Molecular Cloning of the 3' Half of the *Clostridium perfringens* Enterotoxin Gene and Demonstration that This Region Encodes Receptor-Binding Activity

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Clostridium perfringens type A enterotoxin (CPE) causes the symptoms associated with *C. perfringens* food poisoning. To determine whether the C-terminal half of CPE contains receptor-binding activity, the 3' half of the *cpe* structural gene was cloned with an *Escherichia coli* expression vector system. *E. coli* lysates containing the expressed C-terminal CPE fragment (CPE_{frag}) were then assayed for CPE-like serologic, receptor-binding, and cytotoxic activities. CPE_{frag} was shown to contain an epitope located at or near the receptor-binding domain of the CPE molecule. Competitive-binding studies showed specific competition for CPE receptors between CPE and CPE_{frag} lysates. CPE_{frag} lysates did not cause cytotoxicity in Vero (African green monkey kidney) cells. However, preincubation with CPE_{frag} lysates specifically protected Vero cells from subsequent CPE challenge. This indicates that CPE_{frag} recognizes the physiologic receptor which mediates CPE cytotoxicity. Collectively, these studies indicate that the C-terminal half of CPE contains a receptor-binding domain but additional amino acid sequences appear to be required for CPE cytotoxicity.

Clostridium perfringens enterotoxin (CPE) is responsible for symptoms associated with *C. perfringens* food poisoning (26), and CPE may also be involved in other human and veterinary gastrointestinal illnesses (5, 13, 17, 20). CPE is a single polypeptide chain of ≈34 kilodaltons (kDa) (6, 22) which has no significant amino acid homology with other enterotoxins (6).

Recent studies have improved understanding of the apparently unique molecular events involved in CPE action (17). Initially, CPE binds to specific eucaryotic receptors (19, 29) which may include two membrane proteins of 50 and 70 kDa (30). After binding, CPE may be inserted into the plasma membrane (19) and rapidly produces significant changes in membrane ion permeability (19). These changes in ion permeability then produce later secondary CPE effects, such as inhibition of macromolecular synthesis and morphologic damage (17).

Assignment of specific functions to defined regions of toxin molecules is often used to gain insights into toxin action (1, 2, 11, 23). Limited information is available about CPE structure-function relationships. Trypsin removal of the 34 NH₃-terminal residues from CPE increases CPE action (7), indicating that the entire CPE molecule is not required for bioactivity. Recent studies (9, 27) have suggested that a C-terminal CPE fragment (putative amino acids 177 to 309), derived chemically by 2-nitro-5-thiocyanobenzoic acid (NTCB) cleavage of intact CPE, may inhibit CPE specific binding. However, conclusions from those studies should be interpreted cautiously, since freshly trypsinized cells were used for binding-biologic activity experiments with the NTCB-derived fragment and trypsin removes most CPE receptors (19). Further, assignment of this NTCB-derived fragment to the C-terminal half of CPE remains tentative (9), since amino acid sequencing was not performed and conclusive evidence was not presented for complete separation of the NTCB-derived C-terminal frag-

ment away from other possible contaminating CPE fragments before assay for CPE-like activities.

To determine directly which activities are associated with the C-terminal half of CPE, we cloned the 3' half of the *cpe* gene with an *Escherichia coli* expression system. This system provides a defined fragment (CPE_{frag}) which has not been exposed to the harsh denaturing conditions previously used (9) to chemically cleave CPE and is free of possible contamination from intact CPE or other CPE fragments. CPE_{frag} was then tested for serologic, receptor-binding, and cytotoxic activities. Using CPE_{frag}, we demonstrated that the C-terminal half of CPE (amino acids 162 to 309) contains a receptor-binding domain but is insufficient to cause cytotoxicity.

MATERIALS AND METHODS

Bacterial strains and vectors. *C. perfringens* NCTC 8239, which is a high producer of CPE (18), was used as the DNA source for library construction. *E. coli* Y1089 and Y1090, λgt11 DNA and bacteriophage packaging reagents, *E. coli* HB101, and plasmid pGEM4, were purchased (Promega Biotech). Expression plasmid pEV-vrf1 and *E. coli* RR1 (pRK248cIts) (3) were the kind gift of R. Crowl (Hoffmann-La Roche Inc.).

Library construction and screening. DNA was isolated from *C. perfringens* by the method of Marmur (15). A recombinant λgt11 library was generated by random shearing of DNA through a needle, blunting of the ends, protection of endogenous *Eco*RI sites, addition of *Eco*RI linkers, activation of ends with *Eco*RI, and ligation to phage arms as described previously in detail (10, 32, 33). Screening of recombinant λgt11 plaques was performed by immunoblotting (10). The primary antiserum used was an anti-CPE monoclonal antibody MA3C9 (18). Positive-scoring plaques were purified and used to infect *E. coli* Y1089 to obtain temperature-sensitive (*t*⁻) lysogens useful for production of proteins for further analysis (10).

DNA methods. Restriction and modifying enzymes were

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FIG. 1. Construction of plasmid vectors containing the *cpe* gene insert. A 2.3-kilobase (Kb) insert was excised from λ PH161 with *Eco*RI and subcloned into pGEM4 (generating pPH100) or into expression vector pEV-vrf1 (generating pPH300). pPH200 is the result of subcloning of a 200-base-pair *Sma*I (located in the multiple cloning site)-*Hind*III insert from pPH100 into pGEM4. There are at least four additional *Hind*III sites in the 2.3-kilobase insert whose relative positions were not mapped. RBS, Ribosome-binding site; ori, origin of replication; cos, cohesive ends.

purchased from Boehringer Mannheim Biochemicals and used as specified by the manufacturer. Subcloning of the 2.3-kilobase *Eco*RI fragment from λ PH161 into pGEM4 for restriction mapping or into pEV-vrf1 for protein expression was carried out as shown in Fig. 1. DNA sequencing of pPH200 was performed by the dideoxy method (25).

Protein purification, analysis, and radiolabeling. Pure CPE protein was prepared and assayed as previously described (22). CPE was radioiodinated (19) to a specific activity of 2 to 4 mCi/mg of CPE. Cholera toxin (CT; List Biologicals, Inc.) was radioiodinated (29) to a specific activity of 1 to 2 mCi/mg of CT. *E. coli* RR1 containing either pPH300 (producing CPE_{frag} lysate) or pEV-vrf1 (producing negative control lysate) was grown as described by Crowl et al. (3), harvested by centrifugation, and lysed by EDTA-lysozyme treatment, followed by intermittent sonication at 4°C. Debris were removed by centrifugation, and the soluble lysates were then concentrated and partially purified by performing 40% (NH₄)₂SO₄ precipitation, followed by a second precipitation with 15% (NH₄)₂SO₄. At each time, pellet fractions were collected. These concentrated lysates were suspended, analyzed, and radioiodinated by using chloramine T to a specific activity of 0.1 to 1.0 mCi/mg of lysate protein. Protein determinations were by the method of Lowry et al. (14).

Since CPE forms anomalous aggregates when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4, 9, 19), purified CPE and *E. coli* lysates were analyzed by nondenaturing (native) polyacrylamide gel electrophoresis

with 5%, 6%, 7%, and 8% polyacrylamide gels. The gels were stained with Coomassie blue or subjected to immunoblot analysis by using as the primary antiserum either rabbit polyclonal anti-CPE (preadsorbed with *E. coli* RR1 lysates) or previously characterized monoclonal antibodies (31). MAB3C9 neutralizes CPE binding to its receptor, as well as CPE cytotoxicity, while MAB1G7 reacts with CPE but does not neutralize CPE action or affect CPE binding (31).

The molecular weight of the fragment in CPE_{frag} lysate was calculated on the basis of native gel immunoblots by the method of Hedrick and Smith (8) and also by deductive translation of the DNA sequence.

Binding studies. Brush border membranes (BBMs) were prepared from the small intestines of female New Zealand White rabbits (19). BBMs (100 μ g of protein) were incubated for 15 min in 0.5 ml of Dulbecco modified phosphate-buffered saline at 24°C with specified amounts of radiolabeled ligand in the absence (representing total binding) or presence (representing nonspecific binding) of a 100-fold excess of the analogous unlabeled protein sample (19). BBMs were pelleted in a microcentrifuge, washed twice, and counted in a Packard gamma counter. Specific binding was calculated as the difference between total binding and nonspecific binding (19). For competitive-binding studies, BBMs were preincubated with specified amounts of the unlabeled competitor for 15 min at 24°C. Following two washes to remove the unbound competitor, these BBMs were sus-

pended and subjected to specific binding analysis as described above.

Assay for Vero cell cytotoxicity. Confluent cultures of Vero (African green monkey kidney) cells were prepared and labeled with ^3H -labeled nucleotides and toxin treated as previously described (19). CPE-induced release of the label was calculated by the following formula of Thelestam and Mollby (28): % maximal release = [(toxin-induced release - spontaneous release)/(maximal release - spontaneous release)] $\times$ 100. Spontaneous release represents background label release without toxin, while maximal release represents total cytoplasmic radioactivity at the time of toxin challenge. In protection experiments, Vero cells were preincubated for 15 min at 37°C with specified amounts of CPE_{frag} or control lysates and then washed twice before challenge with CPE.

RESULTS

Expression of CPE fragment. When a λ gt11 library of *C. perfringens* DNA was screened with anti-CPE MAb3C9, one positive-scoring plaque was obtained and named λ ph161 (Fig. 1). Since MAb3C9 recognizes an epitope which appears to be at or near the receptor-binding domain of CPE (31), this suggested that λ ph161 contains a CPE gene insert that encodes a CPE receptor-binding domain. This clone was isolated and used to generate a *r*^s lysogen in *E. coli* Y1089 to begin characterization of CPE-like activities encoded by the CPE gene insert. When induced (10), this lysogen expressed a LacZ-CPE fusion protein with an M_r of 135,000 (data not shown) as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12) of cell lysates, followed by immunoblot analysis with either MAb3C9 or a monoclonal antibody to β -galactosidase (Promega Biotech). Since the β -galactosidase moiety of λ gt11 fusion proteins is 116,000 kDa (10), this indicates that approximately 19 kDa of the fusion protein is CPE-specific sequences. The fusion protein had no measurable cytotoxic or binding activities.

Lambda ph161 DNA was then subcloned into appropriate plasmid vectors (Fig. 1) for DNA sequencing and to examine biological activities of the expressed CPE fragment free from possible interference of LacZ protein. Following induction (3), lysates from *E. coli* containing pPH300 (expressing the CPE fragment) or pEV-vrfl (negative control) were immunoblotted (Fig. 2). CPE (lanes a) reacted with all CPE antisera. *E. coli* lysates containing the CPE fragment (lanes b) reacted as a single band with polyclonal anti-CPE and with MAb3C9 but did not react with MAb1G7. Similar immunoblot data (not shown) from four different-percentage polyacrylamide gels were used to calculate (8) the molecular mass of the CPE fragment expressed from pPH300 as approximately 20 kDa, consistent with results obtained from immunoblot analysis of the fusion proteins. Negative control *E. coli* lysates (lanes c) showed no immunoreactivity with any CPE antisera. Coomassie blue staining (Fig. 2) indicated that the fragment and negative control lysates contained similar protein species. By MAb3C9 immunodot blot titration with purified CPE as the standard (data not shown), we estimated that $\approx 0.2\%$ of the concentrated lysate protein was the CPE fragment.

DNA sequence. To identify which portion of the CPE gene had been cloned, DNA sequencing was performed on the 200-base-pair *Sma*I-*Hind*III CPE gene insert contained on pPH200 (Fig. 1). Both strands of this 200-base-pair insert were completely sequenced by the dideoxy method (25) and deductively translated (Fig. 3). The deduced amino acid sequence (Fig. 3) corresponds to the previously reported

FIG. 2. Expression of CPE_{frag}. Samples were subjected to native polyacrylamide gel electrophoresis with 5% polyacrylamide gels and then stained with Coomassie blue (STAIN) or subjected to immunoblot analysis with the following CPE antisera: POLY, rabbit polyclonal anti-CPE; 3C9, MAb3C9; 1G7, MAb1G7. Lanes: a, 5 μ g of purified CPE; b, 50 μ g of protein of concentrated *E. coli* lysates containing CPE_{frag}; c, 50 μ g of protein of concentrated negative control *E. coli* lysate. Immunoblots of 6%, 7%, and 8% polyacrylamide gels were also run to determine the CPE_{frag} M_r (data not shown).

CPE amino acid sequence (6), beginning with amino acid 162. The only difference observed was the presence of Trp in place of Tyr at position 217. The theoretical molecular mass of a fragment from amino acid 162 to the C terminus of CPE is ≈ 20 kDa, which is consistent with direct molecular mass calculations based on immunoblotting (Fig. 2). Collectively, our results indicate that we cloned the 3' half of the *cpe* gene and downstream regions.

Receptor-binding studies. The availability of a clone that expresses only the C-terminal half of CPE permitted investigation of possible activities which may be associated with this region of the CPE molecule. Since fragment purification is ongoing, thorough control studies are presented to confirm that in each experiment CPE_{frag} was the active agent in the *E. coli* lysates. Several experiments were performed to examine whether CPE_{frag} recognizes CPE receptors on rabbit intestinal BBMs (the physiologic site of CPE action).

Initially, *E. coli* lysates containing CPE_{frag} were shown to competitively inhibit [^{125}I]CPE specific binding to BBM

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TTA GGA AAT ATT GAT CAA GGT TCA TTA ATT GAA ACT GGT GAA AGA TGT GTT TTA ACA GTT CCA TCT
Leu Gly Asn Ile Asp Gln Gly Ser Leu Ile Glu Thr Gly Glu Arg Cys Val Leu Thr Val Pro Ser
162                                     170                                     180

ACA GAT ATA GAA AAA GAA ATC CTT GAT TTA GCT GCT GCT ACA GAA AGA TTA AAT TTA ACT GAT CCA
Thr Asp Ile Glu Lys Glu Ile Leu Asp Leu Ala Ala Thr Glu Arg Leu Asn Leu Thr Asp Ala
190                                     200

TTA AAC TCA AAT CCA GCT GGT AAT TTA TAT GAT TGG GGT TCT TCT AAC TCA TAC CCT TGG ACT CAA
Leu Asn Ser Asn Pro Ala Gly Asn Leu Tyr Asp Trp Arg Ser Ser Asn Ser Tyr Pro Trp Thr Cln
210                                     220

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FIG. 3. Nucleotide sequence of the *cpe* gene which encodes a portion of the CPE_{frag} region. The derived amino acid sequence for this region of the *cpe* gene (shown below the DNA sequence) matches exactly the previously reported CPE amino acid sequence (6) for CPE amino acids 162 to 226, except for position 217 (see text).

FIG. 4. Competitive inhibition of [125 I]CPE specific binding to BBMs by *E. coli* lysates. The indicated amounts of concentrated CPE_{frag} lysates or negative control lysates were preincubated with BBMs as described in Materials and Methods. BBMs were then assayed for specific binding of [125 I]CPE (0.5 μ g). The standard error of the mean for each point was <2,000 cpm ($n = 4$).

receptors in a dose-dependent manner (Fig. 4). Complete inhibition of specific binding occurred when BBMs were preincubated with ≈ 400 μ g of lysate protein. In light of our previous estimation that CPE_{frag} represents $\approx 0.2\%$ of total lysate protein, this suggests that at saturation, ≈ 400 pmol of CPE_{frag} binds per mg of BBM protein. This result is consistent with stoichiometric inhibition of CPE binding by CPE_{frag} by competition for CPE receptors, since previous studies (19, 29) have estimated that ≈ 100 to 500 pmol of CPE binds per mg of BBM protein at saturation.

To demonstrate that CPE_{frag} lysates contain an agent which specifically binds to BBMs, *E. coli* lysates were radioiodinated. 125 I-labeled lysates of CPE_{frag} specifically bound to BBMs in a saturable manner (Fig. 5). This binding appeared to be due to CPE_{frag} in lysates, since no specific binding was detected with negative control 125 I-labeled lysates.

Additional preincubation competitive-binding studies

FIG. 5. Specific binding of 125 I-labeled lysates to BBMs. Concentrated lysates containing CPE_{frag} or concentrated negative control lysates were radioiodinated as described in Materials and Methods. Increasing amounts of labeled samples were assayed for specific binding to BBMs. The standard error of the mean for each sample point was <2,500 cpm ($n = 4$).

FIG. 6. Summary of competitive-binding studies. As described in Materials and Methods, BBMs were preincubated as follows with unlabeled competitors (ordinate): NONE, Dulbecco modified phosphate-buffered saline control; CPE, 25 μ g of protein; FRAG, 300 μ g of protein from concentrated CPE_{frag} lysates; NEG, 300 μ g of protein from concentrated negative control lysates; CT, 50 μ g of protein. BBMs were then assayed for specific binding of 125 I-labeled ligands: A, [125 I]CPE (0.5 μ g); B, 125 I-labeled CPE_{frag} lysates (150 μ g); C, [125 I]CT (1 μ g). Unlabeled competitors were also preincubated for 15 min at 37°C with 500 μ g of MAb3C9 (cross-hatched bars) or MAb1G7 (open bars) to demonstrate specificity of fragment binding. Error bars represent standard errors of the means ($n = 4$).

were performed (Fig. 6) to demonstrate further that CPE_{frag} lysates and CPE compete for the same BBM receptor and that CPE_{frag} is the active agent in those lysates. Figure 6A and B confirms that specific binding of [125 I]CPE or 125 I-labeled CPE_{frag} lysates can be specifically inhibited by either unlabeled CPE or unlabeled CPE_{frag} lysates but not by control lysates. Figure 6A and B also shows that preincubation of either unlabeled CPE or CPE_{frag} lysates with MAb3C9 eliminated their subsequent ability to compete successfully against [125 I]CPE or 125 I-labeled CPE_{frag} lysate binding. However, preincubation of these unlabeled competitors with MAb1G7 did not affect their subsequent abilities to compete against [125 I]CPE or 125 I-labeled CPE_{frag} lysate binding. This specific neutralization of binding competition by MAb3C9 (Fig. 6A and B) strongly indicated that CPE_{frag} was the active binding competitor in the lysates.

Results shown in Fig. 6C support the notion that CPE_{frag} lysates specifically recognize CPE receptors. Since CPE and

FIG. 7. Protection of Vero cells from CPE-induced cytotoxicity by preincubation with CPE_{frag} lysates. Vero cells were labeled with ³H-labeled nucleotides (19) and then preincubated with increasing amounts of concentrated CPE_{frag} or negative control lysates. After being washed, Vero cells were challenged with CPE (5 μ g per well). CPE cytotoxicity was calculated as previously described (28). Maximal release = 93,000 $\pm$ 5,000 cpm; spontaneous release = 4,000 $\pm$ 500 cpm ($n \geq 3$).

CT recognize different BBM receptors (29), CPE_{frag} lysates should not compete with [¹²⁵I]CT binding if CPE_{frag} binding is specific for CPE receptors. In Fig. 6C, neither unlabeled CPE_{frag} lysates nor negative control lysates significantly competed for [¹²⁵I]CT-specific binding sites. Conversely, unlabeled CT did not affect either [¹²⁵I]CPE or ¹²⁵I-labeled CPE_{frag} lysate binding (Fig. 6A and B).

Cytotoxicity of CPE_{frag}. When ³H-labeled nucleotide release from Vero cells was used to test for CPE cytotoxicity (16), no cytotoxic effects were detected with CPE_{frag} lysates, even up to 25 mg of protein (containing $\approx$ 2.5 nmol of the CPE fragment) per well (data not shown). However, 5 μ g ($\approx$ 135 pmol) of purified CPE per well caused significant ³H-labeled nucleotide release from Vero cells (Fig. 7).

The results shown in Fig. 7 also demonstrate the specificity of CPE_{frag} for the physiologic receptor on Vero cells which mediates CPE cytotoxicity. Preincubation of Vero cells with CPE_{frag} lysates protected these cells from subsequent CPE challenge in a dose-dependent manner. The protection was essentially complete at 2 to 3 mg of CPE_{frag} lysate protein per well. Negative control lysates had no protective effect at 3 mg of protein per well. Additionally, MAb3C9 pretreatment of CPE_{frag} lysates specifically eliminated the subsequent ability of these lysates to protect Vero cells, while pretreatment of CPE_{frag} lysates with MAb1G7 did not have this effect (data not shown).

DISCUSSION

This study reports the cloning and expression of the C-terminal half of CPE in *E. coli*. By this approach, we obtained a defined CPE fragment (amino acids 162 to 309) free from other CPE contaminants. This permitted examination of the proposed relationship (9) between receptor-binding activity and the C-terminal half of CPE. Competitive-binding studies clearly demonstrated that CPE_{frag} contains a receptor-binding domain. Further studies are required to localize more precisely the binding domain within this CPE fragment and to search for possible binding activity in other CPE regions.

CPE_{frag} lysates did not cause CPE-like Vero cell cytotoxicity. Preincubation of native CPE with negative control *E. coli* lysates did not affect subsequent CPE cytotoxicity (data not shown), suggesting that lysates do not inactivate CPE_{frag} cytotoxicity. Further, the fact that both receptor-binding activity and epitopes are retained on CPE_{frag} indicates that lack of CPE_{frag} cytotoxicity is not due to gross distortions in polypeptide folding. Horiguchi et al. also reported no cytotoxicity with their C-terminal NTCB-derived CPE fragment (9). However, their results are inconclusive, since their cleavage incubation conditions alone (i.e., without addition of NTCB cleavage reagent) can inactivate cytotoxicity of intact CPE (unpublished data).

Our results indicate that additional CPE sequences are required for cytotoxicity. The lack of cytotoxicity does not necessarily imply that the cytotoxic domain(s) is absent from the C-terminal half of CPE, as others have suggested (9, 27). It is also conceivable that either several domains are required for cytotoxicity or only part of the cytotoxic domain is present on the C-terminal half of CPE.

However, the current study does provide some important information about CPE cytotoxicity. Previous studies have demonstrated that while specific binding is essential for CPE bioactivity, CPE-specific binding does not necessarily result in bioactivity (21, 31). Colonic cells specifically bind CPE but do not show CPE effects after binding (21). This suggests a multistep action for CPE (21), i.e., binding followed by biologic activity. Alternatively, the colonic cell receptor may be distinct from the receptor in BBMs or Vero cells which conveys CPE cytotoxicity. This report shows that binding of CPE_{frag} to the biologically relevant CPE receptor does not lead to cytotoxicity in Vero cells. This demonstrates that mere receptor occupancy does not necessarily result in CPE-induced cytotoxicity and suggests a role for the CPE molecule beyond the initial binding activity. Therefore, these results support the multi-step model of CPE action (19, 21).

Lastly, our cloned *cpe* gene sequences can be used as probes for molecular studies of CPE synthesis and action and for epidemiologic studies. Also, CPE_{frag} has desirable properties (reactivity with neutralizing antibodies but lack of cytotoxicity) that warrant further study as a possible vaccine candidate (24).

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ADDENDUM IN PROOF

Van Damme-Jongsten et al. recently reported (Antonie van Leeuwenhoek 56:181-190, 1989) the complete nucleotide sequence of CPE and noted several small differences between their sequence and the previously reported CPE amino acid sequence (6). CPE_{frag} corresponds to amino acids 171 to 320 of the sequence reported by Van Damme-Jongsten et al.

LITERATURE CITED

1. Bishai, W. R., A. Miyahara, and J. R. Murphy. 1987. Cloning and expression of *Escherichia coli* of three fragments of diphtheria toxin truncated within fragment B. *J. Bacteriol.* 169: 1554-1563.

2. Collier, R. J. 1987. Cytotoxic proteins: photoaffinity labeling and site-directed mutagenesis of an active site residue in diphtheria toxin, p. 299-306. In D. Oxender, and C. Fox (ed.), Protein engineering. Allen R. Liss, Inc., New York.
3. Crowl, R., C. Seamans, P. Lomedico, and S. McAndrew. 1985. Versatile expression vectors for high-level synthesis of cloned gene products in *Escherichia coli*. *Gene* 38:31-38.
4. Enders, G. L., and C. L. Duncan. 1976. Anomalous aggregation of *Clostridium perfringens* enterotoxin under dissociating conditions. *Can. J. Microbiol.* 22:1410-1414.
5. Genigeorgis, C. 1975. Public health importance of *Clostridium perfringens*. *J. Am. Vet. Med. Assoc.* 167:821-827.
6. Granum, P. E. 1986. Structure and mechanism of action of the enterotoxin from *Clostridium perfringens*, p. 327-334. In P. Falmagne et al. (ed.), Proceedings of the Second European Workshop on Bacterial Protein Toxins. Gustav Fisher, Stuttgart, Federal Republic of Germany.
7. Granum, P. E., J. R. Whitaker, and R. Skjelkvale. 1981. Trypsin activation of enterotoxin from *Clostridium perfringens* type A. *Biochim. Biophys. Acta* 668:325-332.
8. Hedrick, J. L., and A. J. Smith. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* 126:155-164.
9. Horiguchi, Y., T. Akai, and G. Sakaguchi. 1987. Isolation and function of a *Clostridium perfringens* enterotoxin fragment. *Infect. Immun.* 55:2912-2915.
10. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in λ gt10 and λ gt11, p. 49-78. In D. Glover (ed.), DNA cloning: a practical approach, vol. 2. IRL Press, Oxford.
11. Hwang, J., D. J. Fitzgerald, S. Adhya, and I. Pastan. 1987. Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* 48:129-136.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
13. Larson, H. E., and S. P. Borriello. 1988. Infectious diarrhea due to *Clostridium perfringens*. *J. Infect. Dis.* 157:390-391.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
15. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* 3:208-218.
16. McClane, B. A. 1984. Osmotic stabilizers differentially inhibit permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. *Biochim. Biophys. Acta* 641:401-409.
17. McClane, B. A., P. C. Hanna, and A. P. Wnek. 1988. *Clostridium perfringens* enterotoxin: a mini-review. *Microb. Pathog.* 4:317-323.
18. McClane, B. A., and R. J. Strouse. 1984. Rapid detection of *Clostridium perfringens* type A enterotoxin by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 19:112-115.
19. McClane, B. A., A. P. Wnek, K. Hulkower, and P. C. Hanna. 1988. Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin. *J. Biol. Chem.* 263:2423-2435.
20. McDonel, J. L. 1986. Toxins of *Clostridium perfringens* types A, B, C, D and E, p. 477-517. In F. Dorner and H. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, Inc., Elmsford, N.Y.
21. McDonel, J. L., and G. W. Demers. 1982. *In vivo* effects of enterotoxin from *Clostridium perfringens* type A in the rabbit colon: binding vs biologic activity. *J. Infect. Dis.* 145:490-494.
22. McDonel, J. L., and B. A. McClane. 1988. Production, purification and assay of *Clostridium perfringens* enterotoxin. *Methods Enzymol.* 165:94-103.
23. Pappenheimer, A. M., Jr. 1977. Diphtheria toxin. *Annu. Rev. Biochem.* 46:69-94.
24. Sanchez, J., and J. Holmgren. 1989. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. *Proc. Natl. Acad. Sci. USA* 86:481-485.
25. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
26. Skjelkvale, R., and T. Uemura. 1977. Experimental diarrhea in human volunteers following oral administration of *Clostridium perfringens* type A enterotoxin. *J. Appl. Bacteriol.* 43:281-286.
27. Sugii, S., and Y. Horiguchi. 1988. Identification and isolation of the binding substance for *Clostridium perfringens* enterotoxin on Vero cells. *FEMS Microbiol. Lett.* 52:85-90.
28. Thelestam, M., and R. Mollby. 1975. Sensitive assay for detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts. *Infect. Immun.* 12:225-232.
29. Wnek, A. P., and B. A. McClane. 1986. Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in isolated intestinal brush border membranes. *Microb. Pathog.* 1:89-100.
30. Wnek, A. P., and B. A. McClane. 1989. Preliminary evidence that *Clostridium perfringens* type A enterotoxin is present in a 160,000-M_r complex in mammalian membranes. *Infect. Immun.* 57:574-581.
31. Wnek, A. P., R. J. Strouse, and B. A. McClane. 1985. Production and characterization of monoclonal antibodies against *Clostridium perfringens* type A enterotoxin. *Infect. Immun.* 50:442-448.
32. Young, R. A., and R. W. Davis. 1983. Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* 80:1194-1198.
33. Young, R. A., and R. W. Davis. 1988. Yeast polymerase II genes: isolation with antibody probes. *Science* 222:778-782.