# Enteric Toxins from Bacteria Colonizing Human Gut

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The large and heterogeneous microbial population colonising the human intestinal tract includes a number of aerobic and anaerobic bacteria that produce one or more toxins. While exhibiting very different physico-chemical properties these exotoxins share the ability to penetrate intestinal cells after their binding to a specific surface receptor, thus reaching a subcellular target at membrane or cytoskeleton level. The most relevant *in vitro* and *in vivo* data, reported in the literature, on the mode of action of the major enterotoxins and cytotoxins produced by bacteria belonging to the human gut microflora are reviewed in the light of our recent knowledge on bacteria-host cell interactions.

## INTRODUCTION

Intraluminal flora of the intestinal tract forms an extremely large and heterogeneous microbial ecosystem including aerobic, microaerofilic and strictly anaerobic bacteria. The small intestine represents a transition area between the scarcely colonised stomach and the abundant colonic flora, normally reaching in healthy subjects $10^{11}$ colony forming units/mg of intestinal content. In fact, a third of the faecal dry weight consists in bacteria, the anaerobes outnumbering the aerobic species by a hundred to ten thousand times.

Among the numerous extracellular products, exotoxins produced and released from bacteria belonging to the normal gut microflora appear to be potent weapons whose possible harmful effects on the intestinal mucosa deserve in our opinion wider attention. Even more by the light of the increasing amount of *in vitro* and *in vivo* results obtained in recent years on their mode of action. These toxins are very heterogeneous as far as their physico-chemical properties and bacterial sources are concerned, being produced by anaerobic (*Bacteroides fragilis*, *Clostridium difficile*, *Clostridium perfringens*) and aerobic (*Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*) species (Table I). However, this group of toxins shares the ability of penetrating animal cells after binding to a specific receptor present on the cell surface, thus reaching an intracellular target at membrane and/or cytoskeletal level.

In fact, recent studies on different mammalian cell lines indicate the microfilaments, constituted by the 42 kDa G-actin and several actin binding proteins, as the primary targets for an increasing number of toxins (1–6) produced by enteric bacteria. Recalling the significant role of the actin cytoskeleton in numerous cellular functions (7–11) and the participation of actin as the main constituent in the intestinal brush border cytoskeleton, the direct or indirect role of these toxins as proinflammatory agents and virulence factors needs to be further elucidated. In this regard a review of the most relevant data available so far on the main features and the mode of action of the major enterotoxins and cytotoxins produced by enteric bacteria colonising the human intestinal tract (12) may be of value in identifying unclear aspects, missing data and future research targets.

## BACTEROIDES FRAGILIS TOXIN (BFT)

*Bacteroides fragilis* is an obligately anaerobic bacterium that is part of the normal colonic flora (13). Despite accounting for only about 1% of $10^{11}$ organisms/g of stool, *B. fragilis* is the obligate anaerobe most frequently isolated from patients with intraabdominal abscesses and bloodstream infections. However, neither diarrhoeal disease nor extracellular toxin production due to *B. fragilis* was appreciated until 1984. In fact, the initial suggestion that *B. fragilis* could be a cause of diarrhoea came from studies of diarrhoeal disease in lambs in which Myers and his colleagues (14) noted that stools from diarrhoeic lambs were able to stimulate a secretory response in ligated intestinal loops in healthy lambs. In a series of experiments involving cultures on various media and serial passage of strains in lamb ligated intestinal loops, it was determined that the

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secretory response observed could be attributed only to an obligate anaerobe which was identified as being *B. fragilis*. By experimental inoculation of lambs with the secretion-producing strains of *B. fragilis*, clinical signs of the natural disease were reproduced, whereas with strains that were nonsecretory in lamb ligated intestinal loops no signs of disease were found. Further studies demonstrated that culture filtrates of the pathogenic *B. fragilis* strains, but not of the control *B. fragilis* strains, concentrated 20-fold, also stimulated a secretory response in lamb ligated intestinal loops at 18 hours but not before 6 hours (15). Notably, loops treated for $>18$ hours with concentrated culture filtrates tended to burst, an indication of the potency of the secretory response. On the basis of these observations, strains that stimulate secretion in lamb ligated intestinal loops have been termed *enterotoxigenic B. fragilis* (ETBF).

The fluid response was dependent on the animal model used. In fact, in the ileum this response was greater in lambs than in rabbits and rats, whereas the fluid response in the colon was greater in rabbits than in lambs and rats. Analysis of the intestinal fluid elicited by the enterotoxin revealed an accumulation of sodium and chloride as well as albumin and total protein. After histological examination mild necrosis of epithelial cells, crypt elongation, villus attenuation, and hyperplasia were revealed. Moreover, there was an extensive detachment and rounding of surface epithelial cells as well as an infiltration of neutrophils (16).

Several studies suggest that the intestinal effects of *B. fragilis* are attributable to a protein toxin (17). This toxin is an extracellular heat-labile protein with an estimated molecular weight of about 20000 Da as determined by gel filtration chromatography on Superose-12 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified toxin is stable at $-20$°C and 4°C and upon freeze-drying, but it is unstable at temperatures above 55°C. It is characterised by an isoelectric point of approximately 4,5 and is stable at pHs between 5 to 10. It is resistant to trypsin and chymotrypsin but is sensitive to proteinase K and *Streptomyces* protease (18). Like culture filtrates of ETBF, the purified toxin stimulates fluid accumulation in lamb ligated ileal loops and alters the morphology of intestinal epithelial cells *in vitro* as well. Both of these biological activities are neutralised by monospecific antisera to the purified toxin. The amino acid sequence revealed a zinc-binding consensus motif signature (HEXXHXXGXXH/Met-turn) characteristic of metalloproteases termed metzincins (19) and the sequence comparison shows high identity to matrix metalloproteases (e.g., human fibroblast collagenase) within the zinc-binding and Met-turn region. One g-atom of $Zn^{2+}$ per molecule is contained in the purified enterotoxin and it is capable of hydrolysing gelatin, azocoll, actin, tropomyosin, and fibrinogen. Moreover, the enterotoxin is capable of undergoing autodigestion. Optimal proteolytic activity occurs at

**Table I**

*Bacteria colonising human gut and their major enteric toxins*

| Bacterium | Toxins | MW | Mode of action |
|---|---|---|---|
| *Bacteroides fragilis* | BFT | 20 kDa | Cytotoxic and enterotoxic |
| *Clostridium difficile* | Tox A | 308 kDa | Cytotoxic and enterotoxic<br>Pro-apoptotic |
| | Tox B | 270 kDa | Cytotoxic<br>Pro-apoptotic |
| *Clostridium perfringens* | Enterotoxin | 35 kDa | Enterotoxic and cytotoxic<br>Pore-former |
| *Enterococcus faecalis* | Cytolysin | | Acting both as a haemolysin toxic to eukaryotic cells and as a bacteriocin active against gram-positive bacteria<br>Enterotoxic? |
| *Escherichia coli* | CDTs | 76 kDa | Inhibitor of cell division |
| | CNFs | 110 kDa | Cytotoxic and dermonecrotic<br>Anti-apoptotic |
| | LTs | 85 kDa | Enterotoxic |
| | STs | 2 kDa | Enterotoxic |
| *Staphylococcus aureus* | SEA<br>SEB<br>SEC1<br>SEC2<br>SEC3<br>SED<br>SEE | 22–29 kDa | Enterotoxic, superantigen,<br>indirectly pro-apoptotic via T-cell<br>activation and cytokine secretion |
| | Delta-toxin | 3 kDa | Cytolytic<br>Pore-former<br>Enterotoxic? |

37°C and pH 6.5. Primary proteolytic cleavage sites in actin have been identified, revealing cleavage at Gly-Met and Thr-Leu peptide bonds. Enzymatic activity is inhibited by metal chelators but not by inhibitors of other classes of proteases. Additionally, both, the cytotoxic and enterotoxic activity is inhibited by the metal chelators such as EDTA and 1,10-phenanthroline.

Two isoforms of *B. fragilis* toxin (BFT) have been isolated, one secreted by the lamb (ETBF strain VPI 13784) and the second by the piglet (ETBF strain 86-5443-2-2). Nucleotide sequence analysis studies revealed 92% identity and 95% similarity in the amino acid sequences of these two isoforms of BFT. Based on their biochemical properties, these isoforms represent two distinct proteins and are therefore termed BFT-1 (strain VPI 13784) and BFT-2 (strain 86-5443-2-2). However, the biological activities of these two proteins are very similar. The *bft* gene from ETBF strain 86-5443-2-2 consists of one open reading frame of 1,91 nucleotides encoding a predicted 397-residue holotoxin with a calculated molecular weight of 44,493 Da. BFT is most probably synthesised as a pre-proprotein by ETBF strains as the comparison of the predicted BFT protein sequence with the N-terminal amino acid sequence of purified BFT indicates. These data suggest that BFT is processed to yield a biologically active toxin of 186 residues with a molecular mass of about 20 kDa which is secreted into the culture supernatant. In fact, analysis of the holotoxin sequence predicts a 20-residue amphipathic region at the carboxy terminus of BFT (20, 21). Comparison of the sequences available for the *bft* genes from ETBF 86-5443-2-2 and VPI 13784 revealed two regions of reduced homology.

Hybridisation of oligonucleotide probes specific for each *bft* with toxigenic *B. fragilis* strains revealed that 51 and 49% of toxigenic strains contained the 86-5433-2-2 and VPI 13784 *bft* genes, respectively. No toxigenic strain hybridised with both probes. These two subtypes have been termed *bft* 1 (VPI 13784) and *bft*-2 (86-5433-2-2).

The BFT is detectable (22) by morphological changes on HT29, T84, and Caco-2 cells, continuous intestinal epithelial cell lines derived from human colonic carcinomas. The toxin does not alter the morphology of Chinese hamster ovary (CHO), Y-1 adrenal, or MDCK cells, all of which are nonintestinal cell lines (23). Numerous studies have been performed on HT29/C1 cells, which represent a suitable model for studying the TBF and it constitutes an established cytotoxic assay used to detect enterotoxigenic *B. fragilis* (24). After treatment with crude or purified BFT, cells become rounded with numerous surface blebs and the cell clusters dissociate within 3 hours (25, 26). Such alterations in HT29 cells are completely reverted 24 h after the addition of the toxin (27). Experiments with purified toxin reveal that subconfluent HT29/C1 cells treated with as little as 0.1 ng of purified toxin/ml (5 pM) develop morphological changes. Furthermore, staining of toxin-treated HT29/C1 cells with rhodamine-phalloidin, which specifically binds to filamentous actin (F-actin), reveals dissociation of F-actin after toxin treatment that is consistent with the BFT altering the cytoskeleton of intestinal epithelial cells. In fact, typical effects are the redistribution of F-actin, which become completely marginalised, and the loss of stress fibres, the filamentous and contractile form of actin (27). BFT acts also on cell volume and this response is persistent and dependent on the proteolytic activity of BFT. Intoxicated cells exhibit regulatory volume decrease, suggesting that toxin-treated cells remain physiologically dynamic.

For further assessment of the effect of crude and purified toxin on the physiology of intestinal epithelial cells *in vitro*, HT29/C1, Caco-2, or T84 cells were used for studies in Ussing chambers (28, 29). The Ussing chamber is an experimental technique for investigating the physiology of monolayers of cultured intestinal epithelial cells under conditions of ionic, osmotic, and electrical equilibrium. By assessment of three parameters, i.e., short circuit current (I or Isc), potential difference (V or PD) and monolayer resistance (R), changes in active ion transport and monolayer resistance are measured. Under voltage-clamped conditions, Isc and PD are measured; R is calculated by Ohm's law ($V = IR$). The most consistent observation with both crude and/or purified toxin was a striking decrease in monolayer resistance over time. The onset of this decrease typically occurred 20–50 minutes after treatment of apical or basolateral surfaces of the monolayers with toxin. However, the loss of monolayer resistance occurred more quickly and was more complete with toxin application to basolateral surfaces. Depending on the cell line used, in some experiments an increase in Isc that was separable from the calculated changes in monolayer resistance was also observed. This increase in Isc suggests that BFT may also stimulate chloride secretion. These changes in the physiology of HT29/C1 or T84 monolayers occur without release of intracellular lactate dehydrogenase or changes in protein synthesis, thus indicating that cellular damage is not responsible for the loss of monolayer resistance. In addition, T84 monolayers stained with rhodamine-phalloiding showed alteration in F-actin distribution in the cells that may account for the diminished monolayer resistance observed. However, the relationship of the changes in F-actin distribution to the changes in Isc observed in T84 monolayers in response to the ETBF toxin is presently unknown. Although the real contribution of the BFT to the pathogenesis of diarrhoeal disease is yet unknown, it is postulated that this toxin potentially contributes to diarrhoea by altering the barrier function of the intestinal epithelium and by stimulating chloride secretion.

A recent report demonstrates that BFT specifically cleaves E-cadherin within the extracellular domain of the zonula adherens protein. Cleavage of E-cadherin by BFT

is ATP-independent and essential for the morphologic and physiologic activity of BFT. However, the morphologic changes occurring in response to BFT are dependent on target-cell ATP. E-cadherin is, thus, the cellular substrate for a bacterial toxin and this phenomenon represents the identification of a mechanism of action, cell-surface proteolytic activity, for a bacterial toxin (30).

## *CLOSTRIDIUM DIFFICILE* TOXINS

The Gram-positive anaerobic bacterium, *Clostridium difficile*, is the causative agent of pseudomembraneous colitis and of most cases of antibiotic-associated diarrhoea (31–40).

There are many virulence factors expressed by pathogenic strains of *C. difficile* and involved in the onset of diarrhoea and colitis, including fimbriae, proteases and toxins (41–51). The most important and best studied since the early 1980s (52–55) are two exotoxins: toxin A, a cytotoxic enterotoxin (56–62) and toxin B, a more potent cytotoxin lacking enterotoxic activity (63, 64). Both toxins belong to the group of intracellularly acting bacterial proteins which have to be internalised via receptor-mediated endocytosis to exert their cytotoxicity.

Toxins A and B are very large single-chain polypeptides having molecular weights of 308 kDa and 270 kDa, respectively. The genes encoding *C. difficile* toxins have been cloned and sequenced (65–69). The two toxins have about 60% homology at the amino acid level and share an identical structural composition. The polypeptides are composed of three functional domains: i) the N-terminal catalytic domain; ii) the intermediate translocation domain; iii) the C-terminal receptor binding domain. The N-terminal region is thought to specify toxin activity and the rather small hydrophobic intermediate part is involved in entry of the toxins into cells. Near the C-terminal domain is a region comprising of CROPS (C-terminal repeated oligopeptides), which might be involved in membrane receptor binding. In toxin A these CROPS act as receptor-binding sites for galactose-containing residues (Gal_1-3Gal_1-4GlcNAc) on the surface of the intestinal epithelial cells in hamsters and rabbits (70, 71). This is similar to the receptor in the human intestine where I, X and Y antigens in the membranes of epithelial cells lining the mucosa may bind the toxin (72). All these carbohydrate antigens contain the type 2 core (Gal1_1-4GlcNAc) that appears to be the minimum carbohydrate structure bound by toxin A.

Other common structural features are a hydrophobic region of approximately 50 amino acids in the middle part of the protein and four conserved cysteine residues.

Excellent studies have been performed to investigate the properties of these conserved features by constructing toxin B mutants (73). In particular a significant decrease of cytotoxicity was observed by (i) substituting histidine with glutamine at the nucleotide-binding site, suggesting that this region could be the active site; and (ii) removing the internal hydrophobic region that may be responsible for processing and internalisation.

Despite their similarities, the biological activities of the toxins differ. Toxin A causes tissue damage and changes in permeability that result in fluid accumulation in rabbit ileal and colonic loops (74, 75). By contrast, in the animal model toxin B shows no effects on permeability or intestinal integrity.

The observation that a mixture of toxin B with low amount of toxin A when given to hamsters provokes their death suggests that toxin B acts synergistically with toxin A and that the latter is needed for initial tissue damage (45).

Only recently was toxin B shown to cause greater damage to human colonic epithelium *in vitro* than toxin A. In fact it has been demonstrated that, like toxin A, toxin B perturbs the cytoskeleton of human colonic cancer T84 cell monolayers causing an increase in transepithelial resistance (64). In addition, toxin B and toxin A have been tested on human colonic mucosa mounted in Ussing chambers (76). The results of this study demonstrated that both toxins cause morphologic damage and dose-dependent electrophysiologic alterations in human colonic mucosa and that toxin B is more effective than toxin A. This is the first line of evidence of the activity of toxin B on an undisturbed mucosa and may represent a different behaviour of the human intestine with respect to the animal models.

It is possible to speculate that the different biological effects of toxins A and B in the animal model (77) are related to the different receptors used by the toxins.

The main common activity of both toxins, the cytoxicity in cultured cells, even if toxin A is 1000-fold less active than toxin B, has been widely documented and characterised up to the recent determination of the exact mode of action.

In fact, several lines of mammalian cells respond to both toxins although differences in sensitivity can be noted among cell types (78, 79). As viewed by light and scanning electron microscopy, the morphological effect induced by toxins A and B in cell monolayers mainly consists in the retraction and rounding up of the cell body. These morphological changes are generally irreversible and followed by an inhibition of the cell division, but antioxidants are able to protect epithelial cells against the oxidative imbalance due to *C. difficile* toxins (80). Cytoskeleton appears to be strongly involved in such modifications. As observed by fluorescence microscopy, the early response to *C. difficile* toxins is a dramatic change in the microfilament organisation, evident when the cells are still adhering to the substrate (78, 81–88). Prolonging time of exposure, the progressive alteration in the F-actin pattern leads ultimately to the formation of the rounded cells. Protrusions or blebs on the cell surface are also observed by scanning

electron microscopy, the blebbing phenomenon being dose and time dependent (89). These modifications affect the microfilament network of the cytoskeleton without interfering directly with F-actin formation, leading to a relocalisation of F-actin. The change in F-actin organisation is the earliest visible sign of cellular intoxication and is detectable prior to alteration in the microtubular and intermediate filament networks.

As recently demonstrated, toxins A and B are monoglucosyltransferases which catalyse the incorporation of glucose into the small GTP-binding proteins of the Rho family (90–93).

Three subfamilies belong to the Rho family: Rho, Rac and Cdc42. These are involved in the regulation of the dynamic actin cytoskeleton (7). Each of them, however, regulates distinct structures: Rho governs the formation of stress fibres and focal adhesions, Rac is involved in membrane ruffling and Cdc42 in the formation of filopodia. The best understood functional module is the formation of the stress fibres: Rac and Rho regulate the phosphatidylinositol 4-phosphate-5-kinase to form $PIP_2$, which stimulates actin polymerisation and filament growth through interaction with several actin-binding proteins (e.g. gelsolin, profilin). The stress fibres, a supraorganisation of actin filaments, are governed by the RhoA-dependent Rho-Kinase which phosphorylates the myosin light chain thereby activating the actin-myosin system in non-muscle cells. The membrane attachment of the stress fibres is managed through the ERM proteins (ezrin, radixin, moesin). These bifunctional proteins bind through their N-terminal part to transmembrane proteins (CD44 or ICAM proteins) and interact through their C-terminal part with the actin filaments. This interaction is essential for Rho-governed cytoskeletal changes.

All three subfamilies are monoglucosylated by *C. difficile* toxins (91, 92). In fact, toxins A and B catalyse the incorporation of glucose moiety into Thr 37 of RhoA or Thr 35 of both Rac and Cdc42 (88, 94). This modification renders Rho, Rac and Cdc42 inactive, thereby losing their properties to induce actin polymerisation and filament bundling. Inhibition of these GTPases causes the shutdown of signal transduction cascades leading to: (i) depolymerisation of the cytoskeleton; (ii) gene transcription of certain stress-activated protein kinases; (iii) a drop in synthesis of phosphotidyl-inositol 4,5 biphosphate; (iv) and possibly even the loss of cell polarity. By inhibition of signal transduction cascades, toxins A and B are able to block downstream responses (95–99).

Recently, it has been reported that intestinal cells exposed to *C. difficile* toxins A and B exhibit typical features of apoptosis in that a significant proportion of the toxin exposed cells shows nuclear fragmentation and chromatin condensation (100–103). The inhibition of proteins belonging to the Rho family due to *C. difficile* toxins seems to play a role in the induction of apoptosis in intestinal cells.

It has also been demonstrated that toxin B is a potent activator of human monocytes and toxin A is a potent chemoattractant of granulocytes (104, 105).

Hence, toxin B, shown to be extremely toxic for monocytes, initially induces the secretion of proinflammatory products (IL1, IL6, TNF) contributing to epithelium damage, fluid secretion, mucus release and membrane permeability and then eliminates the monocytes, thus preventing phagocytosis of the bacteria.

It has been demonstrated that toxin A causes a rapid dose-dependant increase of free cytosolic calcium in human granulocytes that stimulates cytokine release from macrophages *in vitro* and may be a mediator in eliciting chemotactic response.

Furthermore, a recent study provided indications of IL8 production in T84 and HT29 cells after exposure to toxin A (103). In particular, it has been demonstrated that cultured, as well as primary human colonic epithelial cells, undergo apoptosis following cell rounding and detachment, and then produce IL8.

As IL8 is a potent chemoattractant for polymorphonuclear cells its production may be pivotal in the induction of the intestinal inflammation seen in pseudomembranous colitis.

All these results certainly demonstrate that these two exotoxins represent the major virulence factors produced by *C. difficile* (46, 106), even if other putative virulence determinants (107) could play an important role in its pathogenicity.

## *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

*Clostridium perfringens* is an ubiquitous anaerobic, spore-forming, gram-positive bacterium resident in human gut and associated to gastrointestinal disorders since 1895 by the German microbiologist E. Klein. However, the first episode of food poisoning caused by *C. perfringens* involving about 250 people was described only several decades later, in 1943, by English epidemiologists. This common foodborne illness (108) is caused by the ingestion of foods contaminated with very high concentrations ($> 10^5$ bacteria/g) of type A vegetative cells. The production of toxin in the digestive tract (or *in vitro*) is associated with bacterial sporulation.

*C. perfringens* enterotoxin (109) is a single polypeptide chain with a molecular weight of 35 kDa, eliciting a cytotoxic activity on intestinal cells. The primary subcellular target of the toxin should be represented by the cell membrane as recently demonstrated by us also for *C. perfringens* epsilon toxin (unpublished data). The following steps should characterise the cytotoxic activity:

(i) Enterotoxin binding to a 50 kDa protein receptor present on the membrane;

(ii) Modification of the toxin-receptor complex of about 90 kDa after interaction with a membrane protein of 70 kDa and following establishment of a larger molecular complex of about 160 kDa;
(iii) Consequent structural and functional damages on cell membrane resulting from pore formation due to the partial membrane insertion of the large 160 kDa complex containing the toxin (110).

The major repercussions of membrane damage on intestinal physiology are the inhibition of amino acid transport and the increased secretion of sodium chloride.

## *ENTEROCOCCUS FAECALIS* CYTOLYSIN

This toxin produced by 60% of clinical isolates of *E. faecalis* (111) is a heterodimeric polypeptide consisting of two different structural subunits which are both required for haemolytic and bactericidal activity (112). In fact, early reports on this cytolysin (113, 114) described its ability to inhibit the growth of gram-positive bacteria.

According to recent results (115) the toxin belongs to subgroup A lantibiotics which are elongated, amphiphilic peptides able to open voltage-dependent pores in the bacterial membrane, interfering with energy transduction and modifying membrane potential.

Because of the ability of the toxin to act against gram-positive bacteria, *E. faecalis* cytolysin-producing strains are expected to be able to protect themselves but the mechanism by which immunity is achieved is not definitively understood (116). Recent results seem to demonstrate, however, that immunity arises from a single open reading frame at the 3′ end of the cytolysin operon and that the immunity gene is co-transcribed with the gene encoding the extracellular cytolysin activator (117, 118). In fact, the cytolysin is encoded by large, pAD1-type, pheromone-responsive plasmids and the genetic organisation of the pAD1 operon has been elucidated by transposon insertional mutagenesis, followed by intracellular and extracellular complementation (112, 119).

The *E. faecalis* cytolysin is the only lantibiotic described so far which is also toxic for mammalian cells.

Furthermore, this toxin has been demonstrated to significantly contribute to the severity and lethality of enterococcal infection in three animal models (120–123) as well as in humans (111, 124, 125).

## ENTEROTOXIGENIC *ESCHERICHIA COLI* CYTOTOXINS

Enterotoxigenic *Escherichia coli* (ETEC) are among the most relevant causes of diarrhoea. ETEC are known to produce 4 cytotoxin types: the heat-labile (LT1 and LT2) and the heat-stable (STa and STb) enterotoxins, the cytotoxic necrotising factors (CNF1 and CNF2) and the cytolethal distending toxins (CDTs).

## *E. COLI* HEAT-LABILE ENTEROTOXINS (LTs)

LT1 enterotoxin is closely related to cholera toxin (CT), sharing 80% sequence identity with the A and B subunits of CT (126). LT1, like CT, is an A-B subunit toxin (A/B ratio, 1/5) where B is the subunit (11.6 kDa) binding the GM1 ganglioside receptor and A is the enzymatic intracellular acting component. The A subunit consists of two components generated by proteolysis. A1 (21.8 kDa) is an ADP-ribosyltransferase and A2 (5.4 kDa) links A1 to the B subunits. X-ray crystallography shows that the structure of LT1 consists of 5 B subunits constituting a pentamer with a central pore, where the carboxy terminus of A2 is located. A2 is linked, by an alpha helix, to the A1 subunit (127). The A1 subunit ADP-ribosylates the Gs alpha chain of a heterotrimeric GTP-binding protein which controls the activity of adenylate cyclase (Gs). ADP-ribosylation of alpha Gs by LT1/CT occurs only in the presence of the small GTP-binding protein ARF and leads to the inhibition of the GTPase activity of alpha Gs (128). This provokes the intracellular activation of adenylate cyclase with consequent elevation of cyclic AMP. Like CT, LT1 enters cells by endocytosis and is transferred from early endosomes to late endosomes where, apparently, the separation between the A1 and the A2/B5 subunits occurs. The A1 subunit is therefore transferred from late endosomes to the trans-Golgi network and, in a retrograde fashion, to the endoplasmic reticulum from where it escapes into the cytosol. The enzymatic moiety of LT1 is thus delivered directly at the cell baso-lateral pole where the Gs alpha subunits are located. The A2/B5 fragment ends in lysosomes where it is degraded (129).

Elevation of cAMP by LT1 activates a cAMP dependent kinase (A-kinase) which up-regulates chloride secretion by phosphorylation of the chloride channel CFTR. However, LT1 activity on intestinal cells may induce others effects. Elevating cAMP levels, LT1 also increases the production of platelet activating factor (PAF), which in turn, stimulates production of prostaglandins PGE1 and PGE2 of the E series (130, 131). Despite the similarity with CT, in terms of receptor binding and enzymatic activity, LT1-producing *E. coli* induce mild disease compared to CT. Like CT, LT1 may also alter the activity of the enteric nervous system. Serotonin (5-HT) and VIP are released into the lumen of the small bowel *in vivo* after treatment with CT (132). There is some evidence that CT (thus probably LT1) can bind and activate VIP-containing neurons in the intestinal sub-mucosa of guinea pigs (133).

LT2 has been isolated essentially from animals. It shares only 55% identity with the A subunit of CT but essentially no homology with the B subunit of CT (134). LT2 can be divided into two subgroups, LT2a and LT2b, on the basis of their amino acid sequences. The LT2 subunit B does not bind to GM1 gangliosides; LT2a preferentially binds to GD1b gangliosides and LT2b to GD1a gangliosides.

## *E. COLI* HEAT STABLE ENTEROTOXINS (STs)

This family of enterotoxins encompasses 2 main members: STa and STb. STa is a cystein-rich peptide of 18 amino-acids (aa), encoded by the transposon-associated *estA* gene located on a plasmid (135). STa residues from 5 to 17 allow full binding to its receptor and enterotoxic activities. STa binds to a receptor, the guanylate cyclase (GC) (a 120 kDa protein), localised in the intestinal brush border membrane of the entire digestive tract. GC decreases from the small intestine to the rectum and is present in large amount in young children, rapidly dropping with increasing age (136). This may explain why STa-induced diarrhoea is more severe in young children than in adults. STa binding activates GC by changing GC conformation, thus inhibiting the kinase activity domain of GC. In fact, genetic deletion of the kinase domain provokes the permanent activation of GC and its unresponsiveness to STa. Activation of GC by STa increases the level of intracellular cyclic GMP which in turn stimulates chloride secretion. This results in the inhibition of NaCl absorption. Activation of chloride secretion may occur via activation of a cGMP-dependent kinase present in the apical membrane of enterocytes which ultimately activates the chloride channel CFTR. It has been reported that the secretory response to STa involves F-actin rearrangements only at the basal pole of T84 cells (137). STb, a 71 amino acid peptide, is plasmid encoded (*estB*) (138). The receptor for STb is still unknown. STb does not activate the guanylate cyclase and, consistent with the infrequent occurrence in human disease of *E. coli* strains expressing STb, seems to have no effect on human small intestine. In mouse intestine STb induces histological changes consisting of a loss of villus epithelial cells. No secretion of chloride has been detected. By acting via a pertussis toxin-sensitive heterotrimeric G-protein, STb stimulates a dose dependent increase in intracellular calcium (126).

## *E. COLI* CYTOTOXIC NECROTISING FACTORS (CNFs)

CNF1, discovered by Caprioli and colleagues (139) as a cell-associated product of *E. coli* strains isolated from young children with diarrhoea (140), causes necrosis of rabbit skin and multinucleation of different types of tissue cultured cells (141–150). A second type of CNF (CNF2) was then found in extracts of certain *E. coli* strains isolated from calves with enteritis (151). CNF1 and CNF2 are immunologically related (152, 153) and similar in apparent molecular weight (110–115 kDa). CNF1 which is also produced by *E. coli* strains isolated from animals (154, 155), is chromosomally encoded in a 620 Kb pathogenicity island (PAI II) containing (156) the $\eta$-hemolysin and a fimbrial gene (*prs*). CNF2 gene is located on a large transmissible F-like plasmid (Vir). CNF1 and CNF2 are encoded by a single structural gene. Analysis of the deduced amino acid sequences of CNF1 and CNF2 shows that the two toxins are quite similar (85% identical and 99% conserved residues over 1014 amino acids). Additionally, CNFs are predicted to have a relatively hydrophobic transmembrane domains overlapping partially two predicted helices and no classical signal peptide sequence is found in 50 N-terminal residues. At least for CNF1, it has been reported that the C-terminal part (30 kDa) is responsible for the catalytic activity of the toxin while the N-terminal part comprises the portion involved in cell receptor binding (157, 158). At the level of mRNA, the region corresponding to the first 48 codons of *cnf*1 is involved in the translational regulation of CNF1 synthesis (158). CNFs share regions of homologous amino acids with two other bacterial toxins: *Pasteurella multocida* toxin (PMT) and the dermonecrotic toxin of *Bordetella pertussis* (DNT). CNFs, PMT and DNT form the new family of dermonecrotic toxins (157).

CNF1 is able to provoke a remarkable reorganisation of F-actin structures in cultured cells (159, 160). This toxin induces intense formation of stress fibers, focal contacts, membrane folding and retraction fibers. Reorganisation of the F-actin cytoskeleton by CNF1 results in the inability of the cells to undergo cytokinesis, giving rise to extremely flat large multinucleated cells. CNF1 induces actin reorganisation (161) by stimulating permanently the Rho protein. The first hint for this activity was observed as follows: when the cytosol from HEp-2 cells previously incubated with CNF1 was ADP-ribosylated with exoenzyme C3 it was observed that the Rho protein displayed a shift of molecular weight to a slightly higher value. This result indicated a possible post-translational modification of the GTP- binding protein in CNF1 treated cells (162) which occurred directly *in vitro* without the need of cellular co-factors. Microsequencing of CNF1-modified Rho showed a single modification in the CNF1-treated GTPase compared to wild type, Rho glutamine 63 changing into a glutamic acid (163). Therefore, CNF1 exerts a specific deamidase activity on Rho glutamine 63 (164). The equivalent amino acid of Rho glutamine 63 in p21 Ras is glutamine 61. An identical activity for CNF1 on Rho has been reported using mass spectrometry (165). Rho glutamine 63 is known to be an important residue for the intrinsic and RhoGAP mediated GTPase of Rho (166). Mutated Rho on glutamine 63 into glutamic acid (RhoQ63E) exhibits a mobility shift, upon electrophoresis, identical to CNF1-treated Rho. CNF1-treated Rho and RhoQ63E nucleotide dissociation rate is increased by 2 orders of magnitude but the RhoGAP activity is totally impaired on both CNF1-treated Rho and RhoQ63E. Thus, CNF1 allows Rho to be permanently bound to GTP, thereby enhancing the activity on Rho effectors. The Rho family of small GTPases encompasses three subfamilies that are differently involved in the regulation of the actin cytoskeleton. The Rho subfamily induces stress

fibre assembly, Rac the ruffling activity and Cdc42 the filopodia extension (7). In addition to deamidation of the glutamine 63 in Rho (163, 165), the other members of the Rho family, Rac and Cdc42, are also activated by CNF1 in glutamine 61 (167). However, although CNF1 may trigger all the Rho proteins, the activation of Cdc42 and Rac does transiently occur at high concentrations of CNF1 (167), Rho being the preferential target of the toxin. CNF1 deamidase activity is borne by the 30 kDa carboxy-terminus end of the molecule, whereas the cell binding moiety of CNF1 is localised in the amino-terminus of the molecule (157, 158). By covalently activating the Rho proteins, CNF1 induces a number of actin-dependent phenomena in epithelial cells, such as contractility, cell spreading and the assembly of focal adhesion plaques (168, 169) as well as the formation of actin stress fibres and an intense and generalised ruffling activity (159). CNF1-induced ruffling is reminiscent of the ruffling elicited by invasive bacteria and is consistent with the ability of epithelial cells to exert macropinocytosis (170).

Treatment of HEp-2 cells with CNF1, for increasing lengths of time (from 4 to 72 h), induces an augmented ability of the cells to ingest latex beads (170). Macropinocytosis is totally blocked when CNF1 treated cells are incubated with the F-actin disrupting drug cytochalasin B, demonstrating clearly that the process is F-actin dependent. In addition, non-invasive bacteria such as *Listeria innocua* are found (170) to be as invasive as *L. monocytogenes* when incubated with HEp-2 cells pretreated with CNF1. Only *L. innocua* harboring a plasmid containing the listeriolysin gene was found to multiply in the cytoplasm of HEp-2 cells treated with CNF1, indicating that macropinocytosis of bacteria was associated with formation of vacuoles.

Incubation of intestinal T84 cells with CNF1 does not influence transepithelial resistance, suggesting that the barrier function and surface polarity are not affected by the toxin (171). Incubation of T84 cells with CNF1 induces F-actin accumulation and impaired PMNs transepithelial migration (in either the luminal-to-basolateral or the basolateral-to-luminal directions). Thus CNF1-activated Rho, by reorganising F-actin structures in intestinal epithelial cells, induces a decreased ability of PMNs to cross the epithelial barrier. Furthermore, CNF1 effaces intestinal cells microvilli allowing a better bacterial adherence and probably also an improved growth of microbes by impairing absorption of nutrients by microvilli.

Strictly correlated with the above mentioned ability to induce an aspecific phagocytic-like behaviour in epithelial cells is the capability of CNF1 to protect cells against apoptosis (172, 173). The significant inverse correlation between the two phenomena suggests that they might be part of a pathogenic mechanism used by bacteria. In the mechanism used by CNF1 to hinder apoptosis, proteins of the Bcl-2 family as well as the mitochondrial homeostasis play a pivotal role. In fact, CNF1 is capable of reducing the mitochondrial membrane depolarisation induced by UVB and modulating the intracellular expression of some Bcl-2 related proteins. In particular, the amount of death antagonists, such as Bcl-2 and Bcl-$X_L$, increases following exposure to CNF1 while the amount of the death agonist Bax remains substantially unchanged (174). Bcl-2 family proteins as well as mitochondria play an essential role in apoptosis and they are linked to each other. By modulating the expression of proteins of Bcl-2 family (probably *via* Rho activation), CNF1 may operate on one of the main regulatory systems which drive a cell towards death or survival. Very recently, evidence that Rho-dependent cell spreading activated by CNF1 is also involved in the protection against apoptosis in epithelial cells has been reported (5). In addition to the impairment of nuclear fragmentation, CNF1 protects cells from the radiation-induced rounding up and detachment and improves the ability of cells to adhere to each other and to the extracellular matrix by modulating the expression of proteins related to cell adhesion. In particular, the expression of integrins such as $a_5$, $a_6$ and $a_{v,}$ as well as of some heterotypic and homotypic adhesion-related proteins such as the Focal Adhesion Kinase, E-cadherin, a and b catenins, is significantly increased in cells exposed to CNF1. Thus, the toxin-induced improvement of cell adhesion and promotion of Rho-dependent cell spreading are mechanisms clearly involved in hindering apoptosis in epithelial cells. This is in accordance with the recent opinion that cell spreading favours cell survival (175). A toxin inducing cell spreading without activating Rho, such as Cytochalasin B, is in fact ineffective in favouring cell survival. Thus, by inducing both phagocytosis and protection against apoptosis CNF1 might allow bacteria to invade epithelial cells and to prolong their survival to permit copious bacterial multiplication within them. Also, protection against apoptosis by CNF1 will allow cells to escape from their elimination by macrophages.

## *E. COLI* CYTOLETHAL DISTENDING TOXINS (CDTs)

Cytolethal distending toxins belong to an emerging toxin family whose members have been found in several unrelated bacterial species including: *E. coli*, *Shigella dysenteriae*, *Campylobacter* sp. and *Haemophilus ducreyi*. Three adjacent or slightly overlapping chromosomal genes (*cdtA*, *cdtB* and *cdtC*) encode A (27 kDa), B (29 kDa) and C (20 kDa) subunits of CDT (176). The biological activity of CDT on cultured cells consists of the induction of giant elongated cells (in CHO cells) and an inhibition of cell division at the G2/M stage of the cell cycle. This mitotic block is irreversible and induces cell death after 3 to 5 days of toxin exposure. Recently, it has been reported that CDT-treated cells accumulate in late G2, because of the

lack of cdc2 protein kinase dephosphorylation (177). CDT might therefore interfere with a cell transduction cascade, initiated in the S phase (during DNA replication), called DNA damage checkpoint. However, the nature of the CDT subunit inducing this effect and its exact molecular intracellular activity is still unknown (for a review see 178).

## *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS AND CYTOLYSINS

Staphylococcus aureus *enterotoxins*

*S. aureus* is a major cause of food-borne illness (179) due to the ingestion of one or more types of pre-formed enterotoxins. Furthermore, staphylococcal enterotoxins have been implicated in the pathogenesis of other diseases such as enteritis, septicaemia, skin infections and a few cases of toxic shock (180). Among the different *S. aureus* enterotoxins (SEs) described so far, five main types (A, B, C, D and E) have been serologically identified since the early 1970s. Thence, on the basis of some differences detected in minor epitopes, the serotype C has been further subdivided into subtypes $C_1$, $C_2$ and $C_3$ and the total number of enterotoxins increased to seven: SEA, SEB, SEC1, SEC2, SEC3, SED, SEE (181). All these toxins are small monomeric proteins with molecular weights ranging from 22 to 29 kDa, generally resistant to heat, acids and proteases.

On the basis of structural homology ranging from 30 to about 90%, two different groups (181–183) are recognised: i) enterotoxins A and E exhibiting higher than 90% homology constitute together with toxin D a strictly related group; ii) a second group is represented by enterotoxin B which shares a higher homology with toxins $C_1$, $C_2$ and $C_3$.

As far as the biological activity is concerned, their binding to enterocytes has been demonstrated (184) even though no damage at tissue level has yet been revealed. This apparent contradiction found a satisfactory explanation in the demonstration by Buxser and coworkers (185) that SEA bound to murine lymphocytes. In the early 1990s *S. aureus* enterotoxins were definitively recognised as superantigens (186) able to massively (5–25%) stimulate T cell proliferation (187); in fact, the only detectable tissue effect in the small intestine of patients is represented by the appearance of an abundant infiltration of lymphocytes. Furthermore, this strong polyclonal stimulation of cellular immune response by enterotoxins should be responsible for the observed massive release of lymphokines, including IL-2, IL-4, IFN and TNF. Staphylococcal enterotoxins are the most potent T-cell activators recognised so far, being able to act at very low concentrations ($10^{-13}$–$10^{-16}$ M) both in stimulation of lymphocytes and cytokine production (183). Studies of the effect of staphylococcal enterotoxins SEA, SEB and SEC on rat, dog and flounder intestine seem to indicate these toxins as inhibitors of absorption and/or stimulators of intestinal secretion (188–190).

## *STAPHYLOCOCCUS AUREUS DELTA*-TOXIN

Almost all *S. aureus* clinical isolates produce a small protein toxin with an estimated molecular weight of about 3 kDa, named delta-toxin. The amphipatic nature of this exotoxin, belonging to staphylococcal cytolysins, is the main support for the hypothesis that an association of six molecules of delta toxin could bring about a transmembrane pore lined by the hydrophilic faces of the monomers (191).

Although the specific contribution of this toxin to the pathogenesis of human diseases is still poorly understood, there are interesting data in the literature (192) indicating a possible role in intestinal disease. In fact, a rapid alteration in intestinal transport has been demonstrated in guinea pig ileum after toxin exposure (193, 194), a high concentration of toxin being able to modify the normal histology of the gut epithelium (195).

## CONCLUSIONS

In conclusion, the overall data currently available on the mode of action of several protein toxins produced by common enteric bacteria could provide in our opinion a promising tool to explain how these toxins, acting separately or in a synergistical way, might severely impair both the actin cytoskeleton and the intercellular tight junctions as well as alter intestinal permeability and damage or even destroy (46, 196) enterocytes and microvilli.

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