Dissecting the Contributions of *Clostridium perfringens* Type C Toxins to Lethality in the Mouse Intravenous Injection Model

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The gram-positive anaerobe *Clostridium perfringens* produces a large arsenal of toxins that are responsible for histotoxic and enteric infections, including enterotoxemias, in humans and domestic animals. *C. perfringens* type C isolates, which cause rapidly fatal diseases in domestic animals and enteritis necroticans in humans, contain the genes for alpha toxin (*plc*), perfringolysin O (*pfoA*), beta toxin (*cpb*), and sometimes beta2 toxin (*cpb2*) and/or enterotoxin (*cpe*). Due to the economic impact of type C-induced diseases, domestic animals are commonly vaccinated with crude type C toxoid (prepared from inactivated culture supernatants) or bacterin/toxoid vaccines, and it is not clear which toxin(s) present in these vaccines actually elicits the protective immune response. To improve type C vaccines, it would be helpful to assess the contribution of each toxin present in type C supernatants to lethality. To address this issue, we surveyed a large collection of type C isolates to determine their toxin-producing abilities. When late-log-phase vegetative culture supernatants were analyzed by quantitative Western blotting or activity assays, most type C isolates produced at least three lethal toxins, alpha toxin, beta toxin, and perfringolysin O, and several isolates also produced beta2 toxin. In the mouse intravenous injection model, beta toxin was identified as the main lethal factor present in type C late-log-phase culture supernatants. This conclusion was based on monoclonal antibody neutralization studies and regression analyses in which the levels of alpha toxin, beta toxin, perfringolysin O, and beta2 toxin production were compared with lethality. Collectively, our results highlight the importance of beta toxin for type C-induced toxemia.

Clostridium perfringens is a gram-positive, anaerobic bacterium that causes histotoxic and enteric infections (including enterotoxemias) in humans and domestic animals by virtue of production of a large number of toxins (25, 33). The differential production of four *C. perfringens* toxins, alpha toxin (PLC, also referred to as CPA), beta toxin (CPB), epsilon toxin, and iota toxin, is the basis for classifying *C. perfringens* isolates into five types, types A to E. By definition, type C isolates must produce PLC and CPB, but they could also produce several other biomedically relevant lethal toxins not used in the current toxinotyping classification scheme, including perfringolysin O (PFO), beta2 toxin (CPB2), and/or enterotoxin (CPE).

Type C isolates cause several enteric diseases in domestic animals and have a significant impact on the agricultural industry, in terms of both livestock loss and vaccination costs (34). The type C infections include acute enterotoxemia in adult sheep (also known as “struck”), hemorrhagic or necrotic enterotoxemias in piglets, lambs, calves, kids, and foals, and necrotic enteritis in poultry. Newborn animals, especially piglets, are particularly susceptible to type C infections, which can result in herd morbidity rates of 30 to 50%. While the pathology and case fatality rates associated with type C infections are different in different species, most animals exhibit obvious

hemorrhage and necrosis of the intestines, followed by death that is generally attributed to beta toxemia and/or the intestinal disorders mentioned above. In adult sheep, evidence of toxemia, including accumulation of protein-rich peritoneal, pleural, and pericardial fluid, is often apparent.

C. perfringens type C isolates are also the only non-type A isolates known to cause human disease (15, 17). Type C isolates are responsible for human enteritis necroticans (also known as pigbel or Darmbrand). The risk factors for enteritis necroticans include low intestinal trypsin levels and/or low intestinal motility due to a protein-poor diet or preexisting health problems (such as pancreatic disease) that affect the gastrointestinal tract. In pigbel, the type C isolates are introduced by consumption of undercooked meat products (often pork) (15). In severe cases of this disease, patients exhibit signs of toxemia, and segmental necrotizing enteritis is visible in the proximal intestine (15). Prior to control with a type C toxoid vaccine (16), enteritis necroticans was the most common cause of death in children who were more than 1 year old in the highlands of Papua New Guinea (22).

It has been commonly assumed, but never directly proven, that CPB (the second most potent *C. perfringens* toxin, which has a mouse 50% lethal dose [LD₅₀] of ~310 ng/kg [27]) is responsible for the deaths resulting from type C infections (34). However, administration of CPB alone does not result in the intestinal pathology or lethality of a type C infection (which requires culture supernatants or bacteria), suggesting that CPB

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alone does not cause classic type C disease (34). Furthermore, the current type C vaccines administered to domestic animals generally contain inactivated, relatively crude culture supernatants prepared from type C isolates (35, 38, 40). Consequently, it has not been definitively established which toxins present in type C supernatants (or the type C bacteria themselves) are needed to elicit protective immunity.

The mouse intravenous (i.v.) injection model is used to assess protection by type C vaccines prepared for domestic animals. This model is believed to mimic the systemic phase of type C infections, in which type C toxins circulate systemically after absorption from the intestine (34). However, since type C supernatants contain a number of lethal toxins, it is not clear which toxin(s) in type C supernatants is responsible for mouse lethality. Furthermore, while the effects of i.v. injection of individual purified toxins into mice have been reported, the contributions of different toxins to type C supernatant-associated lethality have not been rigorously assessed previously.

Therefore, the aim of this study was to use the mouse i.v. injection model to assess the role in lethality of individual toxins present in type C vegetative culture supernatants. Initially, the toxin repertoires (at the genotypic and phenotypic levels) of a large collection of type C isolates were determined. Select isolates were then tested further using the mouse i.v. injection model to assess the relative contributions to lethality of toxins produced during type C vegetative growth. Our results suggest that the CPB toxin is the primary mediator of lethality when type C vegetative culture supernatants are injected i.v. into mice.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 55 putative *C. perfringens* type C strains surveyed in this study either were obtained from our laboratory collection (4 human or domestic animal disease isolates) or were kindly provided by J. Glenn Songer (University of Arizona) (7 domestic animal disease isolates) or Russell Wilkinson (University of Melbourne) (44 isolates originating from the Burroughs-Wellcome collection [BW] of *C. perfringens* human or domestic animal disease isolates). The 11 type A isolates surveyed were obtained from our laboratory collection of environmental and disease isolates. Stock cultures of *C. perfringens* isolates were prepared in cooked meat medium (Difco Laboratories) and were stored at -20°C . Prior to use in experiments, bacteria were initially cultured overnight at 37°C in fluid thioglycolate medium (FTG) (Difco Laboratories). To ensure culture purity, samples of overnight FTG cultures were streaked on TSC agar plates (SFP agar [Difco Laboratories] containing 0.1% D-cycloserine [Sigma-Aldrich]), which were incubated overnight at 37°C under anaerobic conditions. Unless indicated otherwise, bacteria were grown in TGY broth (3% tryptic soy broth [Becton-Dickinson], 2% glucose [Sigma Aldrich], 1% yeast extract [Becton-Dickinson], 0.1% L-cysteine [Sigma Aldrich]) to assess vegetative toxin production and to prepare vegetative culture supernatants for mouse i.v. challenge studies.

Multiplex PCR toxin genotyping of *C. perfringens* isolates. *C. perfringens* isolates were inoculated onto brain heart infusion agar (Difco Laboratories) plates and incubated overnight at 37°C under anaerobic conditions. The resultant colonies were then used to prepare template DNA, as previously described (41). To detect the presence of toxin genes in our putative type C isolate collection, PCR primers designed to amplify internal regions of six *C. perfringens* lethal toxins or lethal toxin components (*plc*, *cpb*, *etx*, *ial/b*, *cpe*, and *cpb2*) were mixed with TaqComplete master mixture (GeneChoice), and PCR amplification was performed as previously described (9). The PCR products were separated on 2% agarose gels and were visualized with ethidium bromide.

PCR detection and sequencing of the *pfoA* gene. Two separate PCR assays designed to amplify sequences in the *pfoA* open reading frame (ORF) were used to determine if selected genotype C isolates carry the *pfoA* gene. All primer annealing positions indicated below are the positions relative to the first nucleotide of the initiation codon. The annealing start site for forward and reverse primers was the 5' end of the primer. The primers used were primers pfoAF1

(5'-ATCCAACCTATGGAAAAGTTTCTGG) and pfoAR1 (5'-CCTCCTAAACTACTGCTGTGAAGG), which amplified the ORF region from position 443 to position 975, and primers pfoAF2 (5'-GTATTGATTCTGGAATATCAAGTTAAG) and pfoAR2 (5'-CTTCAAACCTGTGCAACATAGGCTC), which amplified the ORF region from position 110 to position 1223. The expected product sizes were 532 bp and 1,113 bp for the pfoAF1-pfoAR1 and pfoAF2-pfoAR2 reactions, respectively.

To evaluate the basis for the PFO-negative phenotype of the type C isolates that were PCR positive for *pfoA*, the *pfoA* gene was completely sequenced (including the promoter region and ORF). For this *pfoA* sequencing, the following three separate PCR were performed: RXN1 (756 bp), in which pfoAproF (5'-GATACGTGGAAATGATTTAGAGGTAGA-3') and pfoAF2(R) (5'-CTTAACTTGATA TTCCAGAATCAATAC-3') were used to amplify the ORF region from position -619 to position 138; RXN2 (898 bp), in which pfoAproF2 (5'-CTGAACCTGAATTTAAGTTTAGAGAGAGT-3') and pfoAmidR (5'-GTCTA CTCCAAGTGAGTTTCAAGG-3') were used to amplify the ORF region from position -268 to position 630; and RXN3 (1.29 kbp), in which pfoAF1 and pfoAendR (5'-CTGCTCTTAAATCAATGCCTCAGC-3') were used to amplify the ORF region from position 443 to position +232. The PCR products were then sequenced with the primers used for each PCR (the RXN3 product was also sequenced with pfoAR2). Sequencing was performed at the University of Pittsburgh core sequencing facility (<http://www.genetics.pitt.edu/services.html>), and the resulting sequences were analyzed and aligned using Bioedit (11).

All primers (except pfoAF1 and pfoAR1, which were obtained from Garry Myers, The Institute for Genome Research) were designed by using the strain 13 genome *pfoA* sequence (30). Primers were used at a concentration of $1\ \mu\text{M}$ with TaqComplete master mixture. *C. perfringens* template DNA was prepared as described above for multiplex PCR analysis. Each PCR was performed using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min and a final extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

Optimization of vegetative growth conditions for CPB production. An isolated genotype C colony on a TSC plate was inoculated into FTG, which was then incubated overnight at 37°C . A 0.1-ml aliquot of the overnight FTG culture was transferred into 10 ml of either FTG, TGY medium, brain heart infusion broth (Difco Laboratories), or differential reinforced clostridial broth (EM Science). The cultures were incubated at 37°C until the late log phase, when samples were removed for Western blot analysis of CPB (see below). Growth of the cultures was monitored by determining the optical density at 600 nm (OD_{600}) at hourly intervals. Samples of cultures grown in TGY medium (determined to be the most consistent medium for CPB production [see Results]) were taken at 1-h intervals; after centrifugation of these samples, the supernatants were mixed 1:1 with 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer, boiled for 5 min, and then loaded on 10% SDS-PAGE gels for Western blotting of CPB (see below).

Growth conditions used for preparation of genotype C vegetative culture supernatants. Based on the results of the CPB production optimization studies described above, FTG (10 ml) was inoculated with a single colony of a type C isolate grown on a TSC plate. After overnight incubation at 37°C , a 0.1-ml aliquot was transferred into 10 ml of TGY medium, which was then incubated until the culture reached the late log phase based on measurement of the OD_{600} (the levels of lethal toxins expressed during vegetative growth were maximal during the late log phase, based on pilot experiments and previously described data [see Results]). Samples were removed from the cultures and centrifuged, and the resultant culture supernatants were filtered with 0.45- μm -pore-size filters (Millipore). The amounts of PLC, CPB, PFO, and CPB2 (for *cpb2*-positive isolates) present in the sterile vegetative supernatant filtrates were then determined using either Western blotting or activity assays as described below.

Quantification of toxin levels in genotype C culture supernatants. (i) CPB. Late-log-phase supernatants from three independent cultures of each genotype C isolate were prepared, mixed 1:1 with 2× SDS-PAGE loading buffer, and boiled for 5 min prior to loading on 10% SDS-PAGE gels for Western blot analysis of CPB. Different amounts of purified CPB were also loaded on each gel to create a standard curve for quantifying the CPB toxin present in each vegetative culture supernatant. After electrophoresis, protein was transferred onto nitrocellulose membranes (Bio-Rad Laboratories) and immunoblotted with neutralizing anti-CPB monoclonal antibody (MAb) CPCN10A2 (a gift from Paul Hauer, Center for Veterinary Biologics, Ames, Iowa), followed by a rabbit anti-mouse immunoglobulin G (IgG) peroxidase-conjugated antibody (Sigma). The blots were developed using the Supersignal West Pico (Pierce) substrate. The results of all

Western blot experiments were analyzed using a Bio-Rad ChemiDoc imaging system with the Quantity One quantitation software (Bio-Rad).

(ii) **CPB2.** CPB2 toxin levels in late-log-phase culture supernatants prepared from *cpb2*-positive genotype C isolates were determined as previously described (8). Briefly, cultures were either concentrated 10- to 50-fold using Amicon Ultra-15 centrifugal devices (10,000-molecular-weight cutoff; Millipore) or used without concentration, mixed 1:1 with 2× SDS-PAGE loading buffer, boiled for 5 min, and then electrophoresed on 12% SDS-PAGE gels. Purified CPB2 was electrophoresed on each SDS-PAGE gel to create a standard curve for quantifying the CPB2 in each vegetative supernatant. After transfer to nitrocellulose, the blots were probed with rabbit polyclonal anti-CPB2 antibodies, followed by goat anti-rabbit IgG peroxidase-conjugated antibody (Sigma). Immunoreactivity was detected using the Supersignal West Pico substrate.

(iii) **CPE.** The amounts of CPE in sporulating and vegetative culture supernatants from *cpe*-positive genotype C isolates were determined using methods described previously, with minor modifications (28). To assess CPE production by sporulating cultures, 0.2-ml aliquots of overnight FTG cultures were used to inoculate 10 ml of either Duncan Strong (DS) sporulation medium or modified Duncan Strong (MDS) sporulation medium (13). After growth for 15 h, sporulation was assessed using phase-contrast microscopy. The medium resulting in the highest percentage of sporulating bacteria (DS or MDS medium) for each isolate was used for CPE analysis. DS or MDS medium sporulating cultures were sonicated to disrupt any sporulating cells that remained intact in order to release CPE for immunodetection.

All other methods used to detect CPE in supernatants of sporulating or late-log-phase, vegetative cultures were identical to methods described previously (28). Briefly, supernatants were electrophoresed on 10% SDS-PAGE gels, and protein was subsequently transferred to nitrocellulose for Western blot analysis using a polyclonal rabbit anti-CPE antibody, followed by a goat anti-rabbit IgG peroxidase-conjugated antibody. Purified CPE was electrophoresed on each gel to create a standard curve for quantifying the CPE present in sporulating supernatants.

(iv) **PLC.** PLC production by genotype A or C isolates was assessed as previously described (28). Sterile (filtered) late-log-phase culture supernatants were concentrated 10-fold by lyophilization. Lyophilized material was then resuspended in phosphate-buffered saline and used in a phospholipase C assay in which nutrient agar supplemented with 4% (vol/vol) egg yolk agar was used, as described previously (32). A standard curve used to quantify PLC toxin was created using *C. perfringens* PLC (Sigma). Specific activity was expressed in PLC units/mg of total protein. The total protein in each lyophilized sample was determined using a BCA kit from Pierce.

(v) **PFO.** The levels of PFO activity present in genotype A or C culture supernatants were determined using a limiting dilution hemolysis assay, as previously described (36). Briefly, late-log-phase culture supernatants and Dulbecco's phosphate-buffered saline containing 5 mM dithiothreitol (Roche) were used to create a series of twofold dilutions. PFO-induced hemolytic activity was determined using horse erythrocytes (which were not lysed by other *C. perfringens* toxins under the assay conditions used [36]). PFO activity was then expressed as the reciprocal of the last dilution showing complete hemolysis (defined as the point at which a significant decrease in A_{570} was observed).

Assessing the lethality of genotype C vegetative supernatants using a mouse i.v. injection model. In initial screening experiments, sterile late-log-phase culture supernatants were prepared in duplicate as described above for toxin quantification and stored at -80°C until they were tested. To reduce animal use during lethality testing, the up and down method for calculating mouse LD_{50} was used (3). Briefly, this involved each BALB/c mouse (male or female; ca. 17 to 20 g; two mice per supernatant sample; Charles River Laboratories) receiving an i.v. (tail vein) injection of a 0.5-ml supernatant sample. Death as a routine experimental endpoint was not allowed by our Animal Care and Use Committee protocol. However, in pilot experiments we found that the monitored signs of distress (including nonresponsiveness [mice did not move when they were touched] and neurological distress [rolling over or rapid movement of the rear legs]) were typically followed soon by death when mice were inoculated with genotype C culture supernatants. For routine experiments, mice were observed for 48 h after i.v. injection to monitor development of significant distress as a marker for lethality. In pilot experiments, which were performed for up to 1 week, mice always showed signs of distress within 48 h after i.v. injection.

Vegetative culture supernatants that elicited significant distress signs (lethality) in mice following i.v. injection were titrated to calculate a mouse LD_{50}/ml , as described previously (39). Briefly, supernatants were diluted twofold (between 1:50 and 1:800) in 1% peptone water and injected i.v. into pairs of mice. Positive control mice received twofold dilutions of the same supernatant from a sterile genotype C isolate (*cpb2* negative and *cpe* negative) whose toxicity was known.

As a negative control, some mice were injected i.v. with 1% peptone water, which never induced signs of distress. The supernatant lethality titer was then calculated as twice the reciprocal of the highest dilution that induced lethality within 48 h in at least one of the two paired mice; the result was then expressed as the LD_{50}/ml . For these LD_{50}/ml experiments, two independently prepared batches of each vegetative culture supernatant were injected into pairs of mice (two mice per batch and two batches per isolate resulted in four mice tested per isolate at each dilution). The results were expressed as the average of the LD_{50}/ml values determined for each independent batch.

Both batches of vegetative culture supernatant were retested if there was a >3-fold dilution difference in the calculated supernatant LD_{50}/ml values for supernatant batches from the same isolate. LD_{50}/ml values for a limited number of isolates were determined again using freshly prepared supernatants approximately 2 months after the initial experiments to confirm the reproducibility of the results. Experimental procedures involving animals were approved by the Animal Care and Use Committee of the California Animal Health and Food Safety Laboratory, University of California, Davis (permit 04-11593).

Treatment of genotype C vegetative culture supernatants with neutralizing monoclonal antibodies. To help determine which toxin(s) was responsible for the lethal activity of genotype C vegetative culture supernatants, MAb neutralization experiments were performed. To neutralize CPB activity, two 0.8-ml aliquots of a late-log-phase sterile vegetative culture supernatant were mixed with 0.2 ml of the CPB-neutralizing MAb CPCN10A2 (used at a concentration of 2 mg/ml). Pilot experiments with semipurified CPB (see below) confirmed the neutralizing properties of MAb CPCN10A2. The supernatant-MAb mixtures were incubated at room temperature for 30 min. Mice were then injected i.v. with 0.5 ml of the supernatant-MAb mixture (two mice per neutralization trial) or with a control supernatant prepared similarly except for omission of the MAb. The same process was used to neutralize PLC or CPE activity, except that either 2 mg of an anti-PLC MAb (kindly provided by P. Hauer)/ml or 2 mg of anti-CPE MAb 3C9/ml (42) was substituted for the anti-CPB MAb. The semipurified, ultrafiltered PLC used for neutralization studies was obtained from an ovine *C. perfringens* type A isolate (CSL, Ltd., Melbourne, Australia).

Partial purification of CPB. Genotype C strain CN684 was grown to the late log phase in TGY medium at 37°C , chilled to 4°C , and then centrifuged at $10,000 \times g$ for 20 min. The supernatant was retained, and the protein was precipitated with 40% ammonium sulfate (Fisher Scientific). The precipitated protein was then pelleted by centrifugation at $10,000 \times g$ for 30 min. The pellet was resuspended in 30 mM Tris-HCl buffer (Bio-Rad) (pH 7.5) and dialyzed overnight against the resuspension buffer. The solution was filtered with a $0.45\text{-}\mu\text{m}$ filter and loaded onto a DEAE-CL6B Sepharose column preequilibrated with 30 mM Tris-HCl buffer (pH 7.5). CPB was then eluted from the column using 0.1 M NaCl in 30 mM Tris-HCl buffer (pH 7.5). Fractions were assessed for the presence of CPB by Western blot analysis (as described above), and purity was analyzed using Coomassie brilliant blue staining. Fractions containing CPB were pooled and analyzed with the Lowry assay to determine the protein concentration. A $10\text{-}\mu\text{g}$ aliquot of the pooled CPB sample was then loaded onto a 10% SDS-PAGE gel and stained with Coomassie brilliant blue to assess the purity of the final product. Analysis of the stained gel using a Bio-Rad ChemiDoc imaging system indicated that CPB comprised ~95% of the final purified material.

Statistical analysis. The correlation between toxin production and LD_{50}/ml values was determined using linear regression analysis (performed using Microsoft Excel) and was expressed as an R^2 value. Average toxin production levels were compared using the Student *t* test to determine if differences between the averages were statistically significant ($P < 0.05$). The Instat 2.03 software from Graph Pad was used to perform statistical calculations.

Nucleotide sequence accession number. Nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers DQ673097, DQ673098, DQ673099, and DQ673100.

RESULTS

Identification of toxin genes in putative *C. perfringens* type C isolates. To determine which toxin genes were present in our collection of 55 putative type C isolates, we used a multiplex PCR assay that can detect six major *C. perfringens* toxins. This analysis confirmed that seven isolates obtained from J. Glenn Songer and four isolates obtained from our collection were genotype C isolates (i.e., they possessed the *plc* and *cpb* genes but not the *etx* or *iap* gene). Multiplex PCR analysis of 44

TABLE 1. Type C isolate genotype summary

Genotype	No. of isolates (%)
<i>plc</i> ⁺ <i>cpb</i> ⁺	20 (44)
<i>plc</i> ⁺ <i>cpb</i> ⁺ <i>cpb2</i> ⁺	19 (42)
<i>plc</i> ⁺ <i>cpb</i> ⁺ <i>cpe</i> ⁺	5 (11)
<i>plc</i> ⁺ <i>cpb</i> ⁺ <i>cpb2</i> ⁺ <i>cpe</i> ⁺	1 (2)

putative type C isolates from the BW collection, which had been typed many decades previously using the classical toxin neutralization method, confirmed that 34 of these isolates were genotype C isolates (data not shown). The 10 remaining BW collection isolates that had been initially classified as type C isolates by the toxin neutralization method were identified as genotype A isolates by multiplex PCR because they carried the *plc* gene but not the *cpb*, *etx*, or *iab* gene; these isolates were not studied further.

Nearly one-half (44%) of the 45 confirmed genotype C isolates were shown by multiplex PCR to have a simple *cpb plc* toxin genotype, while 42% had a *cpb cpb2 plc* toxin genotype (Table 1). Interestingly, all seven of the North American veterinary genotype C disease isolates surveyed were positive for *cpb2*. The remaining genotype C isolates surveyed were either *cpb*, *plc*, and *cpe* positive (11%) or *cpb*, *plc*, *cpe*, and *cpb2* positive (2%). It is also noteworthy that both the *cpe* and *cbp2* genes were detected in several BW collection strains isolated in the 1930s and 1940s, well before the CPE and CPB2 toxins were identified.

Presence of lethal toxins in genotype C vegetative culture supernatants. Following multiplex PCR analysis, the verified genotype C isolates were assayed to determine their toxin production phenotypes. The production of PLC and the production of PFO were assessed using either an egg yolk agar hydrolysis assay (which measured the phospholipase C activity of PLC) or a horse erythrocyte hemolytic assay (which measured PFO activity). Currently, there are no functional activity assays for specific measurement of CPB, CPB2, or CPE levels. In lieu of such assays, quantitative Western blotting was used to measure CPB, CPB2, and CPE levels in culture supernatants.

The optimal culture conditions for CPB production have not been well established. Therefore, a few randomly chosen genotype C isolates were initially grown in four vegetative growth media (FTG, TGY medium, brain heart infusion broth, and differential reinforced clostridial broth) to identify optimal vegetative growth conditions for CPB production. Western blot analysis of CPB (using an anti-CPB MAb) showed that TGY medium was the most consistent medium for strong CPB production (data not shown). It was also determined (Fig. 1) that CPB production peaks during the late log phase for most genotype C isolates and then decreases during the late stationary phase. One genotype C isolate, CN3715, did not produce detectable levels of CPB (unless it was concentrated 50-fold) during any growth stage in TGY medium.

Since late-log-phase TGY medium cultures produced maximal levels of CPB and also contained maximal or significant levels of CPB2, PFO, and PLC (as determined in previous studies [28]), these growth conditions were used to determine

toxin levels in our study. The vegetative culture supernatants of selected genotype C isolates were eventually tested to determine their levels of lethality using a mouse lethality model, so toxin levels were compared only using these growth conditions in order to minimize the number of animals that were required later for lethality studies.

Using these optimized conditions, CPB production by our collection of genotype C isolates was quantified by densitometric analysis of Western blot CPB bands compared with a CPB standard curve (Fig. 2A). These analyses indicated that for genotype C isolates there were wide variations in the levels of CPB produced, which ranged from <1 to ~50 µg/ml (Fig. 2B). Interestingly, *cpb2*-positive genotype C isolates produced low levels of CPB (average, 3.9 µg/ml) compared to the levels produced by *cpb2*-negative genotype C isolates (average, 13.3 µg/ml), and only one *cpb2*-positive genotype C isolate was among the 33% of the genotype C isolates surveyed that produced >10 µg/ml of CPB (Fig. 2B). The difference between the averages was statistically significant (*P* < 0.01). There was no association between CPB toxin production levels and the date of isolation or origin of the genotype C isolates.

CPB2 toxin production in vegetative culture supernatants prepared from *cpb2*-positive genotype C isolates was also measured using Western blotting and densitometry (Fig. 3A). The

FIG. 1. Optimization of growth conditions for production of CPB by genotype C vegetative cultures. Four representative genotype C isolates were grown in TGY medium at 37°C. Every ~1.5 h the OD₆₀₀ was determined in order to assess growth (A), and samples were removed to determine the amount of CPB present (B). (B) For CPB detection, vegetative culture supernatant samples were processed for Western blotting using anti-CPB MAb. Purified CPB was loaded in the left lane of each blot as a positive control, and sample times are indicated below the blots.

FIG. 2. Western blot quantification of CPB in genotype C vegetative culture supernatants. Genotype C isolates were grown to the late log phase in TGY medium at 37°C and then processed for Western blot analysis of CPB. (A) Results for representative genotype C isolates (indicated at the top). The genotypes are as follows: CN5388, *plc cpb cpe cpb2*; CN2076, *plc cpb cpe*; JGS1075 and CN3715, *plc cpb cpb2*; and CN2109, *plc cpb*. The positions of molecular weight markers are indicated on the left (in kDa), and a *cpb*-negative type A isolate, as a negative control, was electrophoresed in the right lane. Pure CPB was electrophoresed in the left lane as a positive control. CPB levels in vegetative culture supernatants were determined by densitometric comparisons with a CPB standard curve generated using purified CPB. (B) CPB levels for isolates, expressed in $\mu\text{g/ml}$. Results are shown for each toxin subgenotype (all isolates are also *plc*⁺), and the total numbers of isolates for the different ranges are indicated in parentheses above the bars. ND, toxin could not be detected in culture supernatants.

CPB2 toxin levels were found to vary from nondetectable to 13 $\mu\text{g/ml}$ (Fig. 3B). No association between CPB2 production levels and isolate disease origin was apparent.

Genotype C isolates carrying the *cpe* gene were assessed to determine their abilities to produce CPE under both sporulating and vegetative culture conditions. Consistent with previous studies which showed that *cpe*-positive type A and D isolates produce CPE only during sporulation (7, 28), Western blot analysis of CPE (Fig. 4A) demonstrated that all of the *cpe*-positive genotype C isolates surveyed produce CPE during sporulation but not during vegetative growth (Fig. 4B). The higher-molecular-weight species apparent in the purified CPE lane and supernatant samples tested (Fig. 4B) are CPE aggregates.

FIG. 3. Western blot analysis of CPB2 levels in *cpb2*-positive genotype C vegetative culture supernatants. Genotype C isolates carrying the *cpb2* gene were grown in TGY medium at 37°C to the late log phase. Culture supernatant samples from these cultures were then processed with or without concentration (10- to 50-fold) and used for Western blot analysis of CPB2. (A) Representative Western blot for CPB2. The positions of molecular weight markers are indicated on the left, and pure CPB2 was electrophoresed in the left lane as a positive control. All isolates shown have toxin genotype *plc cpb cpb2*. The amount of CPB2 in each sample was determined by comparing the CPB2 signal density to a CPB2 standard curve generated using purified CPB2. (B) Amount of CPB2 in each sample. ND, toxin could not be detected in culture supernatants.

The CPE aggregation phenomenon, particularly when higher concentrations of CPE are subjected to SDS-PAGE, has been well documented (20).

An analysis of the PFO expression of our genotype C isolate collection, performed using a PFO-specific horse red blood cell hemolysis activity assay, showed that most (83%) genotype C isolates produced PFO (Fig. 5A), although the levels ranged from nondetectable to a log₂ titer of 4 to 5. It is noteworthy that five of the six *cpe*-positive genotype C isolates surveyed (the *plc*-, *cpe*-, and *cpb2*-positive genotype C isolate was the lone exception) did not produce PFO, while PFO was produced by nearly all genotype C isolates carrying only the *plc*

FIG. 4. Detection and quantification of CPE in *cpe*-positive genotype C culture supernatants using Western blotting. Isolates were grown in either MDS or DS medium (for sporulating conditions) or TGY medium (for vegetative conditions) for 15 h at 37°C. Culture supernatants were sonicated, and lysates were subjected to Western blot analysis of CPE. (A) Purified CPE was electrophoresed in the left lane as a positive control, and a *cpe*-negative type A isolate was electrophoresed as a negative control. The positions of molecular weight markers are indicated on the left. Whether samples were obtained from sporulating or vegetative culture supernatants is indicated at the bottom. The amount of CPE in each isolate supernatant was determined by comparing the CPE signal intensity to a standard curve created using pure CPE. (B) CPE levels. ND, toxin could not be detected in culture supernatants.

and *cpb* genes or only the *plc*, *cpb*, and *cpb2* genes. For comparison, PFO production during vegetative growth was also assessed (Fig. 5B) for 11 representative genotype A isolates (genotype A isolates are the predominant *C. perfringens* isolates [34]). These comparisons indicated that the PFO titers of vegetative culture supernatants derived from genotype A isolates and genotype C isolates are similar under the experimental conditions used in our study; i.e., the difference between the averages was not statistically significant.

To determine whether PFO-negative genotype C isolates lack the *pfoA* gene as a possible explanation for why these isolates did not produce detectable levels of PFO, two *pfoA* PCR assays in which different regions in the *pfoA* ORF were amplified were performed. These PCR assays did not result in amplification of either of the expected internal *pfoA* PCR products from the five PFO-negative, *cpe*-positive genotype C

isolates (data not shown). In contrast, *pfoA* PCR products of the expected size were amplified from the three PFO-negative, *cpe*-negative genotype C isolates and from two PFO-positive genotype C positive control isolates (data not shown).

Sequencing of the *pfoA* gene was performed for the three type C isolates that were PCR positive for *pfoA* but did not produce detectable PFO activity. This sequencing, which included the promoter region containing the VirR binding box (5) and the ORF, revealed that there were no nucleotide differences in the promoter region of these isolates compared to the *pfoA* sequence of type A strain 13. Compared to the strain 13 PFO amino acid sequence, an A71T amino acid substitution was present in all genotype C isolates sequenced (including three PFO-negative isolates and one PFO-positive genotype C isolate), while an A215V mutation was also present in one of the PFO-negative isolates (data not shown).

Alpha toxin production by genotype C isolates was determined using an egg yolk agar hydrolysis assay that measures phospholipase C activity. The PLC activity (expressed as U/mg total protein $\times 10^{-3}$) in late-log-phase supernatants of genotype C isolates was found to range from nondetectable levels (for 15% of the genotype C isolates surveyed) to ~ 10 U/mg total protein $\times 10^{-3}$ (Fig. 6A). Most of the *cpb2*-positive genotype C isolates produced lower levels of PLC (PLC activity, < 2 U/mg total protein $\times 10^{-3}$) than *cpb2*-negative genotype C isolates produced (average PLC levels, 0.5 and 3.2 U/mg total protein $\times 10^{-3}$), and *cpb2*-positive genotype C isolates accounted for most (18/31) of the isolates that produced low levels of PLC. The difference between the averages was statistically significant ($P < 0.01$). For comparison, the PLC activities in late-log-phase supernatants from 11 representative genotype A isolates (from environmental [five isolates] and disease [six isolates] sources) ranged from 0.6 to > 18 U/mg total protein $\times 10^{-3}$ (Fig. 6B), and only 1 of the 11 type A isolates produced > 4 U/mg total protein $\times 10^{-3}$. The difference between the average PLC values for type C and type A isolates was not significant.

Assessing the contributions of individual toxins to the lethality of genotype C vegetative culture supernatants using a mouse i.v. injection model. The results shown in Fig. 2 to 6 demonstrated that late-log-phase culture supernatants from genotype C isolates typically contained multiple toxins with lethal activity. To assess the contributions of individual toxins to lethality, late-log-phase culture supernatants from select genotype C isolates were prepared for lethality testing with the mouse i.v. injection model. In the absence of a well-characterized small-animal oral challenge model for type C infection, the mouse i.v. injection model is commonly used to assess the lethality of genotype C vegetative culture supernatants (12, 35). For our mouse lethality experiments, sterile-filtered dilutions of vegetative culture supernatants were injected i.v. into mice to determine an LD_{50}/ml for each isolate. The results were then compared with the amount of each lethal toxin (CPB, CPB2, PFO, or PLC) previously shown to be present in the samples in order to evaluate whether there was a correlation between the level of each toxin and lethality (Fig. 7).

For the 22 genotype C isolates tested, there was a strong positive correlation between mouse lethality and the level of CPB toxin (Fig. 7A) in the late-log-phase supernatants. By contrast, lethality showed only a weak positive correlation with

FIG. 5. PFO titers of genotype C isolates and selected genotype A isolates. Serial twofold dilutions of late-log-phase vegetative culture supernatants were incubated with horse erythrocytes to measure hemolysis. The last dilution that produced complete hemolysis (defined as a significant change in A_{570}) was defined as the log₂ titer. (A) PFO titers for genotype C isolates for each toxin subgenotype (all isolates are also *plc*⁺). The total numbers of isolates in the titer groups are indicated in parentheses. (B) PFO titers for representative type A isolates. ND, toxin could not be detected in culture supernatants.

PLC and CPB2 levels and no correlation with PFO (Fig. 7B to D). Additionally, the lethal properties of the supernatants were rapidly destroyed (data not shown) by treating the vegetative culture supernatants with trypsin (0.5% for 30 min at 37°C). Since CPB is very trypsin sensitive (14), this observation is consistent with the hypothesis that CPB is an important factor in the lethality observed when mice receive injections of genotype C vegetative culture supernatants.

Neutralization of genotype C vegetative culture supernatant lethality with MAb. To definitively discern the role of CPB in mouse lethality, monoclonal antibody neutralization experiments were performed. Fourteen genotype C vegetative culture supernatants were pretreated with neutralizing monoclonal antibodies specific for either CPB or PLC. When these pretreated supernatants were tested for lethality in the mouse i.v. injection model, only the late-log-phase supernatants pretreated with a CPB-neutralizing MAb had lost the ability to induce mouse lethality (Table 2). ATCC 13124, a strain that produces a high level of PLC, was used in this study to demonstrate that we could neutralize lethal amounts of toxins

other than CPB in our mouse model, if they were present. It is worth noting that while mice treated with anti-PLC MAb still died, the time until death was longer when mice were injected with supernatants treated with PLC-neutralized supernatants than when they were injected with nonneutralized supernatants; six of seven mice showed a >1.5-fold increase (range, 1.1- to 11.8-fold) in the time until death.

The specificity of anti-CPB monoclonal antibody neutralization was confirmed by demonstrating that preincubation of semipurified CPB with this MAb protected mice from lethal i.v. challenge (Table 2). However, lethality in mice challenged i.v. with the semipurified CPB was not prevented if the toxin was preincubated with either anti-PLC MAb or anti-CPE MAb (Table 2). To further confirm the specificity of the anti-CPB MAb in these neutralization experiments, we showed that semipurified PLC remained lethal when it was preincubated with the anti-CPB MAb prior to i.v. injection into mice, but that anti-PLC MAb was able to completely neutralize the lethality of this PLC preparation (Table 2).

FIG. 6. PLC levels in genotype C and selected genotype A vegetative culture supernatants. Late-log-phase vegetative culture supernatants were assessed to determine their PLC activities using an egg yolk hydrolysis assay. A standard curve was generated using semipurified PLC. PLC activity is shown for genotype C isolates (A) and selected type A isolates (B). PLC results for genotype C isolates are shown for the different toxin subgenotypes (all isolates carry the *plc* gene). The total numbers of isolates in the PLC activity ranges are indicated in parentheses. ND, toxin could not be detected in culture supernatants.

FIG. 7. Correlation of specific toxin levels of genotype C isolates and mouse lethality. Vegetative culture supernatants from select genotype C isolates were assessed to determine their levels of lethality using a mouse i.v. injection model. Pairs of mice were injected i.v. in the tail vein with dilutions of vegetative culture supernatants. The reciprocal of the last dilution that caused lethality in at least one of the two mice was defined as the LD₅₀/ml (see Materials and Methods). The LD₅₀/ml values were then correlated with the amounts of CPB, PLC, PFO, or CPB2 present in the vegetative culture supernatants. A linear equation was used to draw a best-fit line based on the data points, and the R^2 for this line is indicated on each graph.

DISCUSSION

C. perfringens type C isolates remain an important cause of disease in domestic animals, as indicated by the widespread type C vaccination of domestic animals (24). Type C isolates also are the only non-type A isolates documented to cause human disease (15, 17). Type C-associated human disease is most closely associated with Papua New Guinea, where it was once the second leading cause of death in children who were more than 1 year old, which led to a childhood vaccine program that helped to reduce this problem (22). In addition, type C human necrotic enteritis is now seen in developed countries, typically in patients with pancreatic disease (10, 18, 23, 29, 37).

Despite the obvious importance of type C isolates for both human and animal disease, the virulence of these isolates is poorly understood. While these bacteria must produce at least CPB and PLC in order to be classified as type C isolates, our results indicate that most type C isolates also produce a third lethal toxin (PFO) and that ~40% of these isolates produce a fourth lethal toxin (CPB2) during late-log-phase growth. This complex lethal toxin repertoire raises the possibility that type C-induced disease results from the combined activities of several toxins. Consistent with this possibility, animal model studies have shown that CPB alone cannot elicit the symptoms of

a type C infection (34). Furthermore, the current type C veterinary vaccines are based on crude toxoids or bacterin/toxoid (killed bacteria plus inactivated supernatants) (35, 38, 40). In addition, the human vaccine previously used in New Guinea was not prepared with a pure CPB toxoid but instead was prepared with ammonium sulfate-precipitated supernatants from type C vegetative cultures that were formalin inactivated (40; Gregor Lawrence, personal communication). Therefore, the protective immune response elicited by these type C toxoid vaccines against type C infections could involve protective antibodies not only against CPB (which is the only component for which type C vaccines are validated [19]) but also against any other lethal toxins produced by the type C isolates used for producing the toxoid vaccine. To our knowledge, type C strains used for vaccine preparation do not have a well-characterized toxin repertoire.

As a first step in determining which toxins play a role in type C-induced disease, we determined the roles of individual type C toxins in the mouse i.v. supernatant injection lethality model. In this research we initially determined the toxin genotypes and phenotypes of a large number of putative type C isolates, which confirmed that all isolates previously genotyped by multiplex PCR were genotype C isolates. However, a number of

TABLE 2. Neutralization of type C toxins with MAb

Isolate	Type	Phenotype ^a	Lethality neutralized by:		
			Anti-CPB MAb	Anti-PLC MAb	Anti-CPE MAb
CN685	C	CPB _{8.9} PLC _{1.0} PFO _{2.2}	Yes		
CN5383	C	CPB _{23.5} PLC _{3.6} PFO _{1.6}	Yes		
CN885	C	CPB _{23.6} PLC _{6.3} PFO _{2.6}	Yes	No	No
CN886	C	CPB _{24.1} PLC _{5.1} PFO _{4.2}	Yes	No	
CN1797	C	CPB _{18.9} PLC _{5.5} PFO _{3.9}	Yes	No	
CN2109	C	CPB _{12.8} PLC _{9.2} PFO _{3.1}	Yes	No	
CN3685	C	CPB _{13.0} PLC _{6.0} PFO _{2.9}	Yes	No	
CN3717	C	CPB _{<1} PLC _{ND} PFO ₂	Yes		
CN3955	C	CPB _{30.9} PLC _{9.3} PFO _{3.6}	Yes	No	No
NCTC 10719	C	CPB _{2.7} CPB _{20.9} PLC _{<0.7} PFO _{ND}	Yes		
JGS1071	C	CPB _{2.5} CPB _{27.8} PLC _{0.9} PFO _{3.1}	Yes	No	
CN3686	C	CPB _{6.0} CPB _{21.9} PLC _{1.2} PFO _{1.9}	Yes		
CN5388	C	CPB _{3.0} CPE _{<0.05} CPB _{2ND} PLC _{0.9} PFO _{1.8}	Yes		
CN3758	C	CPB _{14.8} CPE _{11.2} PLC _{5.2} PFO _{ND}	Yes	No	No
ATCC 13124	A	PLC _{18.8} PFO _{5.1}	No	Yes	No
	A	Semipurified PLC (from CSL)	No	Yes	
	C	Semipurified CPB	Yes	No	

^a The subscript numbers indicate the supernatant concentrations ($\mu\text{g/ml}$ for CPB, CPB2, and CPE; U/mg total protein $\times 10^{-3}$ for PLC; and $\log_2$ titer for PFO) of the toxins. ND, not detectable.

isolates assigned to type C many years ago using the classical guinea pig skin test were instead classified as type A isolates by multiplex PCR. The explanations for the difference in classification include the loss of the *cpb*-containing plasmid after toxin neutralization typing and, perhaps, the relatively crude nature of historical guinea pig skin neutralization testing.

Interestingly, our genotypic analyses showed that ~40% of the genotype C isolates surveyed carry the *cpb2* gene, which is a considerably higher percentage than the 18% *cpb2*-positive isolates identified in a recent survey of type D isolates (28). The percentage of *cpb2*-positive genotype C isolates in our collection is lower than the percentage determined by Bueschel et al., who reported that 64% of their genotype C isolates ($n = 178$) were *cpb2* positive (4). The *cpb2* carriage rate for type C isolates is also less than the 80% *cpb2* carriage rate determined for type A isolates carrying a plasmid-borne *cpe* gene (8). The very strong association between *cpb2* and type A isolates carrying a *cpe* plasmid is partially attributable to the fact that many, although not all, of the type A isolates carry *cpb2* and *cpe* on the same plasmid. In this regard, it is interesting that we identified only a single *cpe*-positive, *cpb2*-positive type C isolate in the current survey. Studies are now under way to determine whether *cpb* and *cpb2* reside on the same plasmid in our genotype C isolates.

Phenotypic analyses showed that under our assay conditions, genotype C isolates carrying the *cpb2* gene typically produce lower levels of both CPB and PLC than the *cpb2*-negative genotype C isolates produce. Since the regulation of PLC or CPB in type C isolates has not been studied yet, we have no explanation for these observations. However, the explanation might involve differences in the VirR/VirS two-component system, which positively regulates both *cpb2* and *plc* expression in type A isolates via its ability to regulate expression of VR-RNA

regulatory RNA (2, 21, 31). Another possibility is that genes present on the putative *cpb2* plasmid might specifically down-regulate *cpb* and/or *plc* expression.

Phenotypic analyses also revealed that five of the six *cpe*-positive genotype C isolates surveyed produced no detectable PFO activity. Two PCR analyses indicated that the *pfoA* gene was not present in these isolates. In contrast, similar PCR analyses of the *pfoA* gene in *cpe*-positive *C. perfringens* type A isolates demonstrated that the *pfoA* gene is present in most *cpe* plasmid isolates but is not present in most chromosomal *cpe*-positive type A isolates (unpublished observations). Studies should be performed to address the *cpe* gene location (chromosomal versus plasmid) in *cpe*-positive genotype C isolates, which would reveal whether the genotypes of these isolates resemble the *pfoA*-negative genotype of most chromosomal *cpe* type A isolates.

Sequencing of the *pfoA* gene from the three *cpe*-negative genotype C isolates that failed to produce PFO yet were PCR positive for *pfoA* revealed no nucleotide substitutions in the promoter region that could explain the lack of PFO activity for these isolates (5). Nor could the lack of PFO activity for these isolates be attributed to consistent nucleotide changes in the *pfoA* ORF. However, compared to the *pfoA* gene of type A strain 13, there were consistent nucleotide changes in the *pfoA* ORF of the type C isolates sequenced that resulted in two different amino acid substitutions. The A71T substitution was identified in all four type C isolates sequenced, including a PFO-positive isolate, while an A215V substitution was present in a single PFO-negative isolate. Based on these sequencing and PCR results, it appears that the lack of detectable PFO activity for some type C isolates results either from an inability to make PFO (for most or all *cpe*-negative, type C isolates that were PCR positive for *pfoA*), implying that there is a regulatory

gene mutation, or from the actual absence of the *pfoA* gene (for the *cpe*-positive type C isolates negative in both *pfoA* PCR assays). However, our results do not rule out the possibility that some *pfoA*-positive, PFO-negative isolates could produce PFO under culture conditions different than those tested or that the A215V substitution encoded by the *pfoA* gene of one *pfoA*-positive PFO-negative isolate eliminates its PFO activity.

Finally, the results of our toxin phenotype analyses of genotype C isolates allowed us to compare the toxin production abilities of these isolates with those of genotype D and A isolates. No statistically significant differences were found between the average amounts of PLC or PFO produced when we compared genotype C isolates to genotype A or D isolates (28; this study). However, it should be noted that not all of the genotype A isolates used to measure PFO and PLC levels in this study were isolated from gas gangrene cases, which are believed to be associated with genotype A isolates that produce high levels of PFO and PLC. The average levels of CPE (which is produced only during sporulation) in culture supernatants from sporulating genotype C isolates, genotype A isolates (6), and genotype D isolates (28; this study) were also not significantly different. It is notable that while some genotype D isolates did not produce detectable levels of their typing toxin (ETX) (28) even when their supernatants were concentrated, all genotype C isolates tested produced detectable amounts of CPB toxin, although detection sometimes required concentration of the culture supernatants. It is important to note that these findings are specific for the media and test conditions used in this study; i.e., isolates could make more or less PLC (or other toxins) in brain heart infusion media in the late log phase.

The timing of CPB toxin production has not been well documented. Therefore, in this study we tried to determine when this toxin is produced during *in vitro* growth, because quantifying CPB toxin levels in vegetative culture supernatants is important both for our mouse *i.v.* lethality studies and for preparing type C vaccines. The results of these analyses indicated that CPB toxin production reaches peak levels during late-log-phase growth in TGY medium. The timing of CPB production agrees with the results of a previous study in which the workers measured levels of CPB activity in culture supernatants from a single type C isolate using a guinea pig skin test animal model (26). Interestingly, Sakurai and Duncan also found that fermentable sugars can increase CPB production under non-pH-controlled conditions (26). In the current study, the levels of CPB production were consistently higher in TGY medium (which contained 2% glucose/liter) than in FTG (0.55% glucose/liter), brain heart infusion broth (0.2% glucose/liter), or differential reinforced clostridial broth (0.1% glucose/liter). These analyses also revealed that CPB production varies substantially (>40-fold) among type C isolates, suggesting that there are isolate-dependent variations in *cpb* gene regulation. Furthermore, CPB toxin levels were typically found to decrease during the stationary phase, possibly due to decreased CPB production or protease activity present in stationary-phase cultures. Together, the variability in CPB toxin production observed among type C isolates and the reductions in CPB toxin levels detected during longer growth periods highlight the importance of selecting the proper strain and growth conditions for preparing type C toxoid vaccines. To our knowl-

edge, the identities of genotype C strains and the growth conditions used to prepare type C vaccines have not been clearly reported in refereed publications.

To gain further understanding regarding which toxin(s) present in type C supernatants is responsible for virulence (and therefore must be neutralized for a vaccine to elicit protective immunity), sterile late-log-phase supernatants from approximately one-half of our type C isolates were injected *i.v.* into mice. When the LD₅₀/ml values obtained for these vegetative culture supernatants were correlated with the levels of lethal toxins produced by the isolates, no positive correlation was observed between LD₅₀/ml and PFO levels. In contrast, the levels of three other lethal toxins (CPB, CPB2, and PLC) showed at least some positive correlation with supernatant LD₅₀/ml values, and the CPB levels exhibited a much higher LD₅₀/ml correlation ($R^2 > 0.7$) than either the PLC or CPB2 levels (R^2 , 0.38 and 0.18, respectively). These correlation results suggest that CPB plays a major role in the lethality of type C supernatants in the mouse *i.v.* injection model.

The importance of CPB for type C supernatant lethality in the mouse *i.v.* injection model was then conclusively demonstrated by toxin neutralization experiments using neutralizing monoclonal antibodies specific for CPB, PLC, or CPE. In these neutralization studies, the lethal properties of late-log-phase type C supernatants were consistently neutralized by anti-CPB monoclonal antibodies, but neutralization was not observed using either anti-PLC or anti-CPE monoclonal antibodies. Interestingly, the time until death for mice treated with supernatants incubated with anti-PLC antibodies was longer than the time until death for mice treated with untreated supernatants. This observation along with the CPB neutralization data may indicate that when our experimental conditions are used, PLC is not the primary mediator of lethality (since the mice still died), but it does make a minor contribution to *i.v.* lethality, possibly explaining the small positive correlation between supernatant LD₅₀/mls and PLC concentration ($R^2 = 0.38$) (Fig. 7D).

Previous oral challenge and intestinal loop studies suggested that CPB cannot cause the full pathology of a natural type C infection (34). This finding along with the current *i.v.* challenge results suggests that CPB is required for the systemic lethality of type C supernatants, but a secondary factor(s) may facilitate the intestinal absorption of CPB into the circulation during natural disease. This possibility cannot be conclusively addressed by the mouse *i.v.* challenge model or monoclonal antibody neutralization approaches, which do not address factors such as the breaching of the intestinal permeability barrier or possible synergistic toxin interactions. There is a precedent for such synergistic interactions since previous studies have shown that PLC and PFO act synergistically in the pathogenesis of histotoxic type A infections in mice (1). While the current results provide important insights into type C pathogenesis, further research is clearly needed to determine which toxin(s) (or the bacteria themselves) contributes to the early stages of type C-induced enteric disease. To address this issue, efforts are being made to develop a small-animal oral challenge model that better mimics the natural type C disease. When available, this oral challenge model should be useful for virulence testing of type C toxin knockout mutants in order to dissect the specific roles of various toxins at each stage of type C disease.

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