Aa*CdtC is found both on the cell surface and in the cytosol, and *Aa*CdtB is found at the ER and later at the nucleus (Damek-Poprawa et al., 2012) (Figure 2A).

CdtA and CdtC localization to the host cell surface is in agreement with their role in binding to the plasma membrane (see above). However, the intracellular localization of CdtB and CdtC, and more precisely the observation that the nucleus represents the final destination of CdtB, implicate a programmed CDT holotoxin disassembly to ensure an effective cellular trafficking of CdtB.

We will present in this part the lines of evidence depicting the cellular road taken by CdtB to reach the nucleus.

Importance of Endocytosis

A great variety of bacterial toxins use cellular trafficking to enter host cells (Spooner and Watson, 2010). Apart from phagocytosis, happening in certain cell types (macrophages, monocytes, etc.), different endocytic pathways have been described in eukaryotic cells. Endocytic pathways generally involve several steps, as the membrane invagination and the endocytic vesicle pinch off. The invagination step can define different endocytic processes, mainly clathrin coat dependent endocytosis, clathrin-independent but caveolin-dependent endocytosis and clathrin- and caveolin-independent endocytosis, as in bulk-phase pinocytosis (Doherty and Mcmahon, 2009).

Molecule-specific endocytosis is achieved through receptor-mediated endocytosis. However, one can consider that host receptor binding by the toxin is a hijack from their cellular function. Thus, host cell receptors can be seen as cargos exploited by CDTs to enter host cells (Figure 2A). *Hd*CDT is rapidly internalized from the cell surface and subsequent CDT effects seem dependent on clathrin and dynamin (Cortes-Bratti et al., 2000), respectively involved at the early stage of endocytic vesicle formation and at the pinch-off step; *Hd*CDT may therefore enter host cells through receptor-mediated endocytosis. However, this does not rule out the possibility that CDT can enter through other endocytic pathways, such as caveolae, given the importance of cholesterol-rich domains (Boesze-Battaglia et al., 2006, 2009; Guerra et al., 2005; Lin et al., 2011). Lately, it has been shown that CDT intoxication of CHO cells could occur despite significant depletion of membrane cholesterol, ultimately underlining the potential role of alternative endocytic pathways (Damek-Poprawa et al., 2012).

Retrograde Trafficking

After cell entry by endocytosis, molecules can trafficked through different routes, for example to be degraded in the lysosome, or to be relocated within host cells. Early endosomes can be seen as a traffic hub, where decisions are made: endocytosed molecules can be recycled to the plasma membrane, transferred directly to the trans-Golgi network (TGN) or passed to late endosomes, where traffic pathways lead to TGN or to lysosome. If transferred to TGN, molecules can be retrogradely transported to the endoplasmic reticulum (ER) (Spooner and Lord, 2012).

CDT intoxication is inhibited when fusion of early endosomes with downstream compartments is blocked, or when Golgi is disrupted (Cortes-Bratti et al., 2000). This suggests the involvement of a retrograde endosome-Golgi trafficking route (Figure 2A). As

assessed by glycosylation studies, *Hd*CDT is transported from the TGN to the ER, however, this occurs in the absence of the ER-associated degradation (ERAD) pathway (Guerra et al., 2005). Interestingly, if many ER-translocating toxins exploit the ERAD pathway to transit from the ER to the cytosol, CDT translocation is atypical and does not require protein unfolding. To corroborate this, the CdtB subunit could not be seen freely in the intoxicated cells cytosol by confocal microscopy analysis and biochemical assays (Guerra et al., 2009). Thus, *Hd*CDT translocation to the nucleus does not involve the classic ERAD pathways, usually followed by retrogradely transported toxins, and may be directly translocated from the ER to the nucleus.

Recently, the intoxication mechanisms of CDTs from *Ec*CDT-III and *Hd*CDT, sharing limited sequence homology, were compared (Gargi et al., 2013). If both toxins require the Golgi compartment to traffic, they also present distinct features. For example, endosomal disruption blocks *Hd*CDT transport but has no effect on *Ec*CDT-III localization. In addition, cells expressing dominant negative Rab7 (a small GTPase involved in the late endocytic pathway) present no effects after *Hd*CdtB treatment but are affected by *Ec*CDT-III. These results support that *Hd*CDT, but not *Ec*CDT-III, traffic through the late endosomal compartment. Moreover, *Ec*CDT-III and *Hd*CDT may use different cargos, as competitive binding studies suggest that they bind to different cell surface determinants (Gargi et al., 2013). Altogether, these results reinforce that different CDT toxins may use distinct trafficking pathways, possibly reflecting different cargo interactions at the host cell surface.

CdtB Nuclear Localization

Several studies have tried to characterize how CdtB is translocated to the nucleus, mainly through observations of CdtB localization after cellular microinjection (Lara-Tejero and Galàn, 2000; Nishikubo et al., 2003). Four hours after being microinjected into the cytoplasm, *Cj*CdtB and *Aa*CdtB are exclusively located to the nucleus, demonstrating that CdtB trafficking to the nucleus does not require the two other CDT subunits. In order to identify domains important for this nuclear transport, truncated forms of *Aa*CdtB have been fused to the green fluorescent protein (GFP) and expressed in HeLa cells (Nishikubo et al., 2003). An 11 amino-acid sequence, within the N-terminal region of CdtB, has been found essential for nuclear localization and for toxin-induced cellular effects. The replacement of this N-terminal sequence by the Nuclear Localization Signal (NLS) of the SV40 Large T-antigen allows recovering CDT effects (Nishikubo et al., 2003). Thus, CdtB nuclear entry is crucial for CDT cytotoxicity and is governed by a putative NLS sequence. In addition, two potential NLS have been identified in the C-terminal part of *Ec*CdtB-II (McSweeney and Dreyfus, 2004). Interestingly, deletion of each of these NLS sequences produces a differential localization of the active toxin subunit, suggesting a specific function for each NLS sequence.

The Remaining Black-Boxes

The current model of CDT trafficking implies a stepwise holotoxin disassembly with: 1) CdtA remaining bound to the plasma membrane following initial toxin binding to a yet unidentified receptor, 2) endocytosis of the CdtB-CdtC heterodimer and 3) retrograde

trafficking of CdtB to the nucleus (Figure 2A). However, a clear function for CdtC remains to be defined. CdtC could for example impede the CdtB function, as structural data may suggest (Nesić et al., 2004). Even though, some confusion was brought from CdtC trafficking data, as the intracellular delivery of recombinant *Aa*CdtB or *Aa*CdtC was shown to induce CDT characteristic effects on CHO cells (Mao and DiRienzo, 2002). These characteristic CDT effects were also found after HEp-2 epithelial cells were exposed to both *Aa*CdtB and *Aa*CdtC (Akifusa et al., 2005). Yet the CdtC contribution to CDT effects is obscure, and it could well be due to CdtC-related activity on cell trafficking. In addition, *Aa*CdtC mutants predicted to impair receptor binding failed to show internalization defects, indicating that CdtC may only have a minor role in receptor binding (Damek-Poprawa et al., 2012) and may in fact facilitate CdtB internalization, potentially by helping in the first steps of the CdtB trafficking.

Although the majority of CDT internalization studies have been performed using recombinant purified holotoxin, it should not be forgotten that both *Ec*CDT-II, *Cj*CDT and *Aa*CDT may be secreted through OMVs (Berlanda Scorza et al., 2008; Lindmark et al., 2009; Rompikuntal et al., 2012). After treatment with *A. actinomycetemcomitans* OMVs, the CdtB subunit has been localized inside the nucleus of intoxicated cells (Rompikuntal et al., 2012). Whether the delivery of OMV-associated CDT follows the same trafficking as the soluble toxin is doubtful, as differences between these pathways are already known (Amano et al., 2010; Ellis et al., 2010).

Particularly, Rompikuntal *et al*. have shown that OMV internalization is dependent on the lipid raft microdomains of the host cell membrane but is independent of retrograde transport (Figure 2B) (Rompikuntal et al., 2012). In conclusion, many questions still need to be answered, particularly regarding CDT binding, entry, trafficking and nuclear translocation in host cells, as well as the existence of a common pathway to all CDTs or if specific trafficking pathways exist for each CDT.

The Host Cell Response to CDT

At the cellular level, intoxication with CDT causes cell cycle arrest at G2/M accompanied by nuclear and cytoplasmic enlargement, a phenomenon called cellular distention, which eventually lead to apoptotic cell death. Such cell cycle alteration and cytotoxicity effects have been documented with various CDT toxins, originated from diverse bacteria including *A. actinomycetemcomitans* (Ohguchi et al., 1998; Shenker et al., 1999; Sugai et al., 1998), *E. coli* (Comayras et al., 1997; Pérès et al., 1997), *C. jejuni* (Whitehouse et al., 1998), *H. ducreyi* (Cortes-Bratti et al., 1999; Cortes-Bratti et al., 2001), *H. hepaticus* (Young et al., 2000) or *S.* Typhi (Haghjoo and Galán, 2004), and in a broad range of human cell lines. Interestingly, some cellular features of CDT intoxication can be recapitulated in *S. cerevisiae* (Hassane et al., 2001), suggesting that the mode of action of CDT cytotoxicity involves a host target shared from yeast to human.

So far, several lines of evidence indicate that CdtB, the catalytic component of the toxin, attacks host genomic DNA by inducing strand breaks, activating the DNA damage response (DDR), leading to cell cycle arrest and cell death. In this part, we will detail the data supporting this model.

Cellular Features of CDT Intoxication

Morphological Changes

Regardless of CDT origin, intoxicated cells undergo sequential morphological alterations before dying (Figure 3). The timing of events depends on the cell line that has been intoxicated, probably due to the variability in the sensitivity to the toxin.

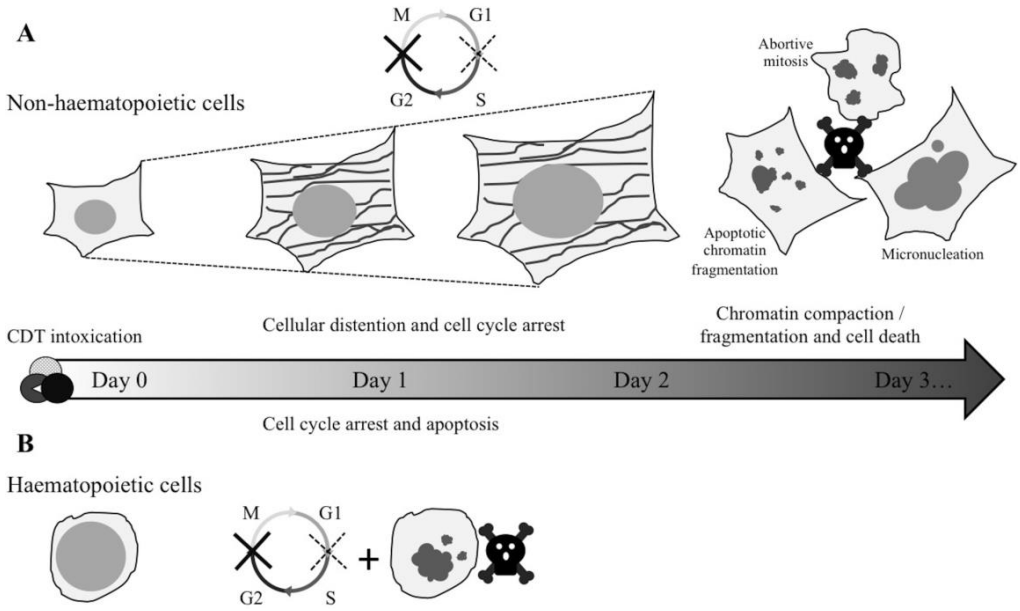

Figure 3. *CDT cellular effects.* This figure depicts the morphological alteration over time of CDT intoxicated cells from non-haematopoietic (A) or haematopoietic (B) lineage. A. Cytoplasmic and nuclear distension occur within the first day of intoxication and extend until three days. At the same time, cells progressively block at G2/M and potentially at G1/S (probably depending on their p53 status). The timing of cell cycle arrest depends on the cell cycle duration and the cellular sensitivity to CDT (i.e. the dose of CDT and the susceptibility of a specific cell line to a defined CDT). After two days, cells dye by either apoptosis or necrosis. A few part of G2/M arrested cells entered an abortive mitosis. Otherwise, cell death is accompanied by chromatin compaction and fragmentation and eventually micronucleation. B. Lymphocytes (and more probably all haematopoietic cells) intoxicated by CDT are much more sensitive than non-haematopoietic cells as they show a rapid apoptotic response even before any obvious cell cycle arrest. As for non-haematopoietic cells, the timing and occurrence of cell cycle arrest and cell death depends on the cellular sensitivity to CDT.

Cellular distension, the first morphological change to be documented, starts to be observable 15 hours after CDT treatment, and increases progressively to reach between four- and sevenfold the normal cell-size (Aragon et al., 1997; De Rycke et al., 2000; Pérès et al., 1997; Sugai et al., 1998; Whitehouse et al., 1998) (Figure 3A). Lymphocytes however represent an exception: albeit these cells are more sensitive to CDT, they do not show any distension phenotype (Shenker et al., 1999) as they die prematurely (Figure 3B). Concomitantly to cytoplasm expanding, the nucleus also enlarges and actin stress fibers are formed (Aragon et al., 1997; Cortes-Bratti et al., 1999; De Rycke et al., 2000; Pérès et al., 1997; Sugai et al., 1998; Young et al., 2000) (Figure 3A). After two days of CDT exposure,

several morphological abnormalities are visualized in a subset of cells (De Rycke et al., 2000; Whitehouse et al., 1998). Thus, a small part of intoxicated cells seems to engage in an abortive form of mitosis, characterized by rounding up, chromatin compaction and fragmentation, granular morphology and distorted spindle structure with centrosome amplification. A more important fraction of cells display irregular chromatin condensation, as well as invagination of the nuclear envelope, progressive lobulation and eventually micronucleation. It has to be noticed that such chromatin condensation and degradation are characteristic of apoptotic cells (Ohguchi et al., 1998).

Cell Cycle Arrest

An overall feature of CDT exposure is cell cycle arrest at the end of the G2-phase, in a variety of proliferating cells including lymphocytes (Cortes-Bratti et al., 2001). Progression through eukaryotic cell cycle requires the sequential activation of heterodimeric CDK-cyclin complexes (Diaz-Moralli et al., 2013) (Figure 4). The CDK1-cyclin B complex, formed during G2, controls the initiation of mitosis upon regulated concentration and localization of cyclin B1, and phosphorylation of CDK1 that dictates its activation state. Cyclin B1 progressively accumulates in the cytoplasm between S and the end of G2, before translocating to the nucleus at early mitosis (Pines and Hunter, 1991). Upon CDT intoxication, cyclin B1 levels increase up to a normal G2 level, but remain outside of the nucleus, confirming that cells accumulate at G2 without proceeding to mitotic entry (Comayras et al., 1997; Fedor et al., 2013). The G2/M transition necessitates CDK1 activation by CDC25-mediated dephosphorylation at T14/Y15 (Shen and Huang, 2012). Cells treated with various CDTs exhibit a strong increase in phosphorylated (inactive) CDK1 (Bielaszewska et al., 2005; Comayras et al., 1997; Cortes-Bratti et al., 1999; Shenker et al., 1999; Whitehouse et al., 1998), which can be reactivated *in vitro* with recombinant CDC25 (Sert et al., 1999). This lack of CDK1 activation can be explained at least in part by the CDT-induced sequestration of CDC25C, out of the nucleus (Alby et al., 2001). These results support that CDT treatment inhibits CDC25 nuclear localization, which in turn fails to activate CDK1, preventing mitotic entry and thus blocking cells in G2. Indeed, overexpression of recombinant CDC25B or CDC25C abrogates CDT-induced G2 arrest, enabling intoxicated cells to enter an abortive mitosis (Escalas et al., 2000).

In addition to the well-documented G2/M block induced by CDT, a subset of cell lines also exhibit cell cycle arrest at the G1/S transition after treatment with CDT from various origins (Cortes-Bratti et al. 2001; Hassane et al. 2003; Belibasakis et al. 2004; Bielaszewska et al. 2005) (Figure 3). This particular response to CDT seems to rely on the G1/S checkpoint that depends on the activation of the p53 tumor suppressor and its downstream effector p21 (Diaz-Moralli et al., 2013). Thus, the variation in the p53 status among the different cell types may explain the discrepancy observed in regards to the cell cycle alterations induced by CDT. Besides, we have recently demonstrated that CDT also induces a slight S-phase slow-down that is difficult to observe by classical flow-cytometry analyses (Fedor et al., 2013). Therefore, the CDT-mediated proliferation arrest may neither be cell-cycle phase nor bacterial species specific, but rather be due to overall cellular responses (like checkpoints) that might differ between cell types (Belibasakis et al., 2004).

Cell Death

The final characteristic of CDT intoxication is generally the programmed cell death by an apoptotic pathway (Figure 3). Apoptosis is an essential process regulating development and tissue homeostasis in multicellular organisms by elimination of unnecessary or damaged cells, in an immunologically silent manner (Gao and Kwaik, 2000). In addition, this mechanism modulates the pathogenesis of different infectious diseases by contributing to an antimicrobial immune response. On the other hand, induction of apoptosis by bacterial pathogens may facilitate colonization, persistent infection and chronic disease (Jinadasa et al., 2011).

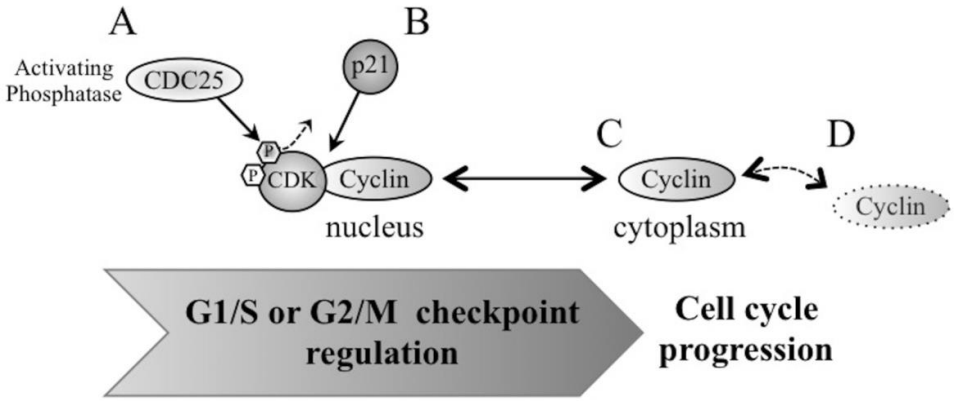

Figure 4. *Model of the DNA damage checkpoints-mediated cyclin-CDK complex regulation*. The cyclin-Cyclin Dependent Kinase (cyclin-CDK) complex is formed by the association of a regulatory cyclin with a kinase, which catalytic activity is highly dependent of the cyclin association. The cyclin-CDK complexes are key components of the cell cycle regulation, particularly at the G1/S and G2/M checkpoints. Specific cyclins and CDK exist throughout the cell cycle phases. A global schema for the regulation of these cyclin-CDK complexes through checkpoint activation can be drawn. Globally, 4 different levels of regulation can be distinguished. A. CDK Phosphorylation status. The activity of the cyclin-CDK complex is controlled by a phosphorylation-dephosphorylation equilibrium. CDC25 proteins are well-documented phosphatases involved in the activation of the cyclin-CDK complexes. CDC25 activation can be regulated by the DNA damage checkpoints. B. p21 CDK inhibitor expression. p21 is able to interact with the CDKs in order to block their activity. p21 expression is induced after the transcription factor p53 is activated, for example after DNA has been damaged. C. The cyclin localization is also a critical step in the cyclin-CDK complex regulation. If bloked in the cytoplasm, cyclins are unable to associate and activate CDKs. D. Cyclin synthesis or degradation. Finally, the synthesis, as well as the degradation of cyclin subunits has been proven key in the regulation of the cyclin-CDK complex.

Apoptosis can be separated into two major pathways (Taylor et al., 2008). The extrinsic pathway is initiated by interaction between extracellular ligands and transmembrane death receptors, resulting in caspase-8 and subsequent caspase-3 and -7 activation, and eventually, cell death. In the intrinsic pathway, activation of the BH3-only pro-apoptotic proteins leads to the alteration of the outer mitochondrial membrane permeability, resulting in the activation of caspase-9 and downstream elements, and subsequent apoptosis. Both pathways imply the activation of caspases that in turn activate specific nucleases (caspase-activated DNases) involved in DNA fragmentation and condensation within the dying cells (Enari et al., 1998; Liu et al., 1997). Cells displaying a wild-type p53 genotype display greater sensitivity to apoptosis, as p53 transcriptionally upregulates many BH3-only proteins (Shibue and

Taniguchi, 2006). However, BH3-only proteins can also be activated by alternative, albeit less efficient, mechanisms in p53-null cells.

CDT-mediated apoptosis has been shown to follow the intrinsic mitochondrial pathway for toxins produced by *H. hepaticus* (Liyanage et al., 2010), *A. actinomycetemcomitans* (Ohara et al., 2004; Shenker et al., 2001), *C. jejuni* (Hickey et al., 2005) and *H. ducreyi* (Wising et al., 2005). Moreover, overexpression of BCL-2, an anti-apoptotic protein involved in the intrinsic pathway, inhibits apoptosis induced by *A. actinomycetemcomitans* CDT (Ohguchi et al., 1998; Shenker et al., 2001). Activation of the mitochondrial pathway-related caspases has been reported in several studies (Shenker et al., 2001; Ohara et al., 2004; Wising, et al., 2005; Liyanage et al., 2010), but among the analyzed cell lines, a few also employ the extrinsic pathway through the activation of caspase-8, which might correlate to their p53 negative status (Hickey et al., 2005; Ohara et al., 2004; Shenker et al., 2001). Finally, a caspase-independent apoptotic pathway has been observed as a late cell-death response to CDT that involves the accumulation of reactive oxygen species and mitochondrial membrane alterations (Ohara et al., 2008).

CDT can induce apoptosis in all cell types tested so far including fibroblasts, epithelial, endothelial or haematopoietic cells, proliferating or non-proliferating primary cells, and established cell lines (Jinadasa et al., 2011). However, haematopoietic cell lines seems more prone to induce a rapid apoptotic response within the first day of CDT exposure, and in some cases before any obvious cell cycle arrest (Ohguchi et al., 1998; Gelfanova et al., 1999; Cortes-Bratti et al., 2001; Ohara et al., 2004). Hence, when tested in identical conditions, epithelial cells, keratinocytes or fibroblasts exhibit a mild apoptotic response compared to monocytes or T cells (Wising et al., 2005). Instead, cells from non-haematopoietic lineages die either by apoptosis or necrosis, the latter being perhaps a consequence of abortive mitosis (see above). The ratio between apoptotic and necrotic cell death is dose-dependent and fluctuate with the timing of treatment. Moreover, apoptotic/necrotic responses of intoxicated cells from similar lineages might depend on the cell types as shown for endothelial cells (Bielaszewska et al., 2005).

To summarize, haematopoietic cells show the most dramatic apoptotic response to CDT, and p53-deficient cells are commonly more resistant to CDT-induced apoptosis, as well as cells exhibiting a high BCL-2 expression level. However, other parameters have to be considered, like cellular susceptibility to the CDT bacterial source, as well as exposure time or toxin concentration. As a matter of fact, all these parameters modulate the balance between the different cellular outcomes after CDT exposure – i.e. prolonged cell cycle arrest, apoptosis, necrosis or even senescence (Blazkova et al., 2010).

CDT Triggers a DNA Damage-Related Response

Interestingly, many studies pointed out that morphological and cell cycle defects induced by CDT are similar to those observed with some DNA damaging agents. As soon as CDT-mediated cell cycle defects were reported, similarities between the checkpoints activated during the DDR have been noticed (Comayras et al., 1997; Whitehouse et al., 1998). The DDR is a multistep process that senses DNA damage and triggers a signaling cascade that activates cell cycle checkpoint arrest at the G1/S or G2/M boundary, to allow sufficient time

to repair DNA lesions or to promote programmed cell death if too much damage has been sustained (Deckbar et al., 2011). In other words, DNA damage-mediated G1/S and G2/M checkpoints interrupt cell cycle progression, in order to prevent the succession of genomic instability at crucial steps including DNA replication during S phase and chromosome segregation during mitosis. In addition, when arising during S phase, DNA lesions stall the replication forks and trigger an intra-S checkpoint that slows down (but does not block) the replication progression (Ghosal and Chen, 2013; Jones and Petermann, 2012). When treated with a lethal dose of etoposide, a topoisomerase II poison that induces DNA double strand breaks (DSBs) by inhibiting the ligation of DNA ends produced after the cleavage intermediate step (Liu, 1989), HeLa cells present patterns similar to those observed after *Ec*CDT-I exposure, including G2/M block, loss of cell viability and morphological alterations (Sert et al., 1999). Furthermore, *Hd*CDT and ionizing radiations induce comparable cell cycle arrests on HL fibroblasts or on HEp-2 cells (Cortes-Bratti et al., 2001). Yet, ionizing radiation-mediated DDR activation is a well-known consequence of DSB induction (Deckbar et al., 2011; Vignard et al., 2013). Finally, De Rycke et al. pointed out that the sequential morphological changes promoted by *Ec*CDT are reminiscent of those due to etoposide or irradiation (De Rycke et al., 2000). Taken together, these reports strongly suggest that CDT intoxication elicits a cellular response related to DSB signaling.

Phosphatidylinositol 3-kinase related protein kinases (PIKK) are central players in the DDR. ATM (ataxia-telangiectasia-mutated) drives the G1/S checkpoint, which also requires active p53, whereas the intra-S checkpoint depends mostly on ATR (ATM- and Rad3-related). Either ATM or ATR can activate the G2/M checkpoint. Whilst ATR responds to single-stranded DNA (ssDNA) that are typically formed at replication forks blocked by a broad range of lesions (Zou and Elledge, 2003), ATM is specifically activated by DSBs through the MRE11-RAD50-NBS1 (MRN) complex (Lee and Paull, 2005) (Figure 5A and B). MRN is a damage sensor that is rapidly localized to DSB sites, where NBS1 recruits inactive ATM dimers (Falck et al., 2005; Lee and Paull, 2005). Autophosphorylation of ATM at serine 1981 induces dimer dissociation, releasing free active monomers that can then phosphorylate downstream substrates including H2AX, CHK2 or p53 (Bakkenist and Kastan, 2003; Matsuoka et al., 2007) (Figure 5C). H2AX is an H2A histone variant that is rapidly phosphorylated on serine 139 (referred to as γH2AX) on several Megabases surrounding DSB sites (Rogakou et al., 1999; Rogakou et al., 1998). The Checkpoint kinase 2 (CHK2) plays a central role in the G2/M checkpoint activation by phosphorylating p53, CDC25A and CDC25C (Ahn et al., 2004), as phosphorylated CDC25 proteins are inactive and cannot activate CDK1, thereby preventing mitotic entry (see above and Figure 4).

CDT Activates the ATM-Dependent DSB Signaling Pathway

The accumulation of DDR proteins to DSB sites has been widely analyzed by immunocytological fluorescent observations of docking structures called foci (Vignard et al., 2013). Strikingly, CDT exposure recapitulates the different steps of DSB signaling (Figure 5). First, the toxin induces foci formation of the three MRN complex members, as shown with CDT from *H. ducreyi* for MRE11 (Li et al., 2002), from *C. jejuni* for RAD50 (Hassane et al., 2003) or from *H. hepaticus* for NBS1 (Guerra et al., 2010) (Figure 5B). Infection of gingivial epithelial cells with *A. actinomycetemcomitans* producing CDT seems to increase ATM

protein levels, suggesting that the host response to CDT intoxication involves the ATM-related pathway (Alaoui-El-Azher et al., 2010). The accumulation of γH2AX is currently the most assessed DSB biomarker, as it signals DSBs very quickly after their formation and disappears once they are repaired. In response to CDT produced by *H. hepaticus, C. jejuni, H. ducreyi* and *E. coli*, many studies have shown that H2AX is phosphorylated and γH2AX foci are formed (Bielaszewska et al., 2005; Blazkova et al., 2010; Fedor et al., 2013; Hassane et al., 2003; Li and Sharipo, 2002; Liyanage et al., 2010) (Figure 5C). Moreover, 53BP1, another well-used DSB marker, also forms foci after CDT exposure (Figure 5D), which colocalize with γH2AX foci, strongly supporting the presence of DSBs (Blazkova et al., 2010; Fedor et al., 2013).

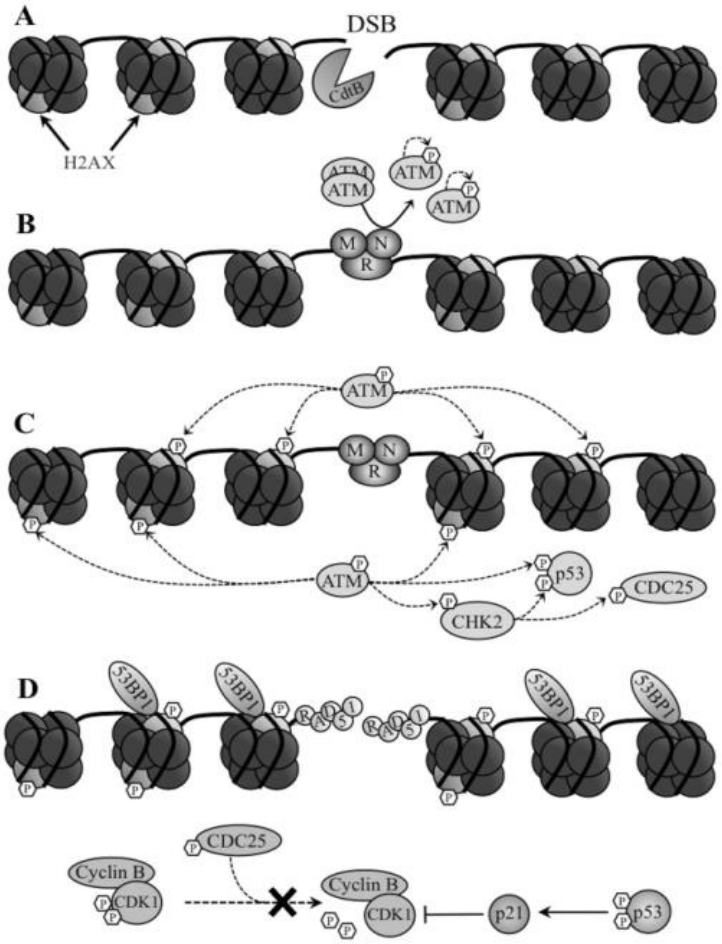

Figure 5. *Activation of the ATM-dependent DSB response induced by CdtB.* A. CdtB induces double-strand breaks (DSB) on host cell chromatin. B. The MRE11-RAD50-NBS1 complex (MRN) binds DSB ends and recruits inactive ATM dimmer through NBS1. ATM autophosphorylation induces release of free active monomers. C. ATM phosphorylates downstream effectors including H2AX (reappointed γH2AX), p53 (stabilization) and CHK2 (activation). Once activated, CHK2 phosphorylates p53 (stabilization) and CDC25 phosphatases (inactivation). D. 53BP1 and RAD51 are recruited to DSB site. Maintaining CDC25 in the inactive form induces the accumulation of phosphorylated (inactive) CDK1. In parallel, p53 transcriptionally upregulates the CDK1 inhibitor p21. CDK1 inhibition prevents mitotic entry.

Finally, CDT intoxication of fibroblasts promotes ATM-dependent CHK2 activation (Cortes-Bratti et al., 2001; Alby et al., 2001; Blazkova et al., 2010; Alaoui-El-Azher et al., 2010). As ATM and CHK2 kinase activities are required for p53 stabilization and DNA damage-related checkpoint activation (Ahn et al., 2004), several studies have shown that CDT induces p53 phosphorylation and stabilization, as well as accumulation of its downstream effector p21, a cyclin-dependent kinase inhibitor (Cortes-Bratti et al., 2001; Sato et al., 2002; Li and Sharipo 2002; Yamamoto et al., 2004; Blazkova et al., 2010) (Figure 5D).

The Cellular Outcomes Induced by CDT Are Associated with the DDR

Thus, the cell cycle defects induced by CDT seem to rely on the ATM-CHK2 pathway that activate p53 and p21, and inactivate CDC25 proteins, both participating to inhibit cyclin-dependent kinase activities and eventually lead to cell cycle arrest (Deckbar et al., 2011). Pharmacological inhibition of PIKK with caffeine or LY294002 prevents CHK2 and CDK1 phosphorylation and partially reverses cell cycle arrest of fibroblasts exposed to CDT produced by *E. coli*, *A. actinomycetemcomitans* or *H. ducreyi* (Cortes-Bratti et al., 2001; Alby et al., 2001; Alaoui-El-Azher et al., 2010). However, theses chemical inhibitors are not specific to ATM, and involvement of other PIKK members in response to CDT cannot be ruled out. Interestingly, use of wortmannin, another PIKK inhibitor, at a concentration that blocks ATM but not ATR kinase activity, does not affect CDT-induced CHK2 phosphorylation (Alby et al., 2001). Whether this response depends on ATR still needs to be established, but recent work from our lab demonstrated that CDT treatment provokes replicative stress prior to blocking cells at G2/M, also supporting an ATR-mediated signaling pathway (Fedor et al., 2013). However, ATM-deficient lymphoblastoid cell lines show a delayed CDT response, characterized by the progression through the G2/M block and a diminution of γH2AX levels, p53 stabilization and RhoA-dependent actin stress fiber formation (Cortes-Bratti et al., 2001; Li and Sharipo, 2002; Frisan et al., 2003). Besides, the apoptotic response to CDT is attenuated when ATM or CHK2 protein levels are depleted (Alaoui-El-Azher et al., 2010). Altogether, these studies clearly link DNA damage signaling to the cellular outcome following CDT intoxication. In agreement with this model, different genetic screens in yeast identified numerous DNA repair genes to be essential for surviving upon CDT intoxication (Guerra et al., 2011; Kitagawa et al., 2007; Matangkasombut et al., 2010). Lastly, HeLa cells deficient for RAD51, a key component of the homologous recombination repair machinery, are hypersensitive to *E. coli* CDT treatment (Fedor et al., 2013), corroborating that DNA damage repair activity is crucial for cells to resist CDT mediated cytotoxicity.

CdtB Supports the CDT Genotoxic Potential

CdtB Delivery in Host Cell Can Mimic CDT Intoxication

As stated above, CDT is an AB toxin, in which CdtB represents the catalytic A-subunit (Nesić et al., 2004). CdtA and CdtC form a heterodimeric B-subunit that mediates toxin

binding to host cells and allows cellular uptake of CdtB. Interestingly, *S.* Typhi, devoided of CdtA and CdtC genes, forms an effective typhoid toxin composed by CdtB, PltA and PltB (Song et al., 2013). Intriguingly, a recombinant *C. jejuni* holotoxin reconstituted *in vitro*, in which *Cj*CdtB has been substituted by *S.* Typhi CdtB, still retains its cyclomodulin and cytotoxic activities (Haghjoo and Galán, 2004). As CdtB from *S.* Typhi and *C. jejuni* are among the most divergent in the CdtB family (Gargi et al., 2012), this result suggests that, although divergent, these CdtB proteins share similar structural properties and mode of action. Moreover, CdtB delivery into host cells is sufficient to elicit the CDT-related cellular defects, as demonstrated by transient expression or direct introduction into host cells of CdtB from *H. ducreyi* (Li and Sharipo, 2002; Wising et al., 2010), *C. jejuni* (Lara-Tejero and Galàn, 2000), *E. coli* (Elwell et al., 2001; McSweeney and Dreyfus, 2004), *A. actinomycetemcomitans* (Mao and DiRienzo, 2002; Nishikubo et al., 2003), *H. hepaticus* (Liyanage et al., 2013) or *S.* Typhi (Haghjoo and Galán, 2004).

Structural and Biochemical Features of CdtB

In silico analyses of the predicted *E. coli* and *C. jejuni* CdtB amino-acid sequences revealed a limited, but relevant, sequence homology with the well documented mammalian deoxyribonuclease (DNase) I (Elwell and Dreyfus, 2000; Lara-Tejero and Galàn, 2000). More precisely, residues from DNase I shown to be essential for enzymatic activity are strictly conserved in all CdtBs homologs − these include residues involved in catalytic activity, divalent cation binding and DNA binding (Pan et al., 1998). Furthermore, the crystal structures of CDT produced by *H. ducreyi* (Nesić et al., 2004), *A. actinomycetemcomitans* (Yamada et al., 2006) and *E. coli* CdtB alone (Hontz et al., 2006) highlighted the structural similarities between CdtB and DNase I, especially concerning their active site and their DNA binding motif. Based on these studies, comparative modeling and structural analyses of CdtB from various origins reinforced the assumption of a conserved three-dimensional feature among all CDTs (Hu et al., 2006) and, by extension with DNase I similarities, of a nuclease-mediated cellular toxicity for CDT.

Therefore, CdtB nuclease activity has been assessed, after incubating plasmid DNA with the CDT holotoxin or with CdtB alone, in order to evaluate the conversion of the supercoiled plasmid to the relaxed (nicking activity) or the linear (DSB activity) form. The nuclease activity of CdtB was first observed with supernatant or periplasmic extracts of bacteria expressing *E. coli* CdtB alone or the CDT holotoxin (Elwell and Dreyfus, 2000). However, a substantial nicking activity, independently of CdtB, demonstrated that a preliminary purification step is necessary to prevent extract-mediated plasmid degradation. Thus, a recombinant *H. hepaticus* CdtB has been purified to homogeneity and biochemical assays emphasize the similarities with DNase I activity, i.e. magnesium and calcium dependencies, and inhibition by zinc and G-actin (Dassanayake et al., 2005). Nevertheless, this study also pointed out that CdtB activity is at least 100 fold weaker than the one observed for the DNase I, a difference corroborated with *H. ducreyi* CDT (Li and Sharipo, 2002). Finally, chimeric constructs between half parts of *Aa*CdtB and human DNase I retain nuclease activity similar to CdtB, strongly supporting a close structural and functional relationship between these two proteins (DiRienzo et al., 2009). Interestingly, it has been shown that *Hd*CdtB activity is inhibited in the context of the holoenzyme, and structural analysis revealed that the N-

terminus tail of CdtC occludes the active site of CdtB, by interacting with several key residues (Nesić et al., 2004). In agreement with that, CdtB nuclease activity is restored when the tripartite complex is formed with a truncated form of CdtC missing the N-terminus tail. Modeling data suggest that this CdtC inhibitory role can be extrapolated to other CDTs (Hu et al., 2006). Thereby, *Aa*CdtB completely lost its nuclease activity when properly complexed with CdtA and CdtC (Cao et al. 2005), suggesting that once within the host cell, and during intracellular trafficking, CdtB is sequestered and has to be freed in order to be activated. Finally, molecular dynamics simulations indicate that CdtB adopts a conformational change when released from the complex, stabilizing the active site by maintaining the DNA binding groove in a closed form (Hu and Stebbins, 2006).

CDT Cytotoxicity Relies on the Nuclease Activity of CdtB

Sequence and structural comparison between CdtB and DNase I enabled the identification of residues important for CdtB nuclease activity. Among them, three catalytic residues of DNase I, namely H134, D212 and H252, are conserved within CdtB bacterial homologs. Mutations of these residues completely abolish CdtB nuclease activity (Elwell and Dreyfus, 2000; Nesić et al., 2004). Moreover, CDT holoenzymes from various origins that contain these CdtB catalytic mutants do not elicit the DDR cellular response nor possess any cytotoxic activity (Elwell and Dreyfus, 2000; Fedor et al., 2013; Haghjoo and Galán, 2004; Hassane et al., 2001, 2003; M. Lara-Tejero and Galàn, 2000; Nesić et al., 2004; Song et al., 2013). In the same way, metal ion binding residues seems essential for nuclease and cytotoxic activities (Elwell and Dreyfus, 2000; Lara-Tejero and Galàn, 2000). The CDT nuclease activity has been investigated *in vivo* by direct visualization of genomic DNA fragmentation after pulse field gel electrophoresis or after single cell gel electrophoresis assays. It appears that the chromosomal fragmentation observed in *Cj*CdtB expressing yeasts depends on an intact catalytic site (Hassane et al., 2001) and the cellular DNA strand breaks rapidly observed after *Ec*CDT exposure are abrogated by the mutation of a key catalytic residue (Fedor et al., 2013). A similar observation has been done with a *Hd*CDT divalent cation-binding mutant, unable to induce DSBs on genomic DNA (Guerra et al., 2005).

Mutations of the putative DNA binding residues lead to a diminution of the nuclease activity, instead of abolition (Nesić et al., 2004), without inducing a typical G2/M arrest, suggesting a dose-dependent cellular defect associated with the CdtB activity. This is supported by the fact that the pathogenic potential of *A. actinomycetemcomitans* isolates correlate to a single nucleotide polymorphism in the *cdtB* gene, modulating the nuclease activity of CdtB (Nishikubo et al., 2006).

Finally, chronic exposure to sublethal doses of *H. hepaticus* or *H. ducreyi* CDTs, that induces minor genotoxic stress without altering cell cycle nor viability, causes mutations and chromosomal aberrations, both being hallmarks of misrepaired DNA lesions (Guidi et al., 2013).

Moreover, these cells present other characteristics of transformed cells: proliferation independent of anchorage signals, inhibition of the DDR leading to uncontrolled proliferation and cell death escape. Altogether, these analyses demonstrate that CdtB-induced DNA damage are strongly associated with cellular toxicity and explain why CDT is classified as a genotoxin and even a potential carcinogen.

The Phosphatase Paradigm

Intriguingly, computational analyses based on sequence homology also predict that CdtB share significant sequence conservation with signaling proteins, including inositol polyphosphate 5-phosphatases (IP5P) (Dlakic, 2001). More recently, structural similarities have been detected between CdtB and the IP5P family, with a strong conservation of key residues in the active site (Shenker et al., 2007). This led the authors to test whether CDT cytotoxicity could be explained by a potential phosphatase activity. Biochemical analyses showed that CdtB hydrolyses phosphatidylinositol-3,4,5-triphosphate (PIP_3) to phosphatidylinositol-3,4-diphosphate. PIP_3 is also the preferential substrate of the PTEN and SHIP1 phosphatases, both playing a regulatory role in the Akt signaling pathway. Interestingly, lymphoid cells that are defective for these phosphatases, and therefore maintain a high level of PIP_3, are highly sensitive to *Aa*CDT. Moreover, CDT intoxication of these cells induces a strong decrease in the basal PIP_3 level. It is therefore tempting to speculate that CdtB-related phosphatase activity may represent an alternative cytotoxic pathway, particularly efficient in lymphocytes as the viability of many leukemic cell lines depends on an elevated level of PIP_3 (Shenker et al., 2007). The higher CDT susceptibility of lymphocytes over fibroblasts, which may be attributable to their specific rapid apoptotic response before any distention phenotype and even before any clear G2/M arrest (Shenker et al., 1999; Cortes-Bratti et al., 2001), reinforces this hypothesis. Mutational analyses indicate that CDT-mediated cell cycle arrest of lymphoid cells specifically involves the CdtB phosphatase activity (Shenker et al., 2007). However, contradictory results came from another study demonstrating that a non-proliferative monocyte cell line is sensitive to an *Aa*CdtB phosphatase mutant, but resistant to the wild type CDT when co-treated with specific PIP_3 phosphatase inhibitors (Rabin et al., 2009). In yeast, a model system devoid of PIP_3, the response to CdtB relies on the DDR machinery and nuclease-dead CdtB mutants show no effect (Hassane et al., 2001; Matangkasombut et al., 2010). More investigations will be necessary to account for the precise function of nuclease and phosphatase CdtB catalytic activities, particularly on different host cell types.

Conclusion

CDT has been the first bacterial toxin known to act as a genotoxin in the nucleus of host target cells. Thus, CDT toxins have been high research subjects for scientists within the last decade. To date, only a few bacterial genotoxins have been described: the CDTs presented in this chapter, the colibactin (Nougayrède et al., 2006) and the *E. coli* Usp (uropathogenic-specific protein) (Nipic et al., 2013). As reported here, CDT intoxicated host cells activate the DDR, in order to counteract the CDT genotoxic activity. This is also the case for colibactin (Cuevas-Ramos et al., 2010). Chronic exposure to genotoxins may lead to genomic instability, as illustrated *in vitro* with CDT (Guidi et al., 2013) or *in vivo* with colibactin (Cuevas-Ramos et al., 2010). Genomic instability has been shown to fuel the transformation process, directly enhancing the risk of inducing tumor development (Janssen and Medema, 2013). However, inflammation is also known to contribute to cancerogenesis (Morrison, 2012), and bacterial pathogens can induce DNA damage in host cells through chronic

inflammation (Grivennikov et al., 2010). A new story is emerging, where bacteria are tightly associated with cancer development, through the expression of genotoxins or through diverse genotoxic processes (Aituov et al., 2012; Guerra et al., 2011). In this context, elucidating the precise mechanisms involved in bacterial pathogenicity-associated carcinogenesis will be of particular interest.

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