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Lethal effects of *Clostridium perfringens* epsilon toxin are potentiated by alpha and perfringolysin-O toxins in a mouse model

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Abstract

Epsilon-toxin (ETX) is the most important virulence factor of *Clostridium perfringens* type D. Two other important toxins, alpha-toxin (CPA) and perfringolysin-O (PFO), are encoded and potentially produced by most *C. perfringens* type D isolates. The biological effects of these toxins are dissimilar although they are all lethal. Since the possible interaction of these toxins during infection is unknown, the effects of CPA and PFO on the lethal activity of 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. However, sublethal or lethal doses of CPA or PFO resulted in reduction of the survival time of mice injected simultaneously with ETX when compared with the intravenous effect of ETX injected alone. When PFO was inoculated intragastrically with ETX, a reduction of the survival time was observed. CPA did not alter the survival time when inoculated intragastrically with ETX. The results of the present study 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.

Keywords

Clostridium perfringens; epsilon toxin; alpha toxin; perfringolysin-O; synergism

1. Introduction

Clostridium perfringens is an important cause of both histotoxic and enteric diseases. The virulence of *C. perfringens* is largely attributable to its ability to produce >15 different toxins, several of which have lethal properties (McClane et al., 2005). However, individual isolates of this bacterium do not express this entire toxin repertoire, providing the basis for a classification scheme that assigns *C. perfringens* isolates to one of five different toxinotypes

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(type A to E) depending upon their production of four lethal toxins (α , β , ϵ and ι) (McDonel, 1986 and Songer, 1996).

In sheep, goats and other animals, *C. perfringens* type D cause enterotoxemias that initiate with the production of toxins in the gut (Songer, 1996). These toxins can act locally producing morphologic and physiologic changes in the gut (Fernandez-Miyakawa and Uzal, 2003; 2005) and/or systemically after intestinal absorption (Finnie, 2003). *C. perfringens* type D enterotoxemia is associated with the production of epsilon toxin (ETX), the most potent clostridial toxin known after botulinum and tetanus neurotoxins (McClane et al., 2005). Intravenous injection of ETX in sheep and goats reproduce many of the lesions usually observed in the natural disease (McClane et al., 2005).

Although ETX is the most important toxin in *C. perfringens* type D enterotoxemia (Finnie, 2003), the potential effects of other toxins secreted simultaneously by this microorganism during the disease are unknown and it has been suggested that at least other toxins produced by *C. perfringens* could act synergistically with ETX to produce disease in animals (Uzal and Kelly, 1998).

The studies reported here were undertaken to evaluate if other toxins produced by *C. perfringens* type D act synergistically with ETX in a mouse model. As alpha toxin (CPA) and perfringolysin-O (PFO) are two important toxins encoded and potentially produced by most *C. perfringens* type D isolates (Sayeed et al., 2005), the effects of both toxins on the lethal activity of ETX were studied.

2. Materials and methods

2.1. Bacteria and growth conditions

C. perfringens strain F4406 type A, *cpe*+, *cpb*2+ was obtained from B.A. McClane, University of Pittsburgh, and was grown on Brain Heart Infusion (BHI, Difco) agar plates, supplemented with 5% bovine blood, at 37°C in an atmosphere of 10:10:80 H₂:CO₂:N₂, or in anaerobic BHI broth supplemented with 0.5% yeast extract and 0.05% cysteine at 37°C. *Escherichia coli* DH5 α (Gibco-BRL) strains were grown at 37°C on Luria-Bertani (LB, Difco) agar or in LB broth with shaking, supplemented with 100 μ g/ml ampicillin, as appropriate.

2.2. DNA techniques

Genomic DNA from *C. perfringens* was isolated by the method of Pospiech and Neumann (1975). *E. coli* plasmid DNA extraction, transformation, DNA restriction, ligation, and agarose gel electrophoresis were performed essentially as described (Ausubel et al., 1994).

2.3. Animals

Conventionally reared, 20–25 g BALB/c mice of either sex were used. Animals were housed in a light cycle, humidity and temperature controlled room. The study was approved by the Animal Care and Use Committee of the California Animal Health and Food Safety Laboratory, University of California, Davis (Permit #34).

2.4. Toxins

Purified epsilon prototoxin was prepared from an overnight culture of *C. perfringens* type D (strain NCTC 8346) in Trypticase-yeast-glucose medium, under anaerobic conditions at 37°C. The overnight cultures were centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant containing ETX was saved for toxin purification. The toxin was then precipitated by ammonium sulfate. Two columns were prepared with DEAE and CM Sepharose (Pharmacia, Sweden), respectively, equilibrated in 10 mM Tris, pH 7.5. The toxin was applied to the DEAE

column, and the effluent was monitored at 220 nm. The initially eluted peak was saved and applied to the CM column. Again the effluent was monitored at 220 nm, and the first peak was collected, dialyzed against phosphate buffer solution (PBS), and freeze-dried. Prior to its use in these experiments, the prototoxin was reconstituted and activated by incubation at 37°C during 30 min with 0.1% trypsin (Sigma).

A semi-purified preparation of CPA was obtained from CSL (Melbourne, Australia).

Recombinant, 6xHIS-tagged PFO was prepared as follows. The *pfo* gene, lacking the coding region for the signal sequence, was amplified from *C. perfringens* F4406 genomic DNA by PCR with primers PFOF (5'-CCAGTAATCTCGAGCTCAAAGGATATAAC-3') and PFOR (5'-CTTACAATTAACTGAATTCAAACTG-3'), containing in-frame mutations encoding *Xho*I and *Eco*RI sites (underlined in the primer sequence), respectively. These primers amplified a 1,450-bp product from base 81 to the stop codon of the *pfo* gene. The PCR fragment was digested with *Xho*I-*Eco*RI and cloned into similarly digested pTrcHis B (Invitrogen), to generate pJGS212. pJGS212 encoded HIS-tagged PFO, a 506 amino acid protein comprising 472 amino acids of the mature PFO, with an N-terminal extension of 34 amino acids encoded by pTrcHis B, including a 6xHIS sequence.

Cultures for preparation of PFO were grown to an OD₆₀₀ of 0.6, prior to induction with 2.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were harvested by centrifugation at 5,000g and the cell pellet was resuspended in 20mM Tris-HCl, 100mM NaCl, pH 8.0. The cells were disrupted by two passages through a French Pressure Cell (Aminco) at 138MPa and the insoluble material was removed by centrifugation at 12,000g. PFO was purified from the soluble fraction using TALON Metal Affinity Resin (Clontech), as per the manufacturer's instructions. HIS-tagged PFO was eluted from the resin with 50mM imidazole, 20mM Tris-HCl, 100mM NaCl, pH 8.0.

The purity of the three toxin preparations used in this study was analyzed by SDS-PAGE. Briefly, PFO, ETX or CPA were mixed 1:1 with SDS-sample buffer (0.2 M Tris-HCl, pH 6.8, 2.5% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.013% bromophenol blue) and boiled for 5 min prior to electrophoresis in a 10% (w/v) SDS-polyacrylamide gel, as previously described (Ausubel et al., 1994). Total protein concentration was determined using Bradford Protein Assay Reagent (Bio-Rad). Also, the PFO and CPA preparations used were analyzed for lecithinase activity as previously described (Sayeed et al., 2005). Endotoxin was analyzed in the three toxin preparations using a commercial kit (Sigma, St. Louis, MO) following the instructions of the manufacturer.

2.5. Intravenous mouse lethality assays

The lethal dose fifty per milliliter (LD₅₀/ml) of CPA, PFO and ETX were determined using pairs of mice that received intravenous injections of two-fold dilutions of each of these toxins in 1% peptone water. The toxin titer was calculated as double the reciprocal of the highest concentration inducing lethality within 48 h in at least one of the two paired mice (Fisher et al., 2006).

For the study of interaction of CPA and PFO on ETX intravenous lethality, groups of three mice were intravenously injected with 0, 0.02, 0.2 or 2 LD₅₀ of CPA or PFO concurrently with 0, 0.12, 0.25, 0.5 or 2 LD₅₀ of ETX diluted in 1% peptone water. All mice were injected with a total volume of 0.5 ml and observed for up to 48 hr to monitor for lethality (defined as death or development of neurological signs). Mice showing neurological distress were immediately euthanized with CO₂.

The effects of intravenously injected CPA or PFO on survival time of mice given ETX intravenous injections were also studied. Groups of four mice were inoculated with 0, 0.2, 0.8, 2 or 20 LD₅₀ of CPA or PFO simultaneously with 20 LD₅₀ of ETX. Control mice were inoculated with different doses of CPA or PFO, but not ETX. Mice were observed during 8 hr to monitor for lethality. The survival time of each mouse was recorded and an average was calculated for each group.

2.6. Intragastric lethality of *C. perfringens* toxins

Mice were deprived of food 18 hr prior to the inoculations, but allowed water *ad libitum* until 2 hr before the experiments. To select the dose that killed approximately 50% of mice, groups of six mice were dosed intragastrically with 0.5 ml of 0, 500, 1,000, 2,000, 4,000 or 8,000 LD₅₀ of ETX in PBS-1.5% NaHCO₃ by stomach gavage with a feeding needle attached to a syringe. The same procedure was employed in other groups of mice inoculated with 0, 40 or 400 LD₅₀ of CPA or 0, 50, 500 or 2,000 LD₅₀ of PFO alone or together with 4,000 LD₅₀ of ETX. All mice were observed for up to 48 hr to monitor for lethality.

3. Results

3.1. Purity of the toxin preparations used

When analyzed by SDS—Page the preparations of ETX and PFO used showed a single band at the level of 32 and 60 KD, respectively (Fig.1). CPA showed a main band at the level of 45 KD and a second very faint band at the level of 58 KD. Only a negligible amount of endotoxin was found in the CPA preparation and no endotoxin was found in the other toxins used. Both CPA and PFO toxin preparations had moderate lecithinase activity.

3.2. Effect of CPA in the lethality of systemic ETX

Sub-lethal doses of CPA injected intravenously in mice concurrently with different doses of ETX did not have any significant effect on ETX lethality (Table 1). However, two LD₅₀ of CPA were lethal when injected alone or with any of the ETX doses assayed in this study. Also, 0.8, 2 and 20 LD₅₀ of CPA reduced the survival time of mice when injected at the same time with 20 LD₅₀ of ETX when compared with mice injected with ETX alone (Fig. 2). Survival times of mice injected with 20 LD₅₀ of CPA alone were longer than survival times of animals injected with 20 LD₅₀ of ETX alone (Fig. 2).

3.3. Effect of PFO in the lethality of systemic ETX

Sublethal doses of PFO injected intravenously with different doses of ETX did not have significant effects on ETX lethality (Table 2). However, two LD₅₀ of PFO were lethal when injected with an 20LD50 of ETX. Survival times of mice inoculated with PFO and ETX were shorter than survival times of animals inoculated with PFO alone (Fig. 3).

3.4. Lethal effects of intragastrically administered ETX and CPA

4,000 LD₅₀ of ETX were lethal for 50% of the mice when inoculated intragastrically, but none of the CPA doses tested intragastrically alone were lethal for the mice. When 40 or 400 LD₅₀ of CPA were inoculated intragastrically with 4,000 LD₅₀ of ETX, no changes in survival time were observed, in comparison with mice dosed with 4,000 LD₅₀ of ETX alone (data not shown).

3.5. Effect of PFO and ETX administered intragastrically

None of the doses of PFO administered intragastrically alone was lethal. However, when 50 or more LD₅₀ of PFO were administered with 4,000 LD₅₀ of ETX, survival times were

significantly reduced and lethality was increased in comparison with mice dosed with 4,000 LD₅₀ of ETX alone (Fig. 4).

4. Discussion

ETX produced by *C. perfringens* type B and D is a lethal and potent neurotoxin that causes edema by damage to endothelial cells (Adamson et al., 2005). This toxin is the key factor of type D enterotoxemia. Overgrowth of *C. perfringens* D in the intestine of susceptible animals produces large amounts of ETX which is absorbed through the intestinal mucosa and produces edema and neurological dysfunction (Songer, 1996). However, *C. perfringens* type B and D are also able to produce other lethal toxins such as CPA and PFO (Sayeed et al., 2005). The objective of this work was to determine if there is any interaction of these toxins.

Although sublethal doses of CPA or PFO did not increase the lethality of ETX, the increase in the ratio CPA/ETX or PFO/ETX resulted in reduction of the survival time of injected mice when compared with those injected with each one of these toxins alone. Although these experiments were performed in mice, it is possible that in the natural hosts (i.e. sheep and goats), both CPA and PFO enhance the ETX lethal effects during enterotoxemia.

The mechanism by which CPA decreased survival time of mice inoculated with ETX is unknown. CPA could act at the cellular level to increase the harmful effects of ETX. In biological membranes, CPA acts as a phospholipase C (PLC), with lethicinase activity (McClane and Rood, 2001; Sakurai et al., 2004). The cleavage of other phospholipids, e.g., sphingomyelin, is also mediated by CPA. This alteration of cellular membranes could increase the interaction of ETX with the cell surface or could also increase the oligomerization of ETX. CPA has also cytolytic, hemolytic, dermonecrotic and lethal activities (Sakurai et al., 2004) and those effects could favor ETX action. CPA induces the production of intercellular mediators in endothelial cells, intercellular adhesion molecule 1, interleukin-8, TNF- α , platelet-activating factor and the endothelial leukocyte adhesion molecule. It appears that these events contribute to the increased vascular permeability and edema (Sakurai et al., 2004). Increased vascular permeability may allow ETX to easily gain access to target organs. As CPA was able to decrease the time necessary for ETX to kill, but did not change the ETX lethal dose, it seems that a physiological alteration could be a more plausible explanation for this augmented lethality.

PFO contributed to the systemic action of ETX by decreasing the survival time of injected mice. PFO is a cholesterol-dependent cytolysin that lyses red blood cells and also has the ability to affect the host inflammatory response (O'Brien and Melville, 2004; Tweten, 1997). The connection of these effects described for PFO on the lethality of ETX is unknown and further work is necessary to clarify this issue.

It has been shown that ETX is lethal when this toxin is administered intragastrically to mice (Uzal and Fernandez-Miyakawa, 2006). In the present study, PFO inoculated intragastrically together with lethal ETX increased the lethality and reduced the survival time of exposed mice. A possible explanation for these observations is that ETX increases the intestinal permeability allowing the unrestricted absorption of macromolecules such as PFO and ETX itself. If lethal concentrations of PFO are absorbed from the intestine, the lethality observed could be produced by PFO instead of ETX since the lethal activity of PFO is faster than ETX.

Awad et al (2001) showed that there was synergism between CPA and PFO in gas gangrene produced by *C. perfringens* type A. However evidence of synergy in the study referred to (Awad et al, 2001) was obtained using an infection model but not purified toxins. We showed that in the model used there was synergism between CPA and PFO, and ETX. However, we have no evidence that the levels of toxin we used, or their relative ratios, are physiological. We

can not therefore, draw from this paper the conclusions that there is synergism between these toxins in the natural infection of sheep and goats.

In a recent study of toxin components of 26 *C. perfringens* type D culture supernatants in mice (Sayeet et al, 2006), it was observed that all the strains studied produced a very small amount of PFO and/or CPA and that these toxin levels did not correlate with toxicity.

The main hosts of natural infection by *C. perfringens* types B and D are sheep and goats. For this study we decided to use laboratory mice instead of the main hosts due to the fact that this species is readily available for research when compared with sheep and goats. ETX has been titrated in cell lines before, and the use of cell cultures (i.e. MDCK) has helped to gain partial understanding of the mechanism of action of ETX. However, MDCK cells are devoid of the many physiological mechanisms present in the living animal and the use of cell lines would have not allowed us to evaluate the whole physiological range of effects of the synergism between the toxins studied in this paper, which as our study has proven, can be very subtle.

The results of the present report show that CPA and PFO can increase the lethal effects of ETX. However, it is unknown if these effects can be observed during natural infections by *C. perfringens*. Quantification of the levels of these toxins in natural cases of type D disease might provide supportive information for this. Also *C. perfringens* type D knockout mutants could be useful to dissect the potential contribution of each toxin during enterotoxemia.

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Figure 1. SDS-PAGE gel stained with Coomassie blue of the three toxin preparations used. Lane 1: molecular weight marker; Lane 2: CPA; Lane 3: ETX; Lane 4: PFO.

Figure 2. Effect of sublethal and lethal doses of CPA in the survival time of mice injected at the same time with 0 or 20 LD₅₀ of ETX. Error bars indicate one standard deviation from the mean; n=4.

Figure 3. Effect of sublethal and lethal doses of PFO in the survival time of mice injected at the same time with 0 or 20 LD₅₀ of ETX. Error bars indicate one standard deviation from the mean; n=4.

Figure 4. Survival time of mice inoculated intragastrically with different doses of PFO and 4,000 LD₅₀ of ETX. Error bars indicate one standard deviation from the mean; n=6.

Table 1

Effect of CPA on ETX intravenous lethality of mice.

ETX (LD ₅₀ /mouse)	CPA (LD ₅₀ /mouse)			
	0	0.02	0.2	2
0	–	–	–	+
0.12	–	–	–	+
0.25	–	–	–	+
0.50	–	–	–	+
2	+	+	+	+

+: lethality

–: no effect

Table 2

Effect of PFO on ETX intravenous lethality of mice.

ETX (LD ₅₀ /mouse)	PFO (LD ₅₀ /mouse)			
	0	0.02	0.2	2
0	–	–	–	+
0.12	–	–	–	+
0.25	–	–	–	+
0.50	–	–	–	+
2	+	+	+	+

+: lethality

–: no effect