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Recent progress in understanding the pathogenesis of *Clostridium perfringens* type C infections

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Abstract

Clostridium perfringens type C causes necrotizing enteritis in humans and several other animal species. Type C isolates must produce at least beta toxin (CPB) and alpha toxin (CPA) and most strains produce several other toxins including perfringolysin O (PFO) and TpeL. However, current evidence indicates that CPB is the main virulence factor for type C infections. Most of this evidence is based upon the loss of virulence shown by isogenic type C CPB knock out mutants on cells, and also in rabbit intestinal loops and in mouse models. This virulence is regained when these mutants are complemented with the wild-type *cpb* gene. Many type C isolates respond to close contact with enterocyte-like Caco-2 cells by producing all toxins, except TpeL, much more rapidly than occurs during *in vitro* growth. This *in vivo* effect involves rapid transcriptional upregulation of the *cpb*, *cpb2*, *pfoA* and *plc* toxin genes. Rapid Caco-2 cell-induced upregulation of CPB and PFO production involves the VirS/VirR two-component system, since upregulated *in vivo* transcription of the *pfoA* and *cpb* genes was blocked by inactivating the *virR* gene and was reversible by complementation to restore VirR expression.

Keywords

Clostridium perfringens; beta toxin; enterotoxemia; pathogenesis; type C

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 toxins, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX). Several other toxins (e.g. enterotoxin [CPE], beta 2 [CPB2] and perfringolysin O [PFO]) can also be produced by some strains of all types of *C.*

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perfringens, but they are not currently used in the classification of this microorganism (Songer and Uzal, 2005) (Table 1).

C. perfringens causes numerous gastrointestinal infections in most mammalian species. These infections are often 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, while others act both locally and systemically (Songer and Uzal, 2005).

C. perfringens type C causes enteritis and enterotoxaemia in several livestock species and also in humans (Table 2). Clinical signs, gross and histopathologic findings in natural type C infections are very similar in most 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 adult animals of some species (e.g. sheep and horses), and is characterized by persistent diarrhea without blood, and dehydration (Songer, 1998; Songer and Uzal, 2005).

At necropsy, the predominant lesions are most frequently observed in small intestine of most species, but cecum and colon can sometimes be involved. Gross and histological lesions are similar in all segments of the intestine. Gross lesions in per-acute cases consist of diffuse or segmental, extensive necrotizing and hemorrhagic enteritis, with emphysema and bloody gut contents (Songer, 1998; Songer and Uzal, 2005). In acute and sub-acute cases, fibrinonecrotic enteritis with the presence of pseudomembranes is usually observed (Jäggi *et al*, 2009).

Histologically, the hallmark of *C. perfringens* type C infection is hemorrhagic necrosis of the intestinal wall, which starts in the mucosa but usually progresses to affect all layers of the intestine. Fibrin thrombi occluding superficial arteries and veins of the lamina propria and sub-mucosa are characteristic of this condition (Songer and Uzal, 2005).

In humans, *C. perfringens* type C isolates cause enteritis necroticans (also known as Darmbrand or Pigbel), disease that occurs sporadically in much of Southeast Asia. This illness was once endemic in Papua New Guinea and is still occasionally observed (Johnson and Gerding, 1997; Lawrence and Cooke, 1980). Although less common, this disease also occurs in diabetic patients elsewhere in the world. Persons suffering from enteritis necroticans often survive less than 48 h after the first appearance of symptoms (Matsuda *et al*, 2007; Petrillo *et al*, 2000; Severin *et al*, 1984; Tonnellier *et al*, 2001).

Several animal models have also been developed to study this condition (Table 2). Until recently, the pathogenesis of *C. perfringens* type C was poorly understood. However, in the last few years, a series of papers have been published providing new insights on the pathogenesis of these infections. We review here the current knowledge on the pathogenesis of *C. perfringens* type C infections, emphasizing recent findings.

Beta toxin

The main properties of the typing toxins produced by *C. perfringens* type C are shown in Table 3. *C. perfringens* type C must produce, at minimum, CPA and CPB, with strains often producing several other toxins (e.g. CPE, CPB2, PFO, TpeL, etc.). However, current evidence (discussed below) indicates that CPB is the most important virulence factor of *C. perfringens* type C. CPB is a 35-kDa protein that forms pores in the membrane of susceptible cell lines, which leads to swelling and cell lysis (Nagahama *et al*, 2003; Sakurai and Duncan, 1978; Steinthorsdottir *et al*, 2000).

CPB is lethal for mice, with a calculated LD₅₀ of 1.87 µg/per kg of body weight when administered via the intraperitoneal route (Sakurai, 1995). The *cpb* gene encodes a prototoxin of 336 amino acids that includes a 27-amino acid signal sequence that is removed during secretion, resulting in a mature toxin of ~35 kDa (Hunter *et al*, 1993; Sakurai and Duncan, 1978). 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 trypsin *in vitro* and *in vivo* (Sakurai and Duncan, 1978; Sayeed *et al*, 2008; Shatursky *et al*, 2000) and pepsin (Uzal *et al*, 2009) treatment. Low pH does not seem to affect its activity (Uzal *et al*, 2009).

The molecular basis of CPB-induced damage during 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 (Nagahama *et al*, 2003a; Steinhorsdottir *et al*, 2000). Studies with recombinant toxin also demonstrated that CPB forms pores in bilayer lipid membranes. Those channels induce K⁺ efflux and Ca²⁺, 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 LD₅₀ dose of CPB for mice by nearly 13-fold (Steinhorsdottir *et al*, 1998), significantly reduced the ability of CPB to form channels. The size of the CPB pore has been calculated to be approximately 12 Å (Shatursky *et al*, 2000).

Purified CPB produces dermonecrosis, edema and plasma extravasation when injected into the skin of guinea pigs or mice (Nagahama *et al*, 2003b; Sakurai and Duncan, 1977). This toxin also provokes the release of TNF-α and IL-1β, and activates tachykinin NK₁ receptors by a still unknown mechanism (Nagahama *et al*, 2008; Nagahama *et al*, 2003b).

Miclard *et al* (2009a) recently showed that CPB specifically binds to vascular endothelial cells in the intestinal mucosa, during peracute or acute cases of type C-induced disease in piglets and humans. Those authors suggested that binding of CPB to endothelial cells is an early event leading to acute endothelial degeneration with subsequent thrombosis; this in turn would be responsible for initiation of the intestinal necrosis characteristic of type C infections (Miclard *et al*, 2009a and b).

Genetics of *cpb* and regulation of CPB production

CPB is encoded by the *cpb* gene, which is carried on virulence plasmids (Katayama *et al*, 1996; Sakurai and Duncan, 1978) ranging in size from ~65 to ~110 kb in size (Gujar *et al*, 2010; Sayeed *et al*, 2010). The gene encoding TpeL, a large clostridial cytotoxin, or the gene encoding CPE can also be present on the *cpb* plasmid. The *cpb* gene has been cloned and sequenced from type B and C isolates, which revealed a very similar ORF of ~1000 bp (Hunter *et al*, 1993; Steinhorsdottir *et al*, 1995).

Despite the proven importance of CPB in type C disease, relatively little has been known about *cpb* gene regulation. However, recent *in vitro* studies demonstrated that, in the presence of human enterocyte-like Caco-2 cells, CPB production is rapidly upregulated under either aerobic or anaerobic incubation conditions (Vidal *et al*, 2009; Ma *et al*, 2011). The VirS/VirR two component regulatory system (TCRS) was shown to be involved in modulating this early CPB production by upregulating *cpb* transcription in the presence of Caco-2 cells. To our knowledge, the rapid Caco-2 cell-induced toxin secretion reported in the study mentioned above (Vidal *et al*, 2009) represents the first identification of host-*Clostridium* cross-talk communication affecting production of classical exotoxins, which are the hallmark of clostridial infections. This phenomenon appears to be widespread among pathogenic *C. perfringens* type C isolates, as several different type C strains isolated from

diseased humans or several domestic animal species were shown to rapidly upregulate their toxin production and secretion in the presence of Caco-2 cells (Vidal *et al*, 2009).

Pathogenesis of diseases produced by *C. perfringens* type C

Disease by *C. perfringens* type C begins with multiplication of the organism in the intestinal tract and production of potent toxins, amongst which CPB seems to be the most important for virulence. While *C. perfringens* type C may be a commensal in the intestine of some animals, this does not always seem to be the case and it is likely that a true infection occurs in at least some cases. However, information on the carrier rate of *C. perfringens* type C in normal animals is scant. 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, produced either by direct action of CPB on epithelial cells and/or indirectly by endothelial damage followed by thrombosis and intestinal ischemia (Miclard *et al*, 2009a and b), might allow CPB and other toxins to be translocated into the circulation to induce its lethal effects.

Several animal models have been used to study type C disease, including pigs, guinea pigs, rabbits, mice, sheep and goats. Experimental piglets were initially extensively used to study the pathogenesis of *C. perfringens* type C infections (Field and Goodwin, 1959; Johansen *et al*, 1986a,b,c; Kohler *et al*, 1979); guinea pigs were also used (Lawrence and Cooke, 1980).

Evidence for the importance of CPB as a virulence factor for *C. perfringens* type C was first provided when a relatively crude beta toxoid was shown to protect animals and humans against type C infection (Walker *et al*, 1979). Also, several studies have proven that purified CPB is highly lethal for mice (Fisher *et al*, 2006; Sakurai and Duncan, 1978; Shatursky *et al*, 2000; Steinhorsdottir *et al*, 1998). A study by Fisher *et al* (2006) 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 that study (Fisher *et al*, 2006), 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, confirming the specificity of CPB intoxication in lethality induced by these preparations.

More recently, a series of *C. perfringens* type C toxin knock out (KO) mutants were tested in a rabbit intestinal loop model (Sayeed *et al*, 2008) (Figs. 1 and 2). In those experiments, late log-phase vegetative cultures of *C. perfringens* type C wild-type isolate CN3685 caused hemorrhagic necrotizing enteritis in rabbit intestinal loops. When isogenic toxin KO mutants prepared using TargeTron[®] technology were inoculated into rabbit intestinal loops, even a double *cpa/pfoA* KO mutant of CN3685 remained virulent. However, two independent *cpb* KO mutants were completely attenuated for virulence in this animal model. Reversal of the *cpb* mutation restored CPB production and intestinal virulence, fulfilling molecular Koch's postulates (Figs. 1 and 2). These results confirmed that CPB is both required and sufficient for CN3685-induced enteric pathology, supporting a key role for this toxin in type C intestinal pathogenesis (Sayeed *et al*, 2008).

Risk factors for developing necrotizing enteritis from type C isolates include low trypsin production due to a protein-poor diet or pancreatic disease and consumption of foods, such as sweet potato, containing high concentration of a TI (Lawrence and Cooke, 1980). Also, the colostrum ingested by newborn animals has powerful inhibitory properties against trypsin (Quigley *et al*, 1995). These risk factors contribute to the persistence of trypsin-sensitive CPB in the gastrointestinal tract during type C infection (McClane *et al*, 2004).

When purified CPB was used to determine the effects of this toxin on different parts of the rabbit gastro-intestinal tract, bloody fluid accumulation and tissue damage were observed in the duodenum, jejunum and ileum. However, the duodenum was found to be less CPB-

sensitive than the jejunum or ileum and the colon was not affected at all (Vidal *et al* 2008.). These regional CPB sensitivity differences provide one explanation for gastro-intestinal aspects of natural type C diseases, which primarily involve the jejunum and ileum of humans and animals (Farrant *et al*, 1996; Matsuda *et al*, 2007; Niilo, 1988; Uzal, 2004). The reduced CPB sensitivity of the duodenum in animal models and natural disease has been attributed, at least in part, to pancreatic secretion producing higher intestinal trypsin levels in the duodenum, which then reduces CPB activity (Zaitlin and Sircus, 1974).

Although the colon is usually not affected in natural or experimental type C disease, there are a few reports of colonic damage in human type C disease (Farrant *et al*, 1996; Severin *et al*, 1984), which may involve another type C toxin (not CPB) possessing colonic activity and/or species-specific differences in colonic CPB susceptibility. Sorting out these possibilities will require additional experiments, but it is notable that another *C. perfringens* toxin, i.e. CPE, resembles CPB by also failing to damage rabbit colonic loops, but yet possessing some limited activity on human colon *in vivo* and *ex vivo* (Bos *et al*, 2005; Fernandez-Miyakawa *et al*, 2005; McDonel and Demers, 1982).

In natural type C disease, lethality is thought to involve not only intestinal lesions produced by CPB but also the absorption of this and maybe other toxins into the general circulation with action on distant organs (e.g. brain). To study the systemic changes and lethality produced by *C. perfringens* type C isolates, two mice models (intragastric [IG] and intraduodenal [ID]) were developed using wild-type isolate CN3685 (Uzal *et al*, 2009). These mouse models reproduced many, but not all, clinical signs reported during natural type C disease in spontaneous type C animal disease. Clinical alterations observed in both spontaneous type C infections of animals and our mice models, included depression, swollen abdomen, respiratory distress and/or neurological signs culminating in lethality. However, neither diarrhea nor significant gross or histological abnormalities in the intestine were observed in either of the mouse models. The absence of significant gross and histological small intestinal lesions in mice might reflect the absence or less abundance of CPB receptors in mouse enterocytes and/or endothelial cells. Although the systemic effects of CPB were only hypothesized before, in a study by Fisher *et al* (2006) neurological alterations were observed in mice inoculated intravenously with CPB. Furthermore, these clinical alterations were eliminated when CPB was incubated with an anti CPB mATB before inoculation. Additionally, neurological alterations were observed in mice receiving an IG or ID challenge with type C isolates, or purified CPB, but no histological lesions were observed in their intestines which rules out that those alterations were non specific effects produced by absorption of other toxins generated in a necrotic intestine (Uzal *et al*, 2009). All together, these results confirm that CPB produces systemic alterations. Elucidating the pathogenesis of the neurological clinical alterations in CPB in toxicated animals requires further research.

When the isogenic CN3685 toxin KO mutants described above were inoculated into mice using either the IG or the ID model, inactivation of the CPB-encoding gene rendered the highly lethal type C strain CN3685 avirulent in the IG model and nearly non-lethal in the ID model. In contrast, inactivation of the genes encoding CPA or PFO only slightly decreased the lethality of CN3685. Mice inoculated with wild type strain CN3685 could also be protected against lethality by intravenous passive immunization with a CPB antibody prior to intragastric challenge (Uzal *et al*, 2009). These results indicated that CPB is also a major contributor to the systemic effects of type C infections.

Conclusions

For many years it has been thought that most deleterious effects of *C. perfringens* infections in animals and humans, including those produce by *C. perfringens* type C, are mediated by one or more of its powerful toxins. The use of *C. perfringens* purified toxins and the recent

development of methods that inactivate toxin genes have provided very useful tools to study the role of several toxins of *C. perfringens* in animal and human disease. These tools have greatly improved our understanding of how these toxins mediate cellular damage. The so-called molecular Koch postulates have now been fulfilled for CPB. These now available research tools are becoming very important in the field and will continue helping researchers: (1) to establish the role of CPB in animal 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. Studies to evaluate the role of the VirS/VirR TcRS and also quorum sensing mechanisms that activate TCRS of *C. perfringens* type C *in vitro* and *in vivo* (i.e. Agr and Lux systems) are also currently under way.

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Figure 1.

Gross pathology of rabbit-ligated intestinal loops inoculated with CN3685, isogenic single- and double-toxin KO mutants, purified CPB or sterile, non-toxic culture medium (TYG) and then incubated for 6 h. Note that loops inoculated with the wild-type strain, the *pfo* KO mutant, *cpa* KO mutant, double *pfo/cpa* KO mutant or purified CPB are severely hemorrhagic and distended with fluid. No significant gross abnormalities are observed in the loops inoculated with *cpb* KO mutant or TYG. Modified from Sayeed *et al*, 2008.

Figure 2.

Histological damage in rabbit ileum treated for 6 h with an 8 h culture of wild-type CN3685, wild-type strain, the *pfo* KO mutant, *cpa* KO mutant, double *pfo/cpa* KO mutant or purified CPB. Control loops, which were inoculated with sterile, non-toxic culture medium (TGY), and loops inoculated with the *cpb* KO mutant showed normal, full-length intestinal villi with a well-preserved epithelium and lamina propria. Loops inoculated with wild-type CN3685, *cpa* KO mutant, *pfoA* KO mutant, double *pfoA/cpa* KO mutant or with cpb complement all showed histological damage consisting of necrosis and loss of epithelium, necrosis of lamina propria, blunting of the villi, haemorrhage of the mucosa and diffuse neutrophilic infiltration of mucosa and sub-mucosa. Sections were stained with hematoxylin and eosin and photographed at 200x magnification. Modified from Sayeed *et al*, 2008.

Table 1

Major toxins produced by the five toxinotypes of *Clostridium perfringens*

Toxinotype	Major Toxin				
	CPA	CPB	ETX	ITX	CPE
A	+	-	-	-	+/-
B	+	+	+	-	+/-
C	+	+	-	-	+/-
D	+	-	+	-	+/-
E	+	-	-	+	+/-

Table 2Main spontaneous and experimental diseases associated to *C. perfringens* type C

Spontaneous disease	Sheep	Lamb dysentery (Struck)
	Goats	Necrotizing enteritis
	Cattle	Necrotizing enteritis
	Pigs	Necrotizing enteritis (mainly piglets)
	Horses	Necrotizing enteritis (mainly foals)
	Humans	Enteritis necroticans (pig bell, dambrand)
Experimental disease	Rabbits *	Necrotizing enteritis affecting mostly jejunum and ileum
	Mice **	Depression, neurological disease, swollen abdomen, death. No significant damage in intestine.
	Pigs	Necrotizing enteritis affecting mostly jejunum and ileum; occasionally colon
	Guinea pigs	Necrotizing enteritis affecting mostly jejunum and ileum
	Sheep	Necrotizing enteritis affecting mostly jejunum and ileum
	Goats	Necrotizing enteritis affecting mostly jejunum and ileum

*
Intestinal loop model**
Intragastric and intra duodenal model

Table 3

Main properties of the major toxins of *C. perfringens* type C (modified from Sakurai, 1995 and Uzal *et al*, 2010)

	CPA	CPB	CPE	CPB2
Molecular weight	42.528 kDa	35 kDa	35 kDa	28 kDa
LD 50* (mice)	3 µg	1.87 µg/ip; 30 ng-50 µg/iv	50 µg	0.3 µg
Biological activity	Lethal Necrotizing Hemolytic Contraction of smooth muscle	Dermonecrosis and edema, enterotoxigenic, cytotoxic	Erythema Lethal Enterotoxigenic	Dermonecrosis and edema, enterotoxigenic, cytotoxic
Thermostability	Stable	Inactivated at 60°C (Sakurai and Duncan, 1978), ~2 month at -80°C	Inactivated at 60°C <1 week at RT ~1 month at -20°C, years lyophilized	Unknown
Reaction to Trypsin	Susceptible	Trypsin sensitive, (inactivated)	Activated (resistant)	Sensitive (inactivated)

*
per kg of mouse

ip intraperitoneal injection

iv: intravenous injection