The Whole Shebang: The Gastrointestinal Tract, *Escherichia coli* Enterotoxins and Secretion

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Abstract

This review focuses on diarrhea caused by toxins released by enterotoxigenic *Escherichia coli.* These bacteria are known to produce toxins that have adverse effects on the intestinal tissue in Man and animals. *E. coli* is contracted through the ingestion of water or food contaminated by this bacterium. Generally, *E. coli* colonizes the intestinal mucosa where it multiplies and causes damage to the target cells or interferes with the homeostasis that prevails in the gastrointestinal tract. Enteropathogens such as *E. coli* are only able to exhibit their effects after colonization of the intestinal mucosa from where they release their toxins. These bacteria mainly affect chloride ions secretion through second messenger pathways resulting in secretory diarrhea. In this review, the association of bacteria with the gastrointestinal tract as pathogens and the resulting effects on the various systems of the intestine, including the nervous system and mediators leading to secretion and diarrhea are examined.

Introduction

In Man, the gastrointestinal (GI) tract is where the most important microbial community is found. Microbial populations of varying sizes and complexity occur throughout the digestive system. Microorganisms that colonize the human GI tract are nowadays collectively described as the microbiota (or microbiome). The most important effect of the microbiota is to make it more difficult for exogeneous pathogenic bacteria to colonize the GI tract and to cause disease (Surawicz, 2010). In humans, 1-2 Kg of bacteria colonize the human body with 90% of these found in the GI tract representing over 1,000 species (McFarland, 2010). *E. coli* represents the most abundant aerobic species in the human intestine (Ron, 2006). As the human microbiota co-evolved with mankind, it is part of the human physiology and so contributes to the body homeostasis. Although anaerobic microorganisms are one hundred times more numerous in the microbiota, *E. coli* strains found in the normal flora are commensals that interact positively with other members of the microbiota and help the host animal in, for example, producing substances like vitamins required for the good health of the individual. Diet is the key factor in shaping the gut microbiota structure (Kallus and Brandt, 2011; Gophna, 2011; Wu et al., 2011).

Pathogenic *E. coli* can be responsible for severe diarrhea in humans and animals and this is produced through the action of toxic proteins known as enterotoxins.

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As diarrheal disease is complex, little is known about the molecular mechanisms imposed by various pathogens to cause disease (Guttman and Finlay, 2008). Research has shown that diarrhea cannot be explained solely by secretion of ions and water by the intestinal tissue in response to toxins elaborated by microorganisms. The enteric nervous system (ENS), as well as numerous mediators, were reported to be involved in secretion observed in diarrhea (Hansen and Skadhauge, 1995; Surawicz, 2010).

The gastrointestinal microbiota

The GI microbiota represents an association that is presumed to have occurred early in human evolution. Although mammals appeared about 65 million years ago, the association between a prokaryotic microflora and the eukaryotic alimentary tract of animals began earlier, around 600 million years ago, when these living creatures first appeared on planet earth. About 10^{14} bacteria inhabit the human alimentary tract which is tenfold greater than the total number of eukaryotic cells making the human body (Campeotto et al., 2007). The human organism can thus be considered as a eukaryotic-prokaryotic consortium.

At birth our alimentary tract is sterile. The establishment of the microbiota begins rapidly after birth and it is based on the contact with the maternal flora and the surrounding environment. All microorganisms enter through the mouth with food or drinks or by direct contact from person-to-person. It is now accepted that more than 1,000 bacterial species colonize the alimentary tract with differences in the type and number of microorganisms found along its longitudinal sections. For now, only 30% of the intestinal microbiota can be cultured.

In the lumen, along the tract, various environmental niches exist for aerobic, facultative anaerobic and anaerobic microorganisms. The low intragastric pH restricts the number of bacteria found in the stomach to 10^3/ mL. Many lactobacilli are present as they can resist this low pH for a few hours. With the discovery of *Helicobacter pylori* by Warren and Marshall in 1983, we realized that this Gram-negative spiral shape bacteria could reside in the human stomach both as a commensal or a pathogen. This bacterium was shown to be responsible for chronic gastritis, some gastric ulcers and could also increase the risk of gastric cancer (Ruggiero, 2010). In the duodenum, the number of bacteria rises to 10^4/ mL with a majority of lactobacilli. In the distal ileum 10^6/ mL microorganisms are found where streptococci constitute a good fraction of the flora. Finally, in the caecum a marked rise of bacteria up to 10^{12}/ g feces are found. *Bacteroides*, *Clostridium* and methanogenic bacteria, representing the anaerobic community, constitute an important fraction of this consortium.

E.coli is the major facultatively anaerobic bacterium in the intestinal tract of most animal species. Typically, in humans it is present in 10^7-10^9 organisms per gram of feces. The commensal microbiota is responsible for controlling enteropathogens as many studies have shown production

of inhibitory substances by these microorganisms (Lievin-LeMoal and Servin, 2006). Today, the knowledge of bacterial antagonism has led to the therapeutic use of probiotics as, for example, lactic-acid producing bacteria. These microorganisms produce lactic acid and short chain fatty acids that lower intraluminal pH and inhibit the growth of acid intolerant organisms. Another approach to promote the growth of indigenous probiotics is to administer substances such as fructose-containing oligosaccharides. The substances acting in this way are known as prebiotics (Wallace et al., 2011).

Anatomy and function of the gastrointestinal tract

The GI tract is composed of four segments: the oesophagus, stomach, small intestine and large intestine. The small intestine is composed of the duodenum, jejunum and ileum. The total length in human is about 7.5 meters. The most proximal portion, the duodenum (about 25 cm) extends from the pylorus to the ligament of Treitz. Forty percent of the remaining intestine (about 2.5 meters) is jejunum and the other 60% is ileum (3.6 meters).

The small intestine is lined by a monolayer of columnar cells. Mucous secreting glands are present and play a role in coating of the epithelium. Villi project off the wall increasing the surface area for adsorption. The crypts between the villi secrete fluid. The small intestine is the primary location in the GI tract for adsorption of nutrients into the blood. The passage through the small intestine provides adsorption of most of the water and electrolytes and essentially all dietary organic molecules. The small intestine thus plays a role in providing nutrients to the body but it also plays a critical role in water and acid-base balance. Perturbing its homeostasis affect the consistency of the intestinal content. The small intestine is the site of action of *E. coli* enterotoxins.

The large intestine or colon function is to absorb water from the indigestible food matter and then pass useless waste material from the body through the anus. The colon is much wider than the small intestine. Its wall is lined with simple columnar epithelium. Instead of having the evaginations of the small intestine (villi), the large intestine has invaginations. Goblet cells are abundant in this organ. The colon is about 1.5 meter long and this structure is responsible for absorption into the blood of the vitamins produced by the bacterial biota (i.e. vitamin K, biotin).

The intestinal epithelium lining has a high permeability for lipid soluble compounds. The epithelial barrier is much less permeable to water soluble substances. The passive transport, also called diffusion, of hydrophilic substances across the epithelium occurs via pores in the tight junctions. A marked difference exists between tight junction permeability in villi and crypts. The villous epithelium is tighter than the crypt epithelium. The villi contain pores with radius of 6Å whereas in the crypts they are of 50-60 Å (Fihn et al., 2000). Water soluble compounds may cross the epithelial barrier when their molecular mass is smaller than 1kDa.

The adsorption and secretion of fluids and nutrients are central functions of the intestines. The movement of electrolytes and other solutes depend on active transport mechanisms which control fluid transport throughout the GI tract (Barrett, 2000). Chloride is the predominant electrolyte that drives fluid secretion and is related to the movement of sodium ions and water. Malfunction of these mechanisms produces symptoms of constipation and diarrhea.

Diarrhea

An estimated 4 billion episodes of diarrhea occur each year worldwide (Kopic and Geibel, 2010). Acute infectious diarrhea is the second most common cause of death in children in developing countries (Kosek et al., 2003) accounting for an estimated 3-4 million deaths annually, the majority of whom are pre-school children. The major agents responsible for this situation are enterotoxigenic *E.coli* (ETEC), *V. cholerae*, Shigella sp. and rotavirus (Qadri et al., 2005). These microorganisms are endemic to the developing countries. These etiological agents are also a major cause of travelers' diarrhea in persons who travel to these areas and, in addition, they can be imported to the developed world through these hosts (Ericsson, 2003). Travelers' diarrhea is due to an intestinal infection and ETEC is the most frequently isolated etiological agent in all parts of the world (Casburn-Jones and Farthing, 2004b). More than 50 million people travel each year from developed countries to developing countries and 20 to 50% of these travelers report having diarrhea during the first two weeks of their stay. Typically, diarrhea occurs on the third day after arrival in a country at risk (Casburn-Jones and Farthing, 2004b). ETEC is responsible for up to 30-60% of all travelers' diarrhea cases (Gascon et al., 1998; Jiang et al., 2002) and two third of ETEC infection involves heat-labile (LT) toxin. ETEC is spread by the fecal-oral route with food and water being the principal sources of infection. Diarrhea prevention advice given to travelers involves abstaining from potentially contaminated food and beverages. However, contamination is difficult if not impossible to avoid. In animals, ETEC infections are associated with watery diarrhea and they are responsible for important diarrheal diseases and economic losses due to growth retardation, treatment and death (Nagy and Fekete, 1999, 2005).

Water accounts for approximately 75% of the mass of healthy human stool. When this percentage rise to greater than 85% with an increase in frequency of bowel movements it is diagnosed as diarrhea (Donowitz et al., 1995). Diarrhea defined as an abnormally frequent discharge of semisolid fluid fecal matter from the bowel can be either acute or chronic. This condition can be caused by a variety of agents including parasites, viruses and bacteria infecting the GI tract, as mentioned before. Many bacteria have been identified in playing a role in the onset of diarrhea by producing and delivering toxins to the target tissue. Some can damage the intestinal epithelial cells as they are cytotoxic and/or hemolytic. Others are cytotonic thus producing their effects without killing the cells.

The main ways by which these microorganisms cause diarrhea include: (a) a decrease in intestinal surface with resulting decrease in adsorption (b) mucosal destruction resulting in a change in mucosal osmotic permeability and (c) a change in fluid and electrolyte homeostasis due to toxin's action on ion channels (Morris and Estes, 2001). As a rule, these pathogens colonize the intestinal mucosa, multiply and affect target cells. Large fluid losses, up to several liters a day, can be seen. Acute watery diarrhea usually resolves within 5-10 days.

Commensals and pathogens

E. coli is one of the most important bacterial species in the human alimentary tract. In healthy humans, these bacteria are harmless commensals and live in a symbiotic relationship

contributing to the welfare of the host. Some strains can also be hostile. In fact, closely related microorganisms can be both commensal and pathogen. The difference between them depends on the presence of acquired genetic material encoding one or more virulence factors. Genetic material on the bacterial chromosome, a plasmid, a transposon or a pathogenicity island can induce the change to virulence. In general, disease-producing microorganisms are outnumbered by commensals. Pathogenic *E. coli* represents a group of disease-producing bacteria with an extensive spectrum of clinical disease including watery diarrhea (Nataro and Kaper, 1998). The various pathogenic subtypes possess additional genetic material that encodes specific virulence factors that determine the nature of the disease. ETEC, for example, possesses two major virulence factors. Specific adherence appendices called fimbriae mediating adherence to the host epithelium (Dubreuil, 2008). Afimbrial adherence factor can also be produced as, for example, the adhesin involved in diffuse adherence (AIDA). The adherence process shows selectivity in its binding characteristics toward human, porcine or bovine epithelial cells. The pathogenic bacteria also possess other genes that encode enterotoxins responsible for fluid homeostasis perturbation including secretion. Heat-labile toxins I and II and heat-stable toxins, STa, STb and EAST1 (enteroaggregative heat-resistant toxin 1) promote chloride ions secretion from the intestinal epithelial cells of the small intestine resulting in diarrhea (Dubreuil, 2008). The emergence of virulent strains of enteric bacteria is probably responsible for the survival of the microorganisms as production of diarrheic stools favor transmission to other humans and/or animals.

Enterocytes and intestinal microbes

Exposing intestinal epithelial cells to various strains of bacteria causes increased expression and secretion of a number of cytokines with chemoattractant and proinflammatory functions (Farthing et al., 2004). Expression and release of some interleukins are also increased by intestinal epithelial cells in response to microbes. Other biological substances released by enterocytes due to microbes are prostaglandins (PG), nitric oxide (NO) and intercellular adhesion molecules. It is interesting to note that the response of enterocytes to luminal bacteria is more or less the same, regardless of the microbe involved or its virulence.

Enteric nervous system of the small intestine

The small bowel has four layers: mucosa, submucosa, muscularis propria and serosa. The mucosa is the adsorptive layer and it is made of villi and the crypts, as previously described. Epithelial cells are differentiated into enterocytes, goblet cells, enteroendocrine cells and Paneth cells. These are recycled every 2 to 5 days. The submucosa carries the blood supply, lymphatics and nerve fibers as well as the ganglion cells of the submucosal plexus. The muscularis propria is the muscle region responsible for gut movement known as peristalsis. It has two layers of smooth muscle, one inner and circular and the outer longitudinal layer. The serosa is a smooth membrane consisting of a secretory epithelial layer and a connective tissue layer. The epithelial layer secretes serous fluid. The connective tissue layer provides blood vessels and nerves for secretory cells. It also serves as the binding layer to adhere to organs and other structures.

The ENS functions independently of the central nervous system (CNS) but is linked to it through parasympathic and sympathic afferent and efferent nerves which assemble as the central autonomic neural network. Cell bodies of enteric neurons are grouped as ganglia. Theses are connected by bundles of nerves that constitute two major plexuses (a) the myenteric plexus and (b) the submucosal plexus. The first is mainly involved in motor control of the gut but also provides secremotor innervation of the mucosa. The submucosal plexus innervates the intestinal epithelium and has a major role in the control of secretory processes. Neurons within the ENS are classified as intrinsic afferent neurons, inter-neurons, and motor secretory neurons. Many of the compounds secreted by bacteria may alone or together, activate afferent neurones in villi (Kirkup et al., 2001).

The ENS functions through numerous neurotransmitters. Activation of enteric nerves by electrical field stimulation identified a role for ENS in intestinal transport processes (Hubel, 1985). It is now well established that the ENS is involved in the intestinal secretory processes There is also increasing evidence that some bacterial enterotoxins produce their secretory effects, at least in parts, via a neuronal arc reflex. The ENS appears to have a central role in enhancing the secretory effects of some bacterial enterotoxins as will be discuss later.

Neuronal activation and secretion

Intestinal secretion in response to luminal distention and feeding are mediated, at least in part, by the ENS, involving the neurotransmitters substance P and acetylcholine and also the release of 5-hydroxytryptamine (5-HT) from enterochromaffin cells (EC) (Jodal, 1990; Cooke, 2000). Neural pathways have been implicated in a variety of intestinal bacterial and viral infections including those due to *V. cholerae*, enterotoxigenic *E. coli*, *Clostridium difficile* and rotavirus (Pothoulakis, 2000; Morris and Estes, 2001)

Some secretory enterotoxins are known to act as reflex arc activators by releasing endogenous mediators such as 5-HT (or serotonin) and PG. Early studies using nerve (tetradotoxin) and ganglion (hexamethonium) blockers indicated the involvement of the ENS in intestinal secretion resulting from enterotoxin action. The question is to know which component of the ENS is involved.

Endocrine cells and the small intestine

The GI tract is the largest endocrine organ in the body and contains numerous hormones. The hormones are contained within specialized cells dispersed among the enterocytes. The two most investigated endocrine cells are the cholecystokinin cells (CCKC) and the EC. CCKC are concentrated in the proximal small intestine. They produce cholecystokinin (CKK), a hormonal regulator of the digestive process (Liddle, 1997). CKK can be produced by endocrine cells and various neurons of the GI tract and CNS functioning either as a hormone or a neuropeptide. EC cells are dispersed throughout the epithelium of the intestinal tract on villi and in crypts. Granules containing 5-HT and/or peptide hormones are concentrated around the basolateral surface of the cells.

Enterochromaffin cells

About 95% of 5-HT in the gut is stored in the secretory granules of EC. These cells originate from the intestinal

stem cells near the base of the crypts and migrate towards the villous tips. The 5-HT release into the lumen or lamina propria then acts either on enterocytes or on mucosal afferents nerves in the lamina propria, initiating both secretion and propulsive motor patterns. Activation of EC by a range of stimuli induces a rise in intracellular calcium which causes the release of prestored 5-HT from the basal granules (Braun et al., 2007). Luminal administration of 5-HT, mimicking a release from the 5-HT containing EC elicits a nervous stimulation of fluid secretion in the rat (Cassuto et al., 1982; Sjöqvist et al., 1992). As observed with electrophysiological techniques, luminal 5-HT activates sensory neurons of the ENS (Bertrand et al., 2000). The ENS contains numerous serotogenic neurons including interneurons and secretomotor neurons as well as mucosal afferents nerves whose activity is modulated by 5-HT. Stimulation of EC which include vomiting and diarrhea can be seen as part of a primitive protective mechanism against infection, designed to remove the infecting organism as seen in diarrheal sickness.

5-HT and PG released from EC or villus of the small intestine mediate secretory effects *in vivo*. Then, there is activation of ENS and release of vasoactive intestinal peptide (VIP). VIP binds to crypt cell receptors and triggers secretion of NaCl and water (Goyal and Hirano, 1996). Overall, ENS amplifies signals originating in the lumen where toxins or other agents react with villus cell receptors.

Bacterial enterotoxins

Enterotoxins are proteins or peptides produced by pathogenic microorganisms. Bacterial enterotoxins are potent molecules that are capable of disrupting normal homeostasis of the bowel at nanomolar concentrations or less. In general, few molecules are sufficient to intoxicate and kill target cells. These molecules are secreted into the intestinal lumen after bacteria have adhered to the epithelium. The end result is the activation of Cl^- ions secretion and inhibition of Na^+ ions adsorption by the enterocytes with an osmosis driven release of water. The net fluid secretion observed results in diarrhea. In certain cases this condition can be fatal.

Escherichia coli

Most *E. coli* are commensals and only a small proportion of strains are pathogenic. *E. coli* causes a wide variety of enteric and extraintestinal diseases in animals and Man. The term pathotype (or pathovar) is used to identify *E. coli* types on the basis of their virulence mechanism (Levine, 1987; Nataro & Kaper, 1998). ETEC represents one of these pathotypes and it is the most common cause of diarrhea in Man and farm animals (Quadri et al., 2005; Fairbrother et al., 2005; Nagy and Fekete, 2005).

ETEC discovery goes back more than 50 years (De et al. 1956) when failure to isolate *Vibrio cholerae* from feces of patients with signs and symptoms typical of cholera led to the observation of pure *E. coli* culture that tested positive in the rabbit loop model. The experiments conducted showed the pathogenicity of these newly isolated *E. coli* strains and production of toxins unknown before. From there followed studies to purify and characterize the newly identified toxins.

ETEC infections are classically associated with acute watery diarrhea. In addition to diarrhea, other signs and symptoms are often reported. These include headache, fever, nausea and vomiting. Some patients may have prolonged diarrhea lasting a week or more (Yoder *et al*., 2006). Infecting ETEC must be able to produce, secrete and deliver effectively the toxins to the target cells.

Some ETEC strains produce a heat-labile toxin that is closely related to the cholera toxin (CT). Likewise, it is a heterohexameric molecule comprising a pentameric B subunit that serves to recognize and bind the receptor and a single A subunit which is the toxic moiety. Heat-stable STa and STb toxins are both small cysteine-rich peptides but share no homology. In fact, they represent different molecules at the structural level and this is reflected by a divergent mechanism of action. EAST1 toxin was initially identified in enteroaggregative *E. coli* (Levine et al., 1988). Recently, it has been associated with some ETEC strains (Savarino et al., 1993; Bertin et al., 1998; Paiva de Sousa and Dubreuil, 2001).

LT

LT is structurally and functionally similar to CT. The effect of LT is irreversible (Table 1). Heat-labile enterotoxin is known to activate adenylate cyclase in enterocytes. LT is

Table 1. *Escherichia coli* enterotoxins characteristics.

Toxin	Subtypes	MW (Daltons)	Organization	Receptor(s)	Binding to	Host	Toxic effect
LT-I	LT-Ih	85,000	AB_5	GM_1, GD_{1b}, GM_2, asialo-GM_1	Galβ1-3GalNac β1-4(NeuAcα2-3)Gal	Human	Irreversible
	LT-Ip	85,000	AB_5	Galactoprotein, Galactose containing Glycolipids	β1-4(NeuAcα2-3)Gal	Pig	Irreversible
LT-II	LT-IIa	85,000	AB_5	GD_1b	Gal(β1-3GalNac) β1-4(NeuAcα2-8 NeuAcα2-3)	Cow, buffalo, pig, human	Irreversible
	LT-IIb	85,000	AB_5	GD_1a	NeuAcα2-3Gal β1-4 GalNac	Cow, buffalo, pig, human	Irreversible
STa	STaH	2,000	Single amino-acid chain (19aa)	Guanylate cyclase C		Human	Reversible
	STaP	2,000	Single amino-acid chain (18aa)			Pig, bovine, human	Reversible
EAST1	Numerous variants*	4,100	Single amino-acid chain (38aa)	Guanylate cyclase C?		Human, bovine, pig	Reversible?
STb	Variant His^{12}→Asn	5,200	Single amino-acid chain (48aa)	Sulfatide	Galactose-$SO_4^=$	Pig, human, bovine, chicken, horse, dog, cat, ferret	Reversible

?: Not experimentally proven
* Ménard and Dubreuil (2002)
GalNac: N-acetylgalactosamine
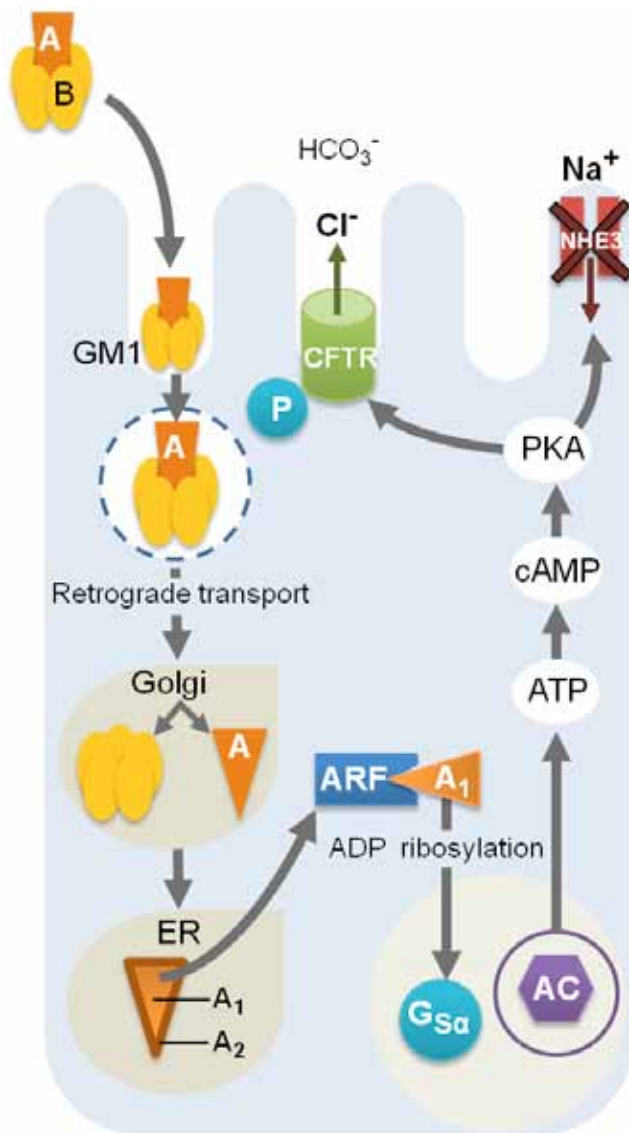NeuAc: N-acetylneuraminic acid

Figure 1. Mechanism of action of LT. LT-I binds to the GM1 ganglioside receptor (or other gangliosides or glycoproteins) (Table 1) via the LT-B subunits. The holotoxin is internalized in vesicles by receptor-mediated endocytosis. Via retrograde transport, the vesicles transits through the Golgi and endoplasmic reticulum (ER). The holotoxin is disassembled in the Golgi and the A subunit transit to the ER. The LT-A subunit is translocated to the ER where it undergoes a cleavage into A_1 ribosylating activity moiety and A_2 moiety involved in linking the LT-B subunits. In the cytoplasm, the A_1 subunit binds to ADP-ribosylating factors (ARF). The activated A_1 subunit ADP-ribosylates the α subunit of a regulatory G_s protein. This modification results in an irreversible activation of the membrane bound adenylate cyclase (AC) which stimulates production of cAMP. Accumulation of this compound in the cytoplasm activates cAMP-dependent protein kinase A (PKA) inducing phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) that results in secretion of Cl- and to a lesser extent of HCO_3^-. PKA at the same time inhibits Na^+ re-absorption by the Na^+/H^+-exchanger 3 (NHE3) upon phosphorylation.

not secreted into the culture supernatant during growth but remains cell associated being localized to either the periplasm or the cytoplasm. Much of LT secreted under laboratory conditions remains associated with outer membrane vesicles which can enter the host cell via lipid raft dependent endocytosis (Kesty et al., 2004). This toxin is tricky to purify and this explain why CT is so often use as a substitute as it is antigenically and functionally related (Clements and Finkelstein, 1979).

Two subtypes of LT have been described. These are referred to LT-I and LT-II. LT-I and LT-II can further be subdivided according to their variation in structure (antigenic variation), hosts, and molecules acting as receptors as well as the binding determinants (Table 1). LT is a high molecular weight toxin (85,000 Da). It belongs to the AB5 toxin family where 5B molecules are involved in the binding to its receptor present on the surface of the enterocytes and one A subunit which is the enzymatically active moiety of the toxin.

The pathogenesis of LT-I is initiated by binding of LT-B subunits to the ganglioside receptor GM1 found in caveolae on the host cell surface (Figure 1) (Lencer et al., 1999). Other gangliosides and glycoproteins can act as receptors for LT-I. After binding, the LT holotoxin is internalized in vesicles through the Golgi and the endoplasmic reticulum (ER) via retrograde transport. In the Golgi, the holotoxin is disassembled. The A subunit is transported to the ER. The A subunit consist of two domains linked by a disulfide bond. Subunit A_1 is the active molecule whereas subunit A_2 anchors the subunit A to the B pentamer. The A_1 subunit translocates through the intracellular membrane to interact with ADP-ribosylating factors (ARFs) in the cytoplasm. The activated A_1 subunit then ADP-ribosylates the α subunit of a regulatory Gs, an intracellular guanine nucleotide protein. The inhibition of Gsα GTPase activity results in the irreversible activation of the transmembranar adenylate cyclase complex leading to cAMP accumulation in the cell. Elevated levels of cAMP activate cAMP-dependent protein kinase A (PKA), inducing phosphorylation of the cystic fibrosis transmembrane regulator (CFTR). Activation of CFTR provokes the secretion of Cl- and HCO_3^- (Vaandrager et al., 1997; Hug et al., 2003). PKA is also responsible for inhibition Na^+ re-absorption by the Na^+/H^+-exchanger 3 (NHE3). Overall, the result is an osmotically-driven increased in permeation of water and electrolytes resulting in fluid accumulation in the intestine.

LT alternative ways to provoke secretion

Research during the past 20 years has shown the importance of neurohumoral mechanism in the pathogenesis of diarrhea, notably the role of 5-HT, substance P, VIP and neural reflexes within the ENS (Berkes et al., 2003). Some *E. coli* enterotoxins are known to invoke these mechanisms in diarrhea pathogenesis.

LT activates adenylate cyclase in enterocytes, as seen earlier. However, LT may also induce secretion via alternative mechanisms such as PG production, the ENS and cytokine activation (Nataro & Kaper, 1998). In fact, as the action of LT is inhibited by the ganglion blocker (hexamethonium) and lignocaine it supports the view that the ENS is involved in LT-induced secretion (Farthing, 2000).

However, LT does not provoke the release of 5-HT from EC and the secretion observed is not abrogated or

diminished by 5-HT$_3$ receptor antagonists (Mourad et al., 1995; Turvill et al., 1996 and 1998). Substance P is an established neurotransmitter in the ENS but LT secretion was not affected by selective substance P antagonists (Turvill et al., 2000).

Sigma receptors and its agonists have been implicated in many cellular functions, biological processes and diseases and there is evidence for a role of sigma receptors in regulation of neurotransmitters release and modulation of neurotransmitter receptor function. The sigma receptor agonist igmesine was shown to reverse LT neurally-mediated secretion in rat jejunum *in vivo*. It was effective if given before and after the establishment of the secretory state (Turnvill et al., 1999). Overall, LT enterotoxin shows an indirect, neurally-mediated secretory action.

STa/EAST1

STa toxins are a family of small (18-19 amino acids) cysteine-rich peptides (Table 1). STa can be subdivided on the basis of the host from which it was isolated as well as the amino acid sequence. STaP was originally isolated from pig and comprises 18 amino acids whereas STaH was originally from human and comprises 19 amino acids. STaH is solely produced by human strains but STaP can be found associated with *E. coli* strains from various animals (bovine and porcine) including man. The amino acid sequences of the two toxin subtypes are not identical but each possesses three disulfide bonds. Although not directly proven, STaP can probably cause disease in humans. The effect of STa is reversible.

STa binds to the extracellular domain of guanylate cyclase C (GC-C) found on the brush border of the intestinal epithelium (Figure 2). Activation of the GC-C intracellular catalytic domain leads to the conversion of GTP to cGMP and its intracellular accumulation. This increase activates cGMP-dependent protein kinase II (cGMPKII) leading to the phosphorylation of the CFTR. Also, elevated cGMP levels inhibit phosphodiesterase 3 leading to cAMP increase and activation of PKA. Then, PKA act on the CFTR and also inhibits the re-absorption of sodium by NHE3 upon phosphorylation. STa produces secretory diarrhea via stimulation of the CFTR channel and causing over-secretion of Cl$^-$ and HCO$_3^-$ which is followed by an osmosis-driven electrolytes and water release by the cells.

EAST1 is a 38 amino acids toxin for which we know many variants (Table 1) (Ménard and Dubreuil, 2002). EAST1 was first identified in an enteroaggregative *E. coli* isolate from a child (Levine et al., 1988). Subsequently, the EAST1 gene (*astA*) has been detected in human, bovine and porcine ETEC, EPEC, STEC and also in Salmonella strains (Savarino et al., 1996; Paiva de Sousa and Dubreuil, 2001). EAST1 shares partial structural similarity to STa showing 50% homology with the enterotoxic domain of this toxin (Veilleux and Dubreuil, 2006). EAST1 was also shown to lead to an increase in cGMP (Savarino et al., 1993; McVeigh et al., 2000) and it is believed that it has the same receptor and mechanism of action as STa (Table 1).

Alternative mechanism of STa secretion induction

For STa, the ENS appears to be involved in the mechanism of action as fluid secretion is inhibited by tetrodotoxin, lignocaine and hexamethonium (Farthing, 2000). However, as observed for LT, 5-HT is not released from EC. In the
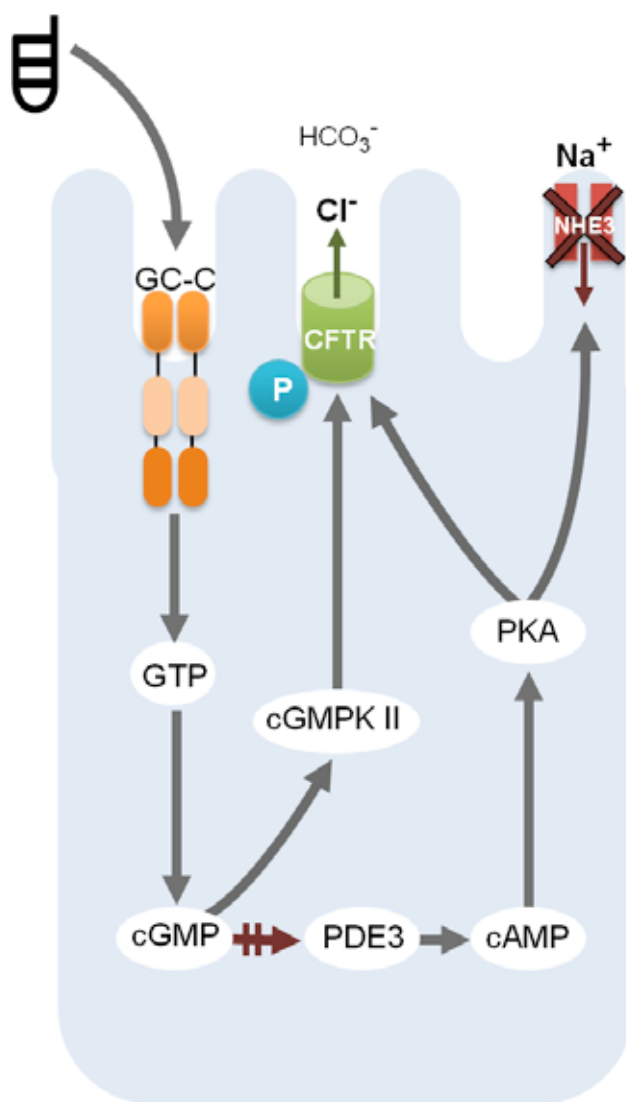

Figure 2. Mechanism of action of STa and proposed mechanism of action for EAST1. STa binds to the extracellular domain of guanylate cyclase C (GC-C). Binding to GC-C leads to activation of its intracellular catalytic domain resulting in conversion of GTP to cGMP and its cytoplasmic accumulation. Higher level of cGMP activates cGMP-dependent protein kinase II (cGMPKII) leading to phosphorylation of CFTR resulting in secretion of Cl$^-$ and to a lesser extent of HCO$_3^-$. In parallel, elevated cGMP inhibits phosphodiesterase 3 (PDE3) that increase the cAMP level and this condition activates protein kinase A (PKA). This enzyme can act on CFTR as well as on the inhibition Na$^+$ re-absorption by the Na$^+$/H$^+$-exchanger 3 (NHE3) upon phosphorylation.

small bowel, the electrogenic Cl$^-$ secretion has been attributed to direct action of STa on the enterocytes via the brush border receptor enzyme guanylate cyclase C (Figure 2). However, fluid secretion could also be largely mediated by a neural mechanism (Eklund et al., 1985). Rolf and Levin (1994) showed that when the intact rat ileum was used *in*

vitro, STa activated electrogenic Cl⁻ secretion through a myenteric reflex with an afferent C fiber component and a NO-mediated afferent arm. In the proximal colon a neural component is also involved since tetrodotoxin (a nerve blocker) inhibits Cl⁻ secretion. STa enterotoxin induces fluid secretion in the rat jejunum, ileum and proximal colon *in vivo*. This fluid secretion was greatly inhibited by the co-presence of luminal capsaicin which is a highly specific neural toxin of afferent C fibers (Nzegu and Levin, 1996). Thus, afferent C fibers appear to be essential for expression of the secretory action of STa. The results indicate that *in vitro* the dominant process causing fluid secretion appears to be neurally mediated by afferent C fibers.

NO probably plays a role in adsorptive and secretory processes (Mourad *et al*., 1996 and 1999). NO is capable of stimulating intestinal epithelial chloride secretion by increasing intracellular cGMP levels (Rolfe and Milla, 1999). Ileal STa stimulates remote secretion in the rat jejunum but not in the colon, probably via nitrinergic, vagal reflex mediated C fibers. This neural pathway amplifies the action of the toxin in its generation of a secretory diarrhea. The results showed clearly that instillation of STa into the rat ileum activated both ileal and jejunal fluid secretion and both could be inhibited by the nitric oxide synthase inhibitor L-NAME but not by the inactive isomer D-NAME (Rolfe and Levin, 1999). NO is involved in STa-induced intestinal secretion and their results showed that NO was implicated in the activation of the remote jejunal secretion. Bilateral vagotomy prevented ileal STa from inducing jejunal secretion but had no effect on the activation of ileal secretion of STa. Thus, luminal STa in the ileum *in vivo* can activate fluid secretion in the jejunum but not in the colon, by a neural mechanism that is inhibited by vagotomy, L-NAME and capsaicin (Nzegwu and Levin, 1996; Lucas et al., 2008).

Mourad and Nassar (2000) showed that the afferent nervous system is important in the pathophysiology of intestinal fluid secretion induced by LT and STa toxins. VIP plays a role in LT and STa induced intestinal secretion and may be the final putative neurotransmitter in the pathophysiology of these toxins. A VIP antagonist partially prevented STa induced secretion demonstrating that VIPergic neurons and NO synthase containing neurons coexist in the ENS (Llewellyn-Smith et al., 1992). In addition, NO may release VIP from nerve terminals (Allescher et al., 1996) and VIP may cause release of NO (Daniel et al., 1994). Thus, we may speculate that NO and VIP interact to induce secretion by STa.

STb

STb has been reported in human isolates (Lortie et al., 1991; Handl and Flock, 1992; Okamoto et al., 1993) but this toxin is mostly associated with porcine ETEC and the majority of porcine ETEC produces STb (Dubreuil, 1997).

For STb, the mechanism of action differs from that of LT and STa. The toxin was shown to permeabilizes piglet jejunal brush border membrane vesicles by forming non-specific pores but poorly active mutants showed no membrane permeabilizing capacity (Gonçalves et al., 2007). In NIH-3T3 cells, FITC-labeleled STb molecules were observed inside the cells and it matched with mitochondria labeling as revealed by confocal microscopy. Mitochondria hyperpolarization was noted as an early intoxication event (Gonçalves and Dubreuil, 2009).
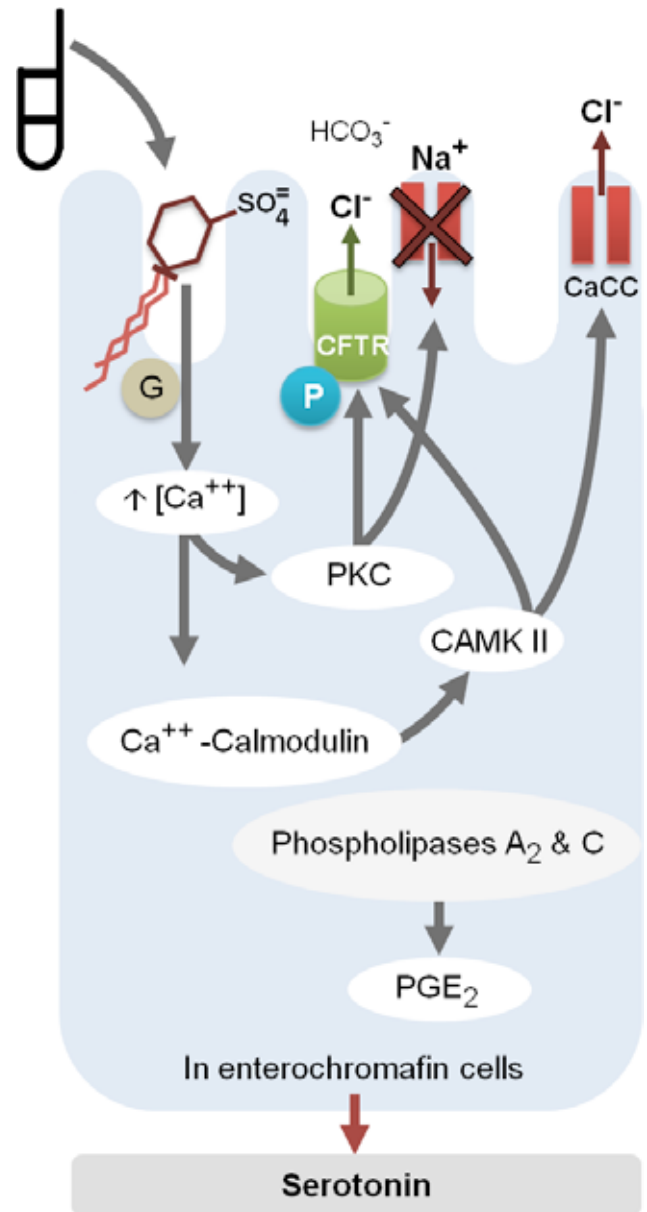

Figure 3. Mechanism of action of STb. STb toxin binds to sulfatide, a glycosphingolipid found on the surface of intestinal epithelial cells. Inside the cell, STb interact with a G protein resulting in a Ca⁺⁺ increase that activates a calmodulin-dependent protein kinase II (CAMKII). This kinase phosphorylates CFTR and this is responsible for Cl⁻ and HCO$_3^-$ secretion. Elevated Ca⁺⁺ also activates protein kinase C (PKC) and this enzyme act on CFTR as well as on inhibition of Na⁺ uptake through an unidentified Na⁺ channel. CAMKII also open a calcium-activated chloride channel (CaCC). At the same time, the initial elevated Ca⁺⁺ level inside the cell influences the activities of phospholipases A$_2$ and C leading to the formation of PGE$_2$ from membrane lipids. In enterochromaffin cells these enzymatic activities result in production of 5-HT (or serotonin). 5-HT can act on ENS to contract smooth muscle of the intestinal cell wall.

A variant of STb was discovered where a histidine at position 12 of the mature toxin is replaced by an asparagine (Taillon et al., 2008). This variant was recently shown to share similar structural properties and binding to its receptor (Taillon et al., 2012). An internalization assay indicated that the variant was more taken up in epithelial cells than the wildtype toxin.

STb induces diarrhea in animals without activating adenylate or guanylate cyclases (Hitotsubashi et al. 1992). The action of STb is reversible (Table 1). Contrarily to STa, the toxin was shown to be internalized to stimulate fluid secretion in the rat model (Labrie et al, 2002). STb binds through its galactose sulfate moiety to an acidic glycosphingolipid, sulfatide, a molecule widely distributed on intestinal epithelial cells (Rousset et al. 1998) (Figure 3). Once STb is inside the cell, a GTP-binding regulatory protein is stimulated resulting in a Ca^{++} level increase activating a calmodulin-dependent protein kinase II (CAMKII) (Dreyfus et al., 1993). Activation of protein kinase C (PKC) is also observed and ultimately CFTR is phosphorylated (Dreyfus et al. 1993; Fujii et al. 1997). PKC also inhibits Na^+ uptake by acting on an unidentified Na^+ channel. CAMKII opens a calcium-activated chloride channel. The increased calcium levels also influence the activities of phospholipases A_2 and C leading to the release of arachidonic acid from membrane phospholipids and formation of prostaglandin E_2 (PGE_2) and 5-HT (or serotonin) from EC. Both compounds mediate the transport of H_2O and electrolytes out of the intestinal cells by a yet unknown mechanism (Hitotsubashi et al., 1992; Harville and Dreyfus, 1995; Peterson and Whipp, 1995).

Alternative mechanism of STb secretion induction
PG synthesis inhibitors significantly reduce the response to STb (Hitotsubashi et al., 1992; Fujii et al., 1995; Dubreuil, 1999). Activation of the ENS by PGE_2 and 5-HT was observed for STb (Sears and Kaper, 1996; Dubreuil, 1997). The release of 5-HT can act on the ENS to contract smooth muscle in the intestinal wall to augment the peristaltism (Guttman and Finlay, 2008). Treatment of rats with ketanserin, a 5-HT-receptor antagonist, reduced intestinal secretion induce by STb (Harville and Dreyfus, 1995). Papaverine which is known to cause relaxation of smooth muscles had also an inhibitory effect on STb (Hitotsubashi et al., 1992). Thus, ENS was shown to be involved in STb-induced secretion through the action of PG and 5-HT.

Conclusions
More than 50 years after the discovery of ETEC, research on the toxins produced by this *E. coli* pathotype is still a greatly active subject. The effects of pathogenic *E. coli* on host intestinal cells are vast and increasing evidence suggests that some bacterial enterotoxins mediate diarrhea not only by acting on enterocytes but also by interfering with or stimulating the ENS (Pothoulakis *et al.*, 1998). As antagonists of specific neurotransmitters could abrogate or markedly reduce the secretion produced by enterotoxins, these were recognized as playing a role in disease. More than 20 neurotransmitters have been identified in the ENS. Some of these were clearly shown to be involved in diarrhea induced by *E. coli* enterotoxins. These act by modulating the motility, absorption and secretion of the intestine (Farthing, 2000 and 2004).

As a result of enterotoxins action diarrhea occurs in the animal and human hosts. This condition can serve two diametrically opposed purposes. On one hand, the potential advantages for the host can be the flushing of the intestinal lumen and the clearing of pathogenic microbes. On the other hand, the advantage to the microbe could be to increase its transmission to new hosts. Our knowledge of the molecular mechanisms underlying ETEC-mediated diarrhea will ultimately help designing new therapeutic approaches to this important disease responsible for so many uncomfortable conditions and/or deaths.

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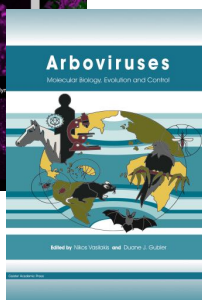
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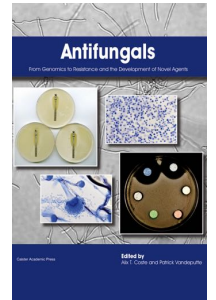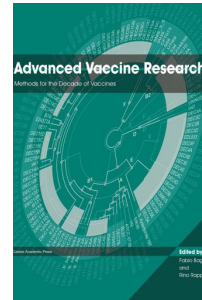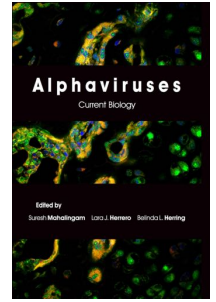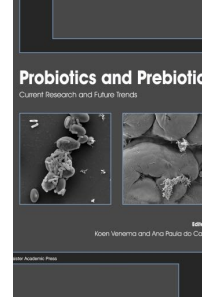
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