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Clostridium Perfringens Toxins Involved in Mammalian Veterinary Diseases

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Abstract

Clostridium perfringens is a gram-positive anaerobic rod that is classified into 5 toxinotypes (A, B, C, D, and E) according to the production of 4 major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX). However, this microorganism can produce up to 16 toxins in various combinations, including lethal toxins such as perfringolysin O (PFO), enterotoxin (CPE), and beta2 toxin (CPB2). Most diseases caused by this microorganism are mediated by one or more of these toxins. The role of CPA in intestinal disease of mammals is controversial and poorly documented, but there is no doubt that this toxin is essential in the production of gas gangrene of humans and several animal species. CPB produced by *C. perfringens* types B and C is responsible for necrotizing enteritis and enterotoxemia mainly in neonatal individuals of several animal species. ETX produced by *C. perfringens* type D is responsible for clinical signs and lesions of enterotoxemia, a predominantly neurological disease of sheep and goats. The role of ITX in disease of animals is poorly understood, although it is usually assumed that the pathogenesis of intestinal diseases produced by *C. perfringens* type E is mediated by this toxin. CPB2, a necrotizing and lethal toxin that can be produced by all types of *C. perfringens*, has been blamed for disease in many animal species, but little information is currently available to sustain or rule out this claim. CPE is an important virulence factor for *C. perfringens* type A gastrointestinal disease in humans and dogs; however, the data implicating CPE in other animal diseases remains ambiguous. PFO does not seem to play a direct role as the main virulence factor for animal diseases, but it may have a synergistic role with CPA-mediated gangrene and ETX-mediated enterotoxemia. The recent improvement of animal models for *C. perfringens* infection and the use of toxin gene knock-out mutants have demonstrated the specific pathogenic role of several toxins of *C. perfringens* in animal disease. These research tools are helping us to establish the role of

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each *C. perfringens* toxin in animal disease, to investigate the *in vivo* mechanism of action of these toxins, and to develop more effective vaccines against diseases produced by these microorganisms.

Keywords

Alpha; beta; Clostridium perfringens; CPB2; CPE; epsilon; iota; toxins

INTRODUCTION

Clostridium perfringens is a gram-positive anaerobic rod that is classified into 5 toxinotypes (A, B, C, D, and E) according to the production of 4 major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) (Table 1). However, this microorganism can produce up to 16 toxins in various combinations, including lethal toxins such as perfringolysin O (PFO), enterotoxin (CPE), and beta2 toxin (CPB2) [1–4].

C. perfringens causes numerous gastrointestinal infections in most mammalian species (Table 2). These infections are generically called enterotoxemias because toxins produced in the intestine may be absorbed into the general circulation. However, while this is true for many *C. perfringens* toxins, some toxins produced in the intestine act only locally. This microorganism can also cause diseases of skin, subcutaneous and muscular tissues (gas gangrene or malignant edema). Most, if not all diseases produced by C. *perfringens* are mediated by one or more of its powerful toxins. We review here the most studied *C. perfringens* toxins involved in mammalian veterinary diseases.

ALPHA TOXIN (CPA OR PLC)

Structure and Mechanism of Action

Alpha-toxin (CPA or PLC) is produced by all *C. perfringens* strains (Table 1), although toxinotype A strains usually produce higher amounts than the other toxinotypes [5]. The main properties of this toxin are summarized in Table 3. CPA is a 43 kDa protein comprised of 370 amino acids. It contains two domains, an alpha-helical N-terminal domain harboring the phospholipase C active site, and an alpha-sandwich C-terminal domain which is involved in membrane binding. CPA has more than 50% identity at the amino acid level, and/or on the basis of antigenic cross-reactivity, to the phospholipases C from *Clostridium bifermentans*, *Clostridium novyi*, *Clostridium baratii* and *Clostridium absonum* [3]. CPA is a classic example of a toxin that modifies cell membranes by enzymatic activity. This toxin is a zinc-dependent phospholipase C which degrades phosphatidylcholine and sphingomyelin, both components of the eukaryiotic cell membranes [6], causing damage on the membrane of erythrocytes and other cells from many animal species. The net result of this action is cell lysis, by degradation of membrane phospholipids [7]. CPA also activates several other membrane and internal cell mechanisms that lead to hemolysis [6–8]. In addition, CPA activates the arachidonic cascade resulting in the formation of thromboxanes, leukotrienes and prostaglandins, which activate the inflammation cascade and produce vasoconstriction [6, 9].

In combination with PFO, CPA allows *C. perfringens* to escape macrophage phagosomes and survive in the host tissue [10]. CPA in sublytic amounts impairs the migration of leukocytes and promotes their aggregation by several different mechanisms including hyperadhesion with leucostasis at the periphery of lesions [11]. These free intravascular aggregates are responsible for the blockade of blood flow in small vessels, and subsequently for the anoxia and necrosis of tissues [12–15] which in turn helps create anaerobic conditions for the proliferation of *C. perfringens*.

Genetics of CPA

All *C. perfringens* strains possess the gene encoding CPA. This gene *(cpa)* is chromosomally encoded and located close to the origin of replication, which is one of the most stable regions within the bacterial chromosome [16]. This chromosomal location of the *cpa* gene contrasts with many of the other major toxin genes of *C. perfringens*, which are plasmid borne [16–19]. Nucleotide sequencing of the *cpa* gene from type A strain ACTC 13124, a high CPA producer, revealed a single open reading frame that includes a putative prokaryotic signal sequence [20]. The *cpa* genes from other type A and types B-E strains of *C. perfringens* have been characterized, showing that they are also located chromosomally [16].

A two component regulatory system named VirS/VirR positively regulates the *in vitro* transcription of *cpa* via a RNA regulatory molecule called VR-RNA [21–24]. Transcription of *cpa* and production of the CPA protein is also regulated by the recently described *C. perfringens* Agr quorum sensing system [25, 26]. Whether those systems act in concert to regulate *cpa* transcription still needs to be clarified.

Role of CPA in Animal Diseases

Intestinal Infections—The role of CPA in intestinal disease of mammals is controversial and poorly documented (Table 2). In sheep, *C. perfringens* type A causes yellow lamb disease [27], a rare form of acute enterotoxemia in lambs. Information about pathogenesis of this disease is minimal and often contradictory, but it is generally assumed that most clinical signs and lesions are due to the effects of CPA [27]. However, no definitive proof of the action of this toxin in the pathogenesis of yellow lamb disease has been provided. The disease is characterized clinically by depression, anemia, icterus, and hemoglobinuria. Necropsy findings include pale and friable liver and spleen and the presence of red urine in the urinary bladder [27]. Histopathological changes include periacinar necrosis of the liver, splenic congestion, nephrosis with hemoglobin casts, and pulmonary congestion and edema [27].

C. perfringens type A has been and is still frequently blamed for enteritis, abomasitis and/or enterotoxemia in cattle [28–32], horses [33–39], goats [40–43] and pigs [44, 45]. However, the role of this microorganism in natural diseases of these species remains controversial and poorly documented [46]. Although it has been suggested that clinical signs and pathological findings in several of these diseases may be the result of CPA action, no definitive evidence has become available that proves the role of CPA in the pathogenesis. While large amounts of CPA are detected in feces of naturally infected cattle [45] (and some of the species

mentioned above), CPA is also present in the intestinal content of many clinically healthy animals by which detection of CPA in intestinal content of sick animals does not have diagnostic relevance for type A disease.

Results of experimental work with *C. perfringens* type A or purified CPA suggest that this microorganism, and probably CPA, can produce disease in several mammalian species [30, 31, 43, 47]. However no definitive proof of this causal relationship has been provided and the role of this microorganism and its major toxin (CPA) in intestinal disease remains controversial. Intraruminal inoculation of *C. perfringens* type A into healthy calves induced anorexia, depression, bloat, diarrhea, and sometimes death [30, 31]. However, enteric disease could not be reproduced by the inoculation of large amounts of *C. perfringens* type A in the small intestine of cattle [46].

Neonatal pigs experimentally inoculated with *C. perfringens* type A developed enteropathy and intragastric administration of CPA to piglets caused disease with symptoms similar to those seen in naturally acquired disease thought to be caused by *C. perfringens* type A [43].

Ligated ovine ileal and colonic loops incubated with *C. perfringens* type A CPA retained more fluid than control loops due to inhibition of water absorption and developed a mild to moderate multifocal infiltration of neutrophils in the lamina propria and submucosa. These results suggested that CPA could be responsible for diseases of intestinal origin in sheep [48].

The intravascular hemolysis and capillary damage, platelet aggregation, hepatic necrosis and cardiac effects seen in several animal species, with presumptive type A infection, are consistent with the action of a circulating toxin such as CPA. Studies with toxin gene mutants have been performed to study the role of CPA in chickens [49]. However, to our knowledge, very few studies have been performed involving gastrointestinal tract challenge exposure of mammals with isogenic *C. perfringens* type A mutants that do or do not produce CPA. For instance, vegetative cultures of *cpe* null mutants of type A that still produce CPA, did not produce damage to the intestinal mucosa in rabbit ileal loops [50]. Cultures of *cpb* null mutants of type C isolates still producing CPA, did not produce fluid accumulation in rabbit ileal loops [51]. Studies with isogenic *C. perfringens* toxin mutants in large animals will be necessary to definitely establish the role of CPA in pathogenesis of intestinal disease in these species.

Gangrene/Malignant Edema—Studies with isogenic mutants strongly suggest that CPA is the major *C. perfringens* toxin involved in gas gangrene in humans [52], which is characterized by extensive local tissue destruction and necrosis progressing to shock and death [53, 54].

C. perfringens type A is also involved in gas gangrene (malignant edema) of domestic mammals including sheep, cattle, goats, and horses, acting alone or in combination with other clostridial species [55]. Although the condition is most frequently described affecting subcutaneous tissue and muscle, gangrenous mastitis produced by *C. perfringens* type A has also been reported in cattle and sheep [56, authors unpublished observation]. Predisposing

factors for this disease in animals include trauma associated to injections, shearing, castration, docking and milking. Although CPA has long been considered the main virulence factor for malignant edema of animals and vaccines containing alpha toxoid seem to protect against this disease, no final evidence of the role of CPA in the pathogenesis of malignant edema has been provided. Also, the fact that most cases of malignant edema in food-producing animals are associated with a mixed clostridial infection (which may or may not include *C. perfringens* type A) suggests that, even if CPA is responsible for some cases of malignant edema, other clostridial toxins can also produce this disease.

Grossly, malignant edema is characterized by severe subcutaneous and muscular edema, skeletal muscle necrosis and generalized petechiae on serous membranes, the latter being a manifestation of terminal disseminated intravascular coagulation due to toxemia. Histologically, in contrast to other bacterial myonecrosis, *C. perfringens* gas gangrene and malignant edema are characterized by a marked absence of inflammatory cells in affected tissues and the accumulation of leukocytes within vessels at the lesion periphery, most likely due to the inhibitory effect that CPA has over leukocyte migration.

Experimentally, CPA is lethal for mice, dermonecrotic and hemolytic. When injected intravenously, CPA suppresses the myocardial contractility and induces hypotension, bradychardia, shock and multiorgan failure. The myotoxic effects could result from a direct activity of CPA on muscle cell membrane, from alteration of local blood flow, and/or inflammatory mediators as a consequence of endothelial cells stimulated by CPA [54]. The role of CPA in gas gangrene was demonstrated by protection of mice against this disease by immunization with recombinant CPA, and by reduced virulence of CPA knock-out mutants of *C. perfringens* when injected into mice [52].

CLOSTRIDIUM PERFRINGENS BETA TOXIN (CPB)

Structure and Mechanism of Action

The main properties of this toxin are summarized in Table 2. The *cpb* gene encodes a prototoxin of 336 amino acids that includes a 27-amino acid signal sequence removed during secretion, resulting in a mature toxin of ~35 kDa [57, 58]. Purified CPB is thermolabile, with >90% of its lethal activity being inactivated by incubation for 1 h at 50°C (or 10 min at 100°C). CPB is also highly sensitive to *in vitro* trypsin [58, 59] and pepsin [60] treatment. Low pH does not seem to affect its activity [60].

The deduced CPB amino acid sequence shares similarity with several pore-forming toxins, including 28% similarity to *Staphylococcus aureus* alpha-toxin and 22, 28 or 18% similarity with the A, B, C components of gamma-toxin, respectively [57]. While those toxins cause hemolysis in erythrocytes from several species [61, 62], CPB is non-hemolytic for rabbit or sheep erythrocytes [63].

It is interesting that while *S. aureus* alpha-toxin, and many other pore forming toxins [64], are cytotoxic for a wide variety of cells [including leukocytes, rabbit kidney cells, smooth muscle cells and fibroblasts [61], CPB-induced cytotoxicity has thus far only been reported

for HL-60 cells, HUVEC (human umbilical vein endothelial cell) and intestinal I407 cells [61,65–68].

Genetics of cpb

CPB is encoded by the *cpb* gene, which is carried on virulence plasmids [58, 69] of different molecular size (authors unpublished observations). The *cpb* gene has been cloned and sequenced from type B and C isolates, which revealed a very similar ORF of ~1000 bp [57, 70]. A possible Shine-Dalgarno region is located 7 bp upstream of the first ATG start codon.

Despite the proven importance of CPB in type C (and probably type B) disease (see discussion later), relatively little is known about regulation of the *cpb* gene *in vitro* or *in vivo*. However, recent studies demonstrated that in the presence of human enterocyte-like Caco-2 cells, CPB production is rapidly upregulated. The VirS/VirR two component regulatory system (TCRS) was shown to be involved in modulating this early production of the CPB protein by upregulating *cpb* transcription [71].

The VirS/VirR system also controls transcription of other *C. perfringens* toxins (i.e. *pfo*A, *plc*/*cpa*, *cpb*2) by either direct binding of the VirR response regulator to VirR boxes located upstream of the target gene, or indirectly through RNA regulatory molecules [23, 24, 72–74]. However, VirR boxes are not detectable within ~800 bp upstream of the *cpb* gene, raising the possibility that a regulatory RNA molecule might control *cpb* transcription via the VirS/VirR system [74].

Quorum sensing mechanisms provide a molecular signal(s) that activates TCRS [75, 76]. *C. perfringens* encodes at least two different quorum sensing systems, i.e. the Lux and the Agr systems [25, 26, 77]. A type C *lux*S mutant still normally regulated its *cpb* transcription and CPB production, both *in vitro* and *in vivo* [26]. Recent studies have found that the *C. perfringens agr* locus regulates *pfo*A and *cpa*/*plc* transcription and PFO and CPA/PLC production, in strain 13 [25, 26]. Additional studies are currently underway to determine the involvement of this novel system in regulating CPB production.

Role of CPB in Animal Diseases

CPB is responsible for diseases in several animal species (Table 2) and it is produced by types B and C of *C. perfringens*. Type B isolates cause an often fatal hemorrhagic dysentery in sheep, and possibly in other species, while type C isolates cause enteritis necroticans (also called pigbel) in humans and necrotic enteritis and/or enterotoxemias in almost all livestock species. Both types B and C animal disease are often accompanied by sudden death or acute neurological signs [2, 4, 78, 79].

Type B disease is a rare occurrence in farm animals and mostly seen in Middle East countries and the UK. Clinical disease can be characterized by diarrhea, neurological signs or both. Post-mortem gross and histopathological findings are similar to those of type C infections (see below), except for those rare cases of focal symmetrical encephalomalacia, believed to be due to the effect of ETX [79, 80].

Clinical signs and histopathologic findings in type C infections are very similar in most livestock animal species. The course of disease can be peracute, acute, or chronic, with signs of the acute and peracute condition including intense abdominal pain, depression, and bloody diarrhea. Chronic disease can occur in older animals of some species (e.g. pigs), and it is characterized by persistent diarrhea without blood and dehydration [43].

At necropsy, the predominant lesions are most frequently observed in small intestine, but cecum and spiral colon can sometimes be involved; occasionally, lesions may be confined to large intestine. Lesions are similar in all segments of intestine, and in acute cases consist of intestinal and mesenteric hyperemia, diffuse or segmental, extensive fibrinonecrotic enteritis, with emphysema and bloody gut contents. Mesenteric lymph nodes are red, an excess of hemorrhagic peritoneal and pleural fluid is found, there may be fibrin strands on intestinal serosa, and adhesions may develop between intestinal loops.

Histologically, the hallmark of acute disease is hemorrhagic necrosis of the intestinal wall which starts in the mucosa but usually progresses to affect all layers of the intestine. Lesions are morphologically similar in all segments of intestine. The luminal surface is covered by a pseudomembrane composed of degenerated and necrotic desquamated epithelial cells, cell debris, inflammatory cells, fibrin, and a variable number of large, thick bacilli with square ends with occasional subterminal spore [81]. The superficial epithelium and layers of lamina propria are necrotic. Fibrin thrombi occluding superficial arteries and veins of the lamina propria are characteristic of this condition. Diffuse edema with variable amounts of protein and inflammatory cell exudate can be seen throughout all intestinal layers, including serosa [81].

There is currently limited information regarding the pathogenic mechanism of type B-associated diseases, but some evidence indicates that both CPB and ETX (another potent toxin produce by type B isolates) may contribute to lethality [82]. The mechanism of pathogenesis of type C disease is under active investigation. We experimentally demonstrated that CPB is necessary and sufficient to cause type C pathology in a rabbit ileal loop model [51]. Type C disease has also been linked to endogenous trypsin deficiency or the presence, in the intestines, of trypsin inhibitors due to diet. CPB is very trypsin sensitive, so low trypsin levels or the presence of trypsin inhibitors likely contribute to disease by favoring CPB persistency in the gastrointestinal tract [2, 51, 83, 84]. Type B and C animal disease is more frequently seen in neonatal animals than at any other age [4, 5, 79]. This age predilection is associated to the inhibitory effect that the colostrum has over intestinal trypsin. This inhibitory effect is an innate mechanism of defense, during the first hours of life, which protect the immunoglobulins present in the colostrum against protease activity of trypsin.

C. perfringens types B and C disease begins in the host intestine [2, 4, 79] with absorption of toxin(s) from the intestines into the circulation that then leads to the death of an infected animal. Different animal models have been developed to help in understanding the mechanism of those diseases involving CPB intoxication [51, 83, 85, 86]. Early work demonstrated that *C. perfringens* type C could cause a pigbel-like disease in guinea pigs fed a persistent low protein diet, combined with dietary protease inhibitors [83]. In that study it

was hypothesized that the low protein diet was a predisposing factor in both humans and guinea pigs, because of low intestinal protease production in malnourished individuals. Enteric lesions similar to those observed in human pigbel cases or animals with type C enteritis were also successfully reproduced by injecting a type C culture, along with a protease inhibitor, into lambs [84].

The role of CPB in type C-induced intestinal disease has recently been experimentally demonstrated by inoculating into rabbit ileal loops a series of *C. perfringens* type C toxin mutants, along with trypsin inhibitor [51]. Experimental type C pathology induced by wild type isolate CN3685 was characterized by accumulation of abundant hemorrhagic fluid (Fig. 1), complete loss of absorptive cells along the villi, and coagulation necrosis of the lamina propria. Confirming the role of CPB, two different *cpb* mutants were unable to cause fluid accumulation or intestinal damage, while complementing back CPB expression to the mutant completely restored virulence (Fig. 1) [51].

For many years, efforts to reproduce intestinal type C disease by injecting purified CPB into animals had been unsuccessful [4]. However, our recent work reproduced type C pathology by injecting native CPB, along with trypsin inhibitor, into rabbit intestinal loops [51, 87]. The CPB-induced intestinal lesions, produced in this model, were similar to those described in type C natural disease [4, 51, 79, 87]. This work was also significant because it established that purified CPB is a very potent toxin in the rabbit small intestine (i.e. one microgram was sufficient to cause severe intestinal damage within 1 h) [87].

As mentioned earlier, the lethality of type C infection has long been attributed to absorption of toxin(s) from the intestine to the circulation. Several studies have proven that purified CPB is highly lethal for mice [58, 59, 63, 85]. A study by Fisher *et al.* [85] later demonstrated, using the mouse intravenous injection lethality model, that CPB is the main lethal factor present in type C late-log-phase culture supernatants. In the study referred to [85], type C isolates growing *in vitro* maximally produced beta toxin during late-log phase. Lethality induced by type C culture supernatants, or purified CPB, could be blocked by preincubation with a monoclonal anti-CPB antibody, but not with a monoclonal anti-CPA antibody (i.e. CPA is also produced by nearly all type C strains) [85].

To study lethality in a more adequate context of infection, we developed two mouse models for type C infection 60]. In those models, mice were inoculated intragastrically or directly in the duodenum with type C isolates, type C toxin mutants or purified CPB, along with trypsin inhibitor. Type C strains or purified CPB reproduced neurological signs and mouse lethality. Prior to the death of challenged mice, animals experienced sudden spastic muscle contraction, tetany and opisthonous, supporting that the toxin(s) acts on the autonomic nervous system. Two different type C null *cpb* mutants produced no lethality in mice, clearly indicating that CPB is the main contributor to type C lethality [60].

A mutational analysis study [63] found that the arginine residue at position 212 of CPB is important for mouse lethality. Tyrosine 191, its counterpart in *S. aureus* alpha-toxin, a metabolite that has 28 % homology with CPB, is located in the putative binding domain

[57]. Whether CPB must bind a specific organ, or cell type, is still unknown. Some evidence has suggested that CPB may target the nervous system [59, 88, 89].

Another study determined that the CPB cysteine residue (at position 265) is not absolutely required for lethal activity. However, this work showed that adding functional groups at cysteine 265 (which induces steric hindrance) or changing residues 266, 268, and 275 caused a complete loss of lethal activity [90]. The authors proposed that the C-terminal region of CPB is either required for beta toxin binding to receptor, if any, or for formation of beta toxin oligomers.

The molecular basis of CPB-induced damage during type B or type C disease is under investigation. Previous reports found that purified CPB forms pores in the membranes of leukocyte-derived HL-60 cells or human umbilical vein endothelial cells [66, 68]. Studies with recombinant toxin also demonstrated that CPB forms pores in bilayer lipid membranes. Those channels induce K^+ efflux and Ca^{2+}, Na^+ and Cl^- influxes, which then produce cell swelling and lysis. The mutation of arginine 212 in CPB to aspartate, previously shown to increase the 50% lethal dose of CPB for mice by nearly 13-fold [63], significantly reduced the ability of CPB to form channels. The size of the CPB pore has been calculated as approximately 12 Å [59].

Purified CPB produces abundant dermonecrosis, edema and plasma extravasation when directly injected into the skin of guinea pigs or mice [91, 92]. CPB also provokes the release of TNF-α and IL-1β, as well as activates tachykinin NK_1 receptors by a still unknown mechanism [86, 91].

Gibert *et al.* (1997) [65] first reported that CPB induces cell rounding and detachment of intestinal I407 cells [65]. *In vivo*, type B and type C isolates produce intestinal lesions consisting of diffuse or multifocal hemorrhagic and necrotizing enteritis, mainly in the ileum, with excess of sanguineous serous fluid in the abdominal cavity [79]. Similar intestinal lesions have been observed after inoculating purified CPB into rabbit intestinal loops [51, 87].

In recent work, Miclard *et al.* showed that CPB specifically binds vascular endothelial cells, during peracute or acute cases of type C-induced disease in piglets or humans. The authors reported that binding of CPB to those cells produced acute endothelial degeneration and vascular necrosis [93, 94].

It is evident that CPB plays an important role in *C. perfringens* type C, and maybe in type B, disease. Those diseases are often lethal with a fatal outcome within 48 h. The first target of CPB during type C infection is the host intestine, where the toxin is highly active. This damage to the intestinal epithelium might allow CPB and other toxins to be translocated into the circulation to induce its lethal effects.

EPSILON TOXIN (ETX)

Structure and Mechanism of Action

The main properties of epsilon toxin (ETX) are summarized in Table 3. ETX is an example of an aerolysin-like, pore-forming toxin. *C. perfringens* ETX and *C. septicum* alpha-toxin are structurally related to aerolysin although ETX shows no significant sequence homology with aerolysin at the amino acid level. ETX and *C. septicum* alpha-toxin form heptameric pores, like aerolysin, and are very potent cytolysins [3].

ETX is considered the major virulence factor of *C. perfringens* types B and D [19]. This toxin causes blood pressure elevation, increased contractility of smooth muscle, vascular permeability increase, as well as brain and lung edema in multiple animal species, while in goats ETX also causes colitis [79, 95]. ETX is the third most potent clostridial toxin after botulinum toxin and tetanus toxin, with a mouse lethal dose of 100 ng/kg [3]. ETX is secreted as a prototoxin (32,981 Da) [96], which is converted into a fully active toxin (~1000 times more toxic than the prototoxin) when activated by proteases such as trypsin, chymotrypsin, and a metalloproteinase named lambda toxin that is produced by *C. perfringens* [42, 97, 98].

ETX is also active on a few cell lines, including Madin Darby Canine Kidney (MDCK) and to a lesser extent human leiomyoblastoma (G-402) [99, 100]. This toxin also kills guinea-pig and rabbit peritoneal macrophages *in vitro*, but had no demonstrable effect on other cells from guinea pigs, rabbits, mice, sheep or goats [101, 102]. When ovine, caprine and bovine aortic endothelial cells were challenged with ETX *in vitro*, no morphological alterations were observed in these cells even when large doses of toxin were used for several hours [102]. In the study referred to [102] it was speculated that the absence of damage to aortic endothelial cells was due to the need for ETX to act in concert with elements present in the living animal but not *in vitro* and/or differences between endothelial cells of the brain and those in the aorta [102].

In MDCK cells, marked swelling is observed in the first phase of intoxication, followed by blebbing and membrane disruption [103]. ETX binds to the MDCK cell surface, preferentially to the apical site, and recognizes a putative specific membrane receptor which is not present in insensitive cells. Binding of the toxin to its receptor leads to formation of large membrane complexes (about 155 kDa) which are very stable when the incubation is performed at 37°C [103]. In contrast, the complexes formed at 4°C are dissociated by SDS and heating [103]. The cytotoxicity in MDCK cells is associated with pore formation that causes a rapid loss of intracellular K^+, an increase of Cl^- and Na^+, with an increase of Ca^{++} occuring later. In polarized MDCK cells, ETX induces a rapid and dramatic increase in permeability and it is thought that pore formation in the cell membrane is likely responsible for the permeability change of cell monolayers. Actin cytoskeleton and organization of tight and adherens junctions are not altered, and the paracellular permeability to macromolecules is not significantly increased upon ETX treatment [104, 105]. Toxin-dependent cell signaling leading to cell necrosis is not yet fully understood, but it includes ATP depletion, AMP-activated protein kinase stimulation, mitochondrial membrane permeabilization, and

mitochondrial-nuclear translocation of apoptosis-inducing factor, which is a potent caspase-independent cell death factor [104].

In the brain, microvascular endothelial damage by this toxin appears to be the fundamental cause of cerebral parenchymal injury and lesions occur in an apparently dose- and time-dependant manner. Large doses of circulating toxin produce a severe, generalized vasogenic cerebral edema and an acute or peracute clinical course to death. With lower doses in partially immune animals, bilaterally symmetrical focal necrosis, occurs in certain selectively vulnerable brain regions [106]. The precise pathogenesis of the brain damage is unresolved, but the following sequence of events was proposed by Buxton and Morgan (1976): ETX reacts with specific receptor sites in the brain endothelium, causing vascular endothelial cells to degenerate. This alters fluid dynamics, which in turn causes the astrocyte end-feet to swell and rupture. Serum proteins and eventually red blood cells leak out and the resultant brain edema and haemorrhage produce parenchymal necrosis, which is responsible for the clinical nervous signs and the histopathological changes seen during acute intoxication of sheep and occasionally in goats.

In addition, degenerative and necrotic changes have been described in brain neurons of rats subacutely intoxicated with ETX even before vascular changes in the brain were evident [107]. Finnie *et al*. (1999) [107] suggested that after gaining access to the brain tissue by producing an increase in vascular permeability, ETX later exerts a direct cytotoxic effect on neurons. It was demonstrated that ETX passes through the blood-brain barrier and accumulates specifically in the brain [106, 108–110]. ETX toxin binds with high affinity to rat brain synaptosomes [111], and limited evidence suggests that the receptor might be a sialoglycoprotein [109, 111, 112]. The neurological disorders observed in several animal species seem to result from ETX action on hippocampus, leading to an excessive release of glutamate [108, 113, 114].

In summary, ETX would act on the brain by affecting vascular permeability but also by direct action on neurons. The precise target cell in host and mechanism of ETX action on neuronal cells remain to be elucidated.

Genetics of etx

The ETX gene (*etx*) is localized on conjugative plasmids [51, 115], with most type B isolates possessing the same ~65 kb *etx* plasmid that also carries the gene encoding beta2 toxin [115]. The *etx* gene in type B, and some type D, isolates is flanked by IS*1151* and a gene related to the transposase (*tnpa*) gene from Tn*3* transposons that lie upstream of *etx*. A region with similarity to transposases from *S. aureus* and *Lactococcus* is located downstream from *etx* [115–117].

Role of ETX in Animal Diseases

ETX-producing *C. perfringens* type D strains are the most common cause of clostridial enterotoxemia in sheep and goats (Table 2). Since the gross and histological changes observed in type D enterotoxemia have been reproduced by intravenous inoculation of ETX

in sheep and goats [118], there is little doubt that the pathogenesis *C. perfringens* type D enterotoxemia is largely mediated by ETX.

C. perfringens type D produces an acute, subacute, or chronic neurological condition in sheep, characterized by sudden death or neurological and respiratory signs, including blindness, opisthotonos, convulsions, bleating, frothing from the mouth, and recumbency with paddling immediately before death. Diarrhea is occasionally observed, although this is not a common clinical sign in sheep [79, 119].

When present, intestinal gross changes consist of hyperemic small intestine mucosa with slight to marked red fluid contents. Colitis may occur, but is not a consistent finding in sheep enterotoxemia. Several gross findings, such as excess pericardial, pleural, and/or abdominal fluids (with or without fibrin strands), which may clot on exposure to air, serosal petechiation, and lung edema are frequently seen [79]. Gross changes in sheep are rarely observed in brain, and consist of herniation of the cerebellar vermis (cerebellar coning) in acute or subacute cases and focal symmetrical encephalomalcia (FSE), in chronic cases. FSE is characterized by dark hemorrhagic foci in corpus striatum, thalamus, midbrain, and cerebellar peduncles and white matter [120–122]. The kidney lesion from which one of the common names of the disease is derived (pulpy kidney disease) is likely to be a postmortem change [80, 123].

Microscopic changes in the brain of sheep with type D infection are unique and pathognomonic, and they are present in approximately 90% of cases [120, 124]. They consist of perivascular proteinaceous edema (microangiopathy) in the brain [120] (Fig. 2). In the rarely observed chronic form of the disease, necrosis of white matter, grossly known as FSE, can be observed [122]. This lesion is usually multifocal and characterized by degeneration of white matter, hemorrhage, and astrocyte and axonal swelling. Perivascular edema and degeneration, and necrosis of brain parenchyma, are always bilateral and symmetrical, and they have been described most frequently in corpus striatum, thalamus, midbrain, cerebellar peduncles, and cerebellar white matter [120, 121]. These areas are not exclusively affected, and lesions can sometimes be seen in other parts of brain, such as cortex and hippocampus [120]. Usually, no significant histological changes are found in the intestine of sheep dying from enterotoxemia [124, 125]. Histological changes were not observed in kidneys of experimentally inoculated lambs necropsied immediately after death [124], supporting suggestions that these lesions are due to postmortem change.

In goats, type D produces acute, subacute, or chronic disease as well. The acute form occurs more frequently in young, unvaccinated animals and is clinically similar to the acute disease in sheep [126, 127]. The subacute form is more frequently seen in adult goats [128], vaccinated or not, and is characterized by hemorrhagic diarrhea, abdominal discomfort, severe shock, opisthotonos, and convulsions. The disease may result in death 2–4 days after onset [41, 127], but some animals recover. Adult animals, often vaccinated, can also exhibit chronic disease [128], which is characterized by profuse, watery diarrhea (often containing blood and mucus), abdominal discomfort, weakness, anorexia, and agalactia [126]. This chronic form may last for days or weeks [129].

Gross changes in acute caprine type D enterotoxemia are similar to those in the ovine disease. In the chronic form of the disease in goats, fibrinohemorrhagic colitis with occasional involvement of the distal small intestine seems to be the most consistent lesion [126]. A combination of the findings in acute and chronic forms of disease are frequently seen in subacute forms. The so-called pulpy kidney has not been reported in caprine enterotoxemia, nor has cerebellar herniation or FSE.

In goats, there are few descriptions of histological changes in type D enterotoxemia, and changes in brain are not considered a consistent feature of caprine enterotoxemia. Nevertheless, perivascular edema and degeneration of white matter, similar to those observed in sheep, can be observed in cases of acute and subacute caprine enterotoxemia [130].

In addition, subacute and chronic type D enterotoxemia in goats are characterized by fibrinonecrotic (pseudomembranous) colitis with large numbers of intralesional Gram-positive bacilli [128]. Microscopic lesions in kidney are not characteristic of goat enterotoxemia.

In cattle there are few reports about natural cases of type D enterotoxemia, and information about clinical and pathologic findings of the disease in this species is scant and frequently contradictory [131–133]. A condition called enterotoxemia of cattle, allegedly produced by *C. perfringens* type D, is described in textbooks [55,134] but confirmation of the etiology of this condition remains unestablished. The occurrence of FSE, similar to the lesion observed in type D enterotoxemia of sheep, has been described by several authors in cattle [134–139] but to date no causal relationship between these lesions and *C. perfringens* type D has been established. Recently, natural type D enterotoxemia was described in two young calves, in which the disease was confirmed by the presence of perivascular proteinaceous edema in the brain and detection of ETX in intestinal contents [140]. These seem to be the first confirmed cases of type D enterotoxemia in cattle.

The effects of intravenous ETX have been studied in sheep [118, 120], goats [118], cattle [46, 141], mice [85, 142, 143] and rats [107]. The toxin produces increased vascular permeability in many tissues, the most significant effects of which are acute pulmonary and cerebral edema with albumin extravasation. This has been demonstrated by immunohistochemistry, which shows strong perivascular immunoreactivity around blood vessels showing perivascular edema. Also, in rats given ETX intravenously, widespread upregulation of aquaporine 4 (a member of the membrane water-channel proteins, which is important in the regulation of water balance in the brain and facilitates reabsorption of excess fluid) was observed in astrocytes [144]. These results suggest that aquaporin 4 has a role in the clearance of edema fluid from brains damaged by ETX. The administration of formalinized epsilon prototoxin to mice prevented leakage of horse radish peroxidase from the brain vasculature of mice given ETX via the same route [145, 146]. Based on these results, it was suggested that ETX exerts its effects through specific receptor sites on the vascular endothelium and that antigenically similar formalinized epsilon prototoxin blocks this effect [145].

In mice given intravenous ETX the granular layer of the cerebellum seems to be the area most frequently affected and lesions progress from an initial vasogenic edema to malacic foci with bilateral symmetry, and with a predilection for the white matter [142]. Ultrastructurally the initial findings include swelling of astrocytes, especially their perivascular extensions and astrocytes in the cerebellum appeared to be particularly sensitive to ETX. These changes are quickly followed by severe endothelial damage with the endothelial cytoplasm becoming attenuated, vacuolated and very electron-dense [143]. Studies with immunohistochemistry demonstrated that after ETX administration to rats, there is rapid opening of the blood-brain barrier to albumin [147]. Renal tubular lesions were described in mice inoculated experimentally with ETX, but not in ruminants.

Niilo *et al*. [46] induced disease by intraduodenal inoculation of whole cultures of *C. perfringens* type D and dextrin in eight calves. Two of the animals presented acute neurological signs and severe pulmonary edema, hydrothorax, hydroperitoneum and serosal hemorrhages, while the other six did not show clinical or pathological changes. However, no histologic lesions were described in any of the animals [146]. Uzal *et al*. [141] reported acute loss of consciousness, hyperaesthesia, intermittent tonic and clonic convulsions, recumbency and dyspnea in calves inoculated intravenously with *C. perfringens* type D ETX. These calves showed severe acute pulmonary edema and varying degrees of perivascular proteinaceous edema in the internal capsule, thalamus and cerebellum. While the gross and histologic findings described in the calves inoculated with ETX were very similar to changes in acute enterotoxemia of sheep, the experimental procedures involved intravenous injection of ETX and this cannot, therefore, be considered a model of natural disease. Recently, a disease clinically and pathologically identical to ovine enterotoxemia was reproduced in cattle by intraduodenal inoculation of whole cultures of *C. perfringens* type D [148], which seems to fulfill Koch's postulates for type D disease in cattle.

IOTA TOXIN

Structure and Mechanism of Action

The main properties of iota toxin (ITX) are summarized in Table 3. *C. perfringens* ITX is a clostridial binary toxin. These toxins have a common structure consisting of two independent protein components that are not covalently linked, one being the binding component (Ib, 100 kDa), and the other the enzymatic component (Ia, 45 kDa) [149]. Both components are required for biological activity. The crystal structure of Ia has been solved, which revealed some resemblance to the ADP-ribosylating *B. cereus* vegetative insecticidal protein [150].

The ITX family components are synthesized during exponential growth phase and secreted by means a signal peptide as inactive proteins. They are proteolytically activated by trypsin or chymotrypsin, removing a 20 kDa N-terminal peptide from the binding component (80 kDa for the active form) and 9 to 11 N-terminal residues from the enzymatic component [151]. Ib triggers the internalization of Ia into the cell by receptor-mediated endocytosis [152, 153]. The mature binding component recognizes a specific cell membrane receptor [154] and then heptamerizes to form ion permeable channels, allowing the enzymatic component to escape from endocytic vesicles into the cytosol upon an acidic pulse [65, 133,

149, 155, 156]. Once activated by trypsin, Ib forms heptamers that insert into the membrane and form pores permitting the passage of molecules such as ions. Ib remains on the cell surface up to 3 h and mediates the endocytosis of Ia molecules [157].

The most prominent effects of ITX include depolymerization of actin filaments and increase of G-actin monomers. This effect results from Ia-mediated ADP-ribosylation of G-actin at Arg-177 [150]. The intermediate filaments are also disorganized, whereas the microtubules are not affected. The final result of this is change in morphology (rounding), inhibition of migration and activation of leucocytes [158], inhibition of smooth muscle contraction, impairment of endocytosis, exocytosis, and cytokinesis. Depolymerization of the actin cytoskeleton by ITX induces disorganization of tight and basolateral intercellular junctions with a subsequent increase in permeability of cultured intestinal cell monolayers [157].

Genetics of itx

The components of ITX are encoded by two genes, *iap* (~1160 nt) and *iab* (~2,630 nt). These genes form an operon that also includes an intermediate region composed of 243 non-coding nucleotides. Transcription of both ITX genes is driven by a promoter located upstream of the *iap* gene [152]. Molecules that regulate the transcription of the ITX genes have not yet been discovered.

The *iap* and *iab* genes have been found on large, potentially conjugative, plasmids of ~97 kb or ~135 kb. Those plasmids typically carry a silent enterotoxin (*cpe*) gene, the *IS*1151 element and a *tcp* locus (18). Interestingly, a study by Li *et al.* [18] suggested that a mobile genetic element, carrying *iap* and *ibp* genes, could have inserted onto a *tcp*-carrying enterotoxin plasmid in a type A isolate to create a progenitor ITX-plasmid. This plasmid then may spread via conjugation to other type A isolates, converting them to type E [18].

Role of ITX in Animal Diseases

Toxinotype E enteric infection of domestic animals was first reported in the late 1940s, and these infections have generally been considered a rare occurrence. As with type A infections and CPA, infections by *C. perfringens* type E are usually assumed to be mediated by ITX (Table 2), although no definitive evidence in this regard has been provided. *C. perfringens* type E produces enterotoxemia in rabbits. A very similar disease, thought to be mediated by a iota-like toxin, is produced in rabbits by *Clostridium spiroforme*. However, because of cross reactivity between *C. perfringes* type E and *C. spiroforme* toxins with the diagnostic methods currently available, it is thought that some previously reprted outbreaks of entropathies associated with C. perfringensmay have been due to *C. spiroforme*.

Both diseases are clinically characterized by diarrhea and loss of condition and characteristic necropsy changes include hemorrhages of the cecal serosa and mucosa, sometimes with involvement of the distal ileum and proximal colon, and the presence of watery mucoid content in these organs. Histologically, there is an acute necrosis of the superficial epithelium with relative sparing of the crypt epithelium, and submucosal hemorrhage and transmural hemorrhage [159].

Toxinotype E is an occasional cause of hemorrhagic enteritis and sudden death in beef calves [32, 160]. Type E disease has rarely been described in sheep and goats [43]. The role of ITX in these infections has not been elucidated.

Clostridium Perfringens Beta 2 Toxin (CPB2)

In recent years, a new *C. perfringens* toxin known as beta2 toxin (CPB2) has been widely reported as a potential contributor to *C. perfringens* related enteric diseases. The *cpb2* gene can be carried by all *C. perfringens* types [18, 85, 161]

Structure and Mechanism of Action

The main properties of CPB2 are summarized in Table 3. Gibert *et al*. [65] first cloned the CPB2 toxin gene (*cpb2*) from *C. perfringens* CWC245, which was isolated from a piglet suffering from necrotizing enterocolitis [65]. The cloned sequence revealed an open reading frame of 265 amino acids, including a 30 amino acid signal sequence absent from the 28 kDa mature toxin [65, 162]. The novel CPB2 has no significant homology with beta toxin or other known bacterial toxins [65, 163, 164]. To date very few studies have been conducted to determine the biological activity of CPB2. An early report suggested that the toxin behaves similarly to CPB [165] and Gibert *et al*. (1997) [65] demonstrated the cytotoxic effects of CPB2 on Chinese hamster ovary and human intestinal embryonic 1407 cedll lines. This cytopathology included cell rounding, membrane blebbing and detachment from the culture matrix [65]. The toxin did modify small G proteins such as actin, either by ADP-ribosylation or by UDP- glucosylation [65]. Other cell lines, such as CaCo2 cells have also been shown to be sensitive to the cytotoxic effects of CPB2 [161]. The cytopathic effects demonstrated by Fisher *et al*. [85] were similar to those described previously and the toxin induced cell death 50 (TCD_{50}) was 0.3 – 4 μg/ml for the purified native CPB2, and a recently described variant CPB2 [65]. Based on these studies, it was suggested that CPB2 toxin could act as a potential pore-forming toxin similar to other enterically-active clostridial toxins. The toxin was shown to be highly susceptible to proteolytic cleavage by trypsin [65].

The minimal lethal dose (LD_{50}) of CPB2 for mice, as described previously [65], was 0.3 μg by the i.v. injection route. A mouse i.v. injection model demonstrated a weak correlation between CPB2 levels in late log-phase culture supernatants of *C. perfringens* type C isolates and the LD_{50} [85], suggesting that CPB2 could be a minor contributor to the lethality of *C. perfringens* type C isolates. Gibert *et al*. [65] showed that CPB2 toxin caused intestinal hemorrhage and necrosis in the guinea pig ligated loop model.

Detailed structure-function studies for CPB2 have not been demonstrated. However, a recombinant *Clostridium perfringens* CPB2 derivative has been purified using Glutathione sepharose affinity chromatography. That toxin was then crystallized using the batch-under-oil technique. Crystallographic analysis of this CPB2 crystal showed that the triangular prism shaped crystals measuring 200 microns belonged to primitive rhombohedral space group *R3*, diffracting up to 2.9 Å [166].

Genetics of CPB2

The *cpb2* toxin gene is transcribed during vegetative growth, especially during the exponential phase and regulation by the VirS/VirR two-component regulatory system [73]. The *cpb*2 gene is present on *C. perfringens* type D isolates on plasmids 48 kb to 110 kb in size [19]. In these isolates, the gene is either present on the same plasmid carrying the *etx* gene or on a different plasmid. In type E isolates, *cpb*2 gene is present on plasmids sizes varying from 70 kb to 90 kb. The gene is always present on different plasmid carrying the iota toxin gene (*itx*) [18]. In recent studies (authors' unpublished observations), the *cpb*2 gene was found to be present on a 65 kb plasmid that also carries the *etx* gene, in *C. perfringens* type B isolates.

Role of CPB2 in Animal Disease

Over the past decade CPB2 has been associated with enteric diseases in a wide range of animals (Tables 3 and 4) including swine [65, 162, 167–170], cattle [28, 32, 171–174], horses [33, 35–39], sheep and goats [175, 176], and wild animals such as deer [177] and bears [178, 179]. The clinical signs observed in CPB2-mediated enteric diseases range from pasty to watery diarrhea with blood in feces, abdominal pain, and loss of body condition. It is hypothesized [165] that CPB2 toxin may act in synergy with other major toxins of *C. perfringens* in the production of necrotic and hemorrhagic enteritis. Circumstances provoking enteric dybiosis, and other predisposing factors, could lead to diseases in these animals [165].

Based on the reported findings related to the pathology of *C. perfringens* encoding CPB2-related enteritis seen in food producing and wild animals [28, 32, 33, 35, 81, 169, 171, 175, 177, 178], the gross pathology is characterized by hemorrhage and necrosis of the small and large intestines. Other prominent gross pathologic lesions include swollen, hemorrhagic mesenteric lymph nodes, as well as thickened and congested abomasal walls. The characteristic histological lesions include degenerated and necrotic desquamated epithelial cells, cell debris, inflammatory cells, fibrin and variable numbers of bacterial vegetative cells and spores. The pathologic lesions observed suggest that CPB2 toxin is a primary enteric toxin affecting the nutrient absorptive capacity of the intestine, thereby debilitating the affected animal.

Despite several recent publications suggesting that CPB2 might be implicated in intestinal diseases of several animal species, most of the evidence to support this is based on isolation of *cpb2*- positive *C. perfringens* strains from affected animals. However, because *cpb2* positive strains can also be found in the intestine of many normal animals, the significance of this finding is not clear. A much larger proportion of strains carrying the *cpb2* gene was found in the intestine of pigs with diarrhea than in normal control animals, which strongly suggests that CPB2 is implicated in pig enteritis. However, the same does not seem to be true for other animal species and the role of CPB2 in enteric disease of those animals remains, therefore, undetermined.

CLOSTRIDIUM PERFRINGENS ENTEROTOXIN (CPE)

Structure and Mechanism of Action

The main properties of CPE are summarized in Table 3. CPE is a 319 amino acid protein consisting of an N-terminal domain, which is important for pore formation and cytotoxicity, and a C-terminal domain that mediates receptor binding [180]. Although CPE lacks sequence homology with other toxins, its C-terminal receptor-binding domain structurally resembles that of some *Bacillus thuringiensis* cry toxins [181].

CPE action starts when the toxin binds to claudin receptors (claudins are important components of the tight junctions made by epithelial and endothelial cells), forming a small complex [182]. Several small complexes then oligomerize on the membrane surface to form a prepore named CH-1 [183]; CH-1 minimally contains six copies of CPE, claudin receptor, and some non-receptor claudins that are likely interacting with the claudin receptors [183]. After its formation, the CH-1 prepore inserts into the membrane bilayer to generate a cation-selective pore [182]. The resultant calcium influx activates apoptotic and oncotic cell death pathways that cause morphologic damage to the CPE-treated cells. This morphologic damage exposes the basolateral surface of the cell, allowing CPE to interact with occludin and additional claudins to form a second CPE-complex named CH-2 [183]. Formation of CH-2 triggers internalization of occludin (and probably claudins), which may contribute to paracellular permeability changes and diarrhea.

All tested mammalian species are sensitive to CPE [184]. This toxin affects all small intestinal regions but is most active in the ileum [184, 185]. CPE has only weak effects on the colon of rabbits [184] or human colonic tissue *ex vivo* [48]. In the ileum, CPE causes villus shortening and epithelial desquamation [50, 184], and such damage appears necessary for accumulation of fluids and electrolytes in the lumen [186].

Genetics of cpe

The gene (*cpe*) encoding CPE can reside on either the chromosome or on plasmids [187, 188]. Most human food-poisoning isolates carry *cpe* on the chromosome [180], and recent multi locus sequence typing (MLST) studies suggest these chromosomal *cpe* isolates are relatively divergent from other *C. perfringens* isolates [189]. In contrast, *cpe*-positive isolates recovered from diseased animals or humans suffering from nonfoodborne human gastrointestinal disease typically carry their *cpe* gene on large plasmids [187, 190].

In type A isolates, there are two major *cpe* plasmid families [189]: i) a family of ~75 kb plasmids that also carry the beta2 toxin gene (*cpb2*); ii) a family of ~70 kb plasmids lacking the *cpb*2 gene. These two *cpe* plasmid families in type A isolates share a conserved 35 kb region. Some, if not all, *cpe* plasmids of type A isolates can transfer between *C. perfringens* via conjugation [191], probably because they possess the same *tcp* locus that mediates transfer of *C. perfringens* tetracycline resistance plasmid pCW3 [192]. Some type C and D isolates also carry a plasmid-borne *cpe* gene [19, 85], although those plasmids do not appear to be closely related to the *cpe* plasmids of type A isolates (unpublished). Interestingly, most type E isolates carry silent *cpe* sequences on their iota toxin-encoding plasmids, which are often related to *cpe* plasmids of type A isolates [18].

During human food poisoning, CPE-positive strains are ingested and then sporulate in the intestines [180, 193]. CPE is expressed during sporulation [180], in a process involving regulation by the SpoOA master regulator of sporulation [194] and alternative sigma factors [195]. The toxin then accumulates intracellularly until lysis of the sporangium, releasing CPE into the intestinal lumen.

The Role of CPE in Animal Diseases

Experiments using isogenic mutants have clearly shown that CPE production is important for *C. perfringens* type A food poisoning or nonfoodborne gastrointestinal (GI) disease isolates to cause diarrheal symptoms in experimental animals [50]. Coupled with extensive epidemiologic evidence, these findings support CPE as an important virulence factor for *C. perfringens* type A food poisoning or CPE-associated nonfoodborne human GI disease [180].

In contrast to the well-established role of this toxin in human GI disease, the data implicating CPE in animal disease remains more ambiguous (Table 2). Animals with diarrhea are rarely tested for the presence of CPE in their feces and diagnostic criteria for establishing CPE-mediated animals disease are lacking [81]. Nevertheless, there are case reports [4, 34, 82, 196–200] suggesting CPE may cause GI disease in both domestic animals (including dogs, pigs, horses, and goats) and possibly wild animals (such as penguins, leopards and tortoises). For example, a relatively recent study definitively showed the presence of CPE-positive, type A isolates and CPE in the small intestines of a goat kid suffering from necrotic enteritis [189]. Additionally, fecal CPE and CPE-positive fecal isolates have been associated with canine diarrhea [197] and it has been suggested that CPE-positive strains can cause recurrent diarrhea in dogs [199].

Finally, in horses, fecal CPE was detected in ~20% of adults with diarrhea and ~30% of foals with diarrhea [201]; thus supporting a role for this fecal toxin contributing to disease in those animals, while no fecal CPE was detected in healthy adult horses or foals.

PERFRINGOLYSIN O OR THETA TOXIN (PFO)

Structure and Mechanism of Action

The main properties of PFO (also called theta toxin) are summarized in Table 3. PFO is a 54 kDa cytolytic toxin that binds to cholesterol-containing eukaryotic membranes. This toxin forms a large oligomeric prepore complex on the membrane surface prior to insertion into the cell membrane [202]. Structurally, PFO is comprised of 4 domains. The C-terminal domain (domain 4) binds cholesterol and then a conformational change in domain three exposes a β–hairpin that spontaneously inserts into the lipid bilayer [78, 202].

It has been proposed that the high affinity of PFO for cholesterol concentrates the toxin on the target membrane, promoting oligomerization and membrane insertion [203]. As mentioned, the proposed model of PFO pore formation includes the binding of water-soluble PFO monomers to cholesterol of a lipid bilayer mediated by domain 4. However, domain 4 does not insert deeply into the membrane and is not directly involved in creating the pore. Instead, PFO monomers bound to cholesterol and orientated perpendicularly to the

membrane assemble and oligomerize to form a prepore complex [78]. The oligomers consist of 40 to 50 monomers, forming on the membrane surface large arcs and rings that, after insertion of their domain three loops, lead to large pores between 300 Å and 450 Å in diameter [204].

Genetics of PFO

PFO is produced by all *C. perfringens* strains and the *pfo*A gene is located on the chromosomal DNA near the origin of replication [163, 164, 205]. PFO is synthesized with a 27 amino acid signal peptide, and the mature protein consists of 472 amino acids (53 kDa) [206].

The *pfo*A gene is directly regulated by the VirS/VirR two-component regulatory system [22–24]. Once activated by the VirS sensor kinase, the VirR response regulator binds to specific VirR boxes (VirR box 1 and VirR box 2) located upstream of the *pfo*A promoter, to activate its transcription [207].

The signal(s) that activates the VirS/VirR two-component regulatory system is unknown. The presence of enterocytes rapidly upregulates PFO production and transcription of the *pfo*A gene, via the same VirS/VirR two-component regulatory system [26, 71]. More recently, the *C. perfringens* Agr quorum sensing system was shown to regulate PFO toxin levels and transcription of the *pfo*A gene *in vitro* [25, 26, 71]. Whether the Agr-secreted factor activates, *in vitro* or in the presence of eukaryotic cells, the VirS sensor kinase remains unknown.

Role of PFO in Animal Diseases

Gangrene—PFO participates with CPA in the production of local lesions of gangrene/malignant edema in humans and animals [208]. When CPA and PFO knock-out mutants of *C. perfringens* type A were used in a mouse model, elimination of both CPA and PFO toxin genes removed most of the histopathological features typical of clostridial myonecrosis. These effects were completely restored when the double mutant was complemented with the alpha-toxin structural gene. However, restoring only PFO activity produced variable results. Reconstitution of both toxins produced histopathology similar to that observed with the alpha-reconstituted strain (1). Studies using single PFO knock-out mutants of *C. perfringens* have shown that PFO induces tissue destruction and an anti-inflammatory response. However, the effects were less pronounced than those elicited by CPA and both toxins seem to have a synergistic action in gangrenous lesions [209].

Since PFO knock-out mutants still cause murine myonecrosis, although less severe than wild type type A strains, PFO is not considered essential for disease. Instead, PFO has the ability to affect the host inflammatory response, particularly the PMNL influx into the myonecrotic lesions. PFO has been implicated in the vascular accumulation of leukocytes within blood vessels and the extracellular matrix of host tissues [11, 210, 211].

PFO and CPA stimulate leucocyte adherence, probably by increasing vascular leucostasis and local ischemia. PFO is leukocytotoxic at high doses, and at a sublethal concentration it significantly stimulates the production of ICAM-1 and adherent glycoprotein CD11b/CD18

in endothelial cells, which contributes to leukostasis in vessels adjacent to gangrenous lesions [11, 210]. The toxin effects on endothelial cells participate in disruption of endothelial integrity resulting in local edema and ultimately in systemic shock and multiorgan failure. Thus, PFO and CPA exhibit synergistic effects [52, 212].

Intestinal Infections—PFO might also have a synergistic effect with ETX. The effects of purified CPA and PFO on the lethal activity of purified ETX were studied in a mouse model. Mice were injected intravenously or intragastrically with CPA or PFO with or without ETX. Sublethal doses of CPA or PFO did not affect the lethality of ETX when either was injected together with the latter intravenously [213]. However, sublethal or lethal doses of CPA or PFO reduced survival times of mice injected simultaneously with ETX versus animals given ETX alone. When PFO was inoculated intragastrically with ETX, there was a reduction in survival time. CPA did not alter the survival time when inoculated intragastrically with ETX. These results suggest that both CPA and PFO have the potential to enhance the ETX lethal effects during enterotoxemia in natural hosts such as sheep and goats [213].

CONCLUSIONS

For many years it has been thought that most deleterious effects of *C. perfringens* infections in animals, and humans, are mediated by one or more of its powerful toxins. Toxins purified from *C. perfringens* culture supernatants have greatly improved our understanding of how these toxins mediate cellular damage. However, the specific role of individual toxins in *C. perfringens*-mediated diseases has just recently started to come to light. The recent improvement of animal models for *C. perfringens* infection and methods that inactivate toxin genes (generating toxin knock outs) have now demonstrated the specific pathologic role of several toxins of *C. perfringens* in animal and human disease. By observing a reduced or lack of virulence in knock-out strains and then full virulence with the complemented strains, the so called molecular Koch postulates have been fulfilled for several *C. perfringens* toxins, including CPB, CPA, CPE and others. These now available research tools are becoming very important in the field and are helping us: (1) to establish the role of each *C. perfringens* toxigenic type in animal or human disease; (2) to investigate the *in vivo* mechanism of action of their toxins; and (3) to develop more effective vaccines that will prevent lethal diseases produced by these microorganisms.

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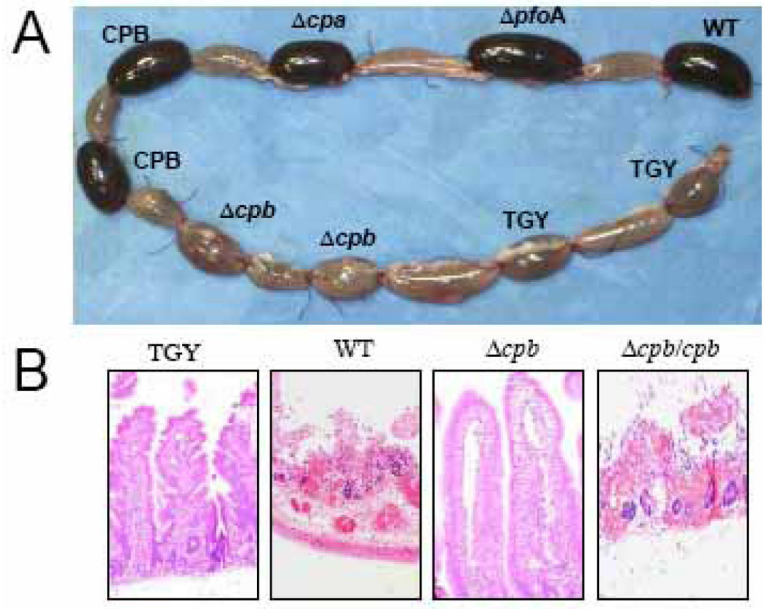
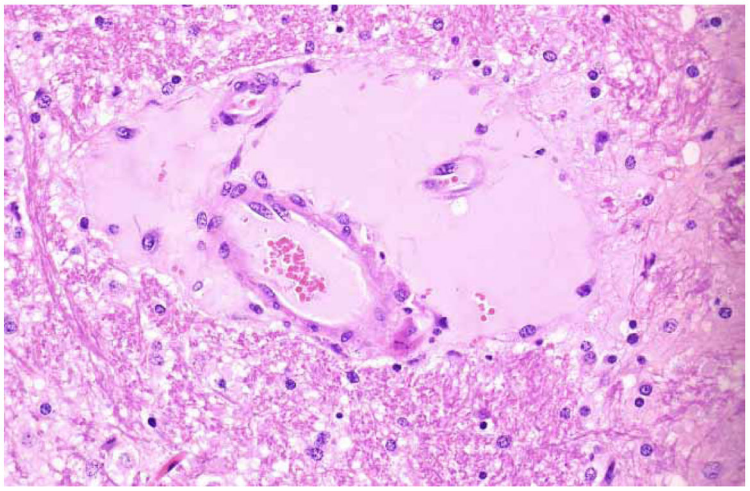


Fig. 1.

CPB-mediated gross pathology and histological damage of rabbit ileal intestinal loops. Rabbit ligated intestinal loops were inoculated with a type C CN3685 isolate (WT), isogenic single toxin mutants, purified CPB or medium (unioculated TGY medium) and then incubated for 6 h. **A**) Gross pathology. Loops inoculated with the WT, Δ*pfo*A, Δ*cpa*, or purified CPB are severely hemorrhagic and distended with fluid. No significant gross abnormalities are observed in the loops inoculated with two different Δ*cpb* mutants or sterile TGY medium. **B**) Histology. Control loops inoculated with sterile TGY, and loops inoculated with the Δ*cpb* mutant showed normal intestinal villi with a well-preserved epithelium and lamina propria. Loops inoculated with wild type CN3685 (WT) or the complemented Δ*cpb/cpb* showed histological damage consisting of necrosis and loss of epithelium, necrosis of lamina propria, blunting of the villi, hemorrhage of the mucosa and diffuse neutrophilic infiltration of mucosa and sub mucosa (Reproduced from Sayeed *et al.* 2008, with permission from Wiley Blackwell publisher).

A



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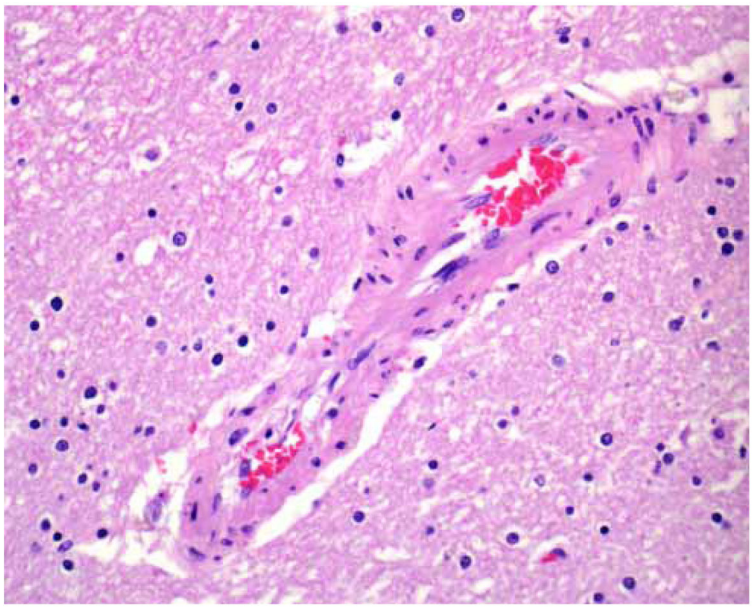

Fig. 2.

A) Proteinaceous perivascular edema in the brain of a lamb with type D enterotoxemia. The edema of the brain in this disease is mediated by the action of ETX. HE, 250x. (Reproduced from Uzal F.A., Ortega Porcel J. and Corpa Arenas J.M., 2008. Enterotoxemia ovina y caprina. Cuadernos de Campo, Merial Ed., Barcelona, Spain). **B**) Brain from a normal lamb is shown for comparison. HE, 250x.

Table 1

Major Toxins Produced by the Five Toxinotypes of *Clostridium perfringens*

MAJOR TOXINS						
Toxinotype	**CPA**	**CPB**	**ETX**	**ITX**	**CPE**	**CPB2**
A	+	−	−	−	+/−	+/−
B	+	+	+	−	+/−	+/−
C	+	+	−	−	+/−	+/−
D	+	−	+	−	+/−	+/−
E	+	−	−	+	+/−	+/−

Table 2

Main Diseases Associated to *C. perfringens* Major Toxins in Domestic Mammals

	Sheep	Goat	Cattle	Pigs	Horses	Other species
CPA	Yellow lamb disease	Not confirmed	Not confirmed	Not confirmed	Not confirmed	Not confirmed
CPB	Lamb dysentery Struck	Necrotizing enteritis	Necrotizing enteritis	Necrotizing enteritis (mainly piglets)	Necrotizing enteritis (mainly foals)	Necrotizing enteritis in several species
ETX	Enterotoxemia	Enterotoxemia	Possible Enterotoxemia	Not reported	Not reported	Not reported
ITX	Enteritis	Not reported	Enterritis	Not reported	Not reported	Enterotoxemia in rabbits
PFO	Possible synergism with CPA in gangrene and ETX in enterotoxemia. No directly responsible for disease.					
CPE	Not reported	Colitis	Not reported	Enteritis	Enteritis	Enteritis in dogs
CPB2	Not confirmed	Not confirmed	Not confirmed	Neonatal diarrhea	Possible enterocolitis	Not confirmed

Table 3

Main Properties of the Major Toxins of *C. perfringens* (Modified from Sakurai, 1995)

	CPA	CPB	ETX	ITX	PFO	CPE	CPB2
Molecular weight	42.528 kDa	35 kDa	34 kDa	Ia: 47,500 Ib: 71,500	54 kDa	35 kDa	28 kDa
LD 50* (mice)	3 μg	1.87 μg/ip; 30 ng-50 μg/iv	100 μg	Ia: 620 μg Ib: 940 μg	Not reported	50 μg	0.3 μg
Biological activity	Lethal Necrotizing Hemolytic Contraction of smooth muscle	Dermonecrotic Edema, Enterotoxic, Cytotoxic	Dermonecrotic Lethal Edema Contraction of smooth muscle	Lethal Necrotizing	Lethal, Necrotizi ng	Erythema Lethal Enterotoxic	Dermonecrotic Edema, Enterotoxic, Cytotoxic
Thermostability	Stable	Inactivated at 60°C (Sakurai and Duncan, 1978), ~2 month at −80°C	Up to 1 week at room temperature, several months at −20C years at −80 C	Ia. Stable Ib: Labile	Unknown	Inactivated at 60°C <1 week at RT ~1 month at − 20°C, years lyophilized	Unknown
Reaction to Trypsin	Susceptible	Sensitive (inactivated)	Activated (resistant)	Activated (resistant)	Susceptible	Activated (resistant)	Sensitive (inactivated)

* per kg of mouse

ip: intraperitoneal injection

iv: intravenous injection

Table 4

Epidemiological Studies Linking CPB2 and Diseases in Different Animals

Toxinotype	Species	Disease	Reference
A	Swine	Necrotic enteritis	Gibert *et al*. (1997)
A	Swine	Diarrhea	Klaasen *et al*. (1999)
A	Swine	Diarrhea, gastroenteritis	Waters *et al*. (2003)
A	Swine	Diarrhea	Bueschel *et al*. (2003)
A	Swine	Enteritis	Jost *et al*. (2005)
A	Swine	Enteritis	Jost *et al*. (2006)
A	Swine	Neonatal diarrhea	Hendriksen *et al*. (2006)
A	Bovine	Enterotoxaemia	Manteca *et al*. (2002)
A	Bovine	Hemorrhagic bowel syndrome	Dennison *et al*. (2005)
A	Bovine	Enterotoxaemia	Lebrun *et al*. (2006)
A	Bovine	Hemorrhagic bowel syndrome	Ceci *et al*. (2006)
A	Bovine	Hemorrhagic enteritis	Songer & Miskimins (2005)
A, E	Bovine	Neonatal diarrhea	Ferrarezi *et al*. (2008)
A	Equine	Fatal and Non fatal Typhlocolitis	Herholz *et al*. (1999)
A	Equine	Typhlocolitis	Bacciarini *et al*. (2003)
A	Equine	Type A myonecrosis	Choi *et al*. (2003)
A	Equine	Gastrointestinal diseases	Waters *et al*. (2005)
A	Equine	Typhlocolitis	Vilei *et al*. (2005)
A	Equine	Enterocolitis	Timoney *et al*. (2005)
A	Caprine	Enterotoxaemia	Dray *et al*. (2004)
D	Caprine	Enterotoxaemia	Uzal *et al*. (2008)
A	Cervidae	Clostridial enterotoxaemia	Embury-Hyatt *et al*. (2005)
?	Ursidae	Necrotic and Hemorrhagic enteritis	Greco *et al*. (2005)
A	Ursidae	Normal flora	Jores *et al*. (2008)