INFECTION AND IMMUNITY, Dec. 1991, p. 4338–4342
0019-9567/91/124338-05$02.00/0

Vol. 59, No. 12

Epitope Mapping of the Alpha-Toxin of *Clostridium perfringens*

ALISON J. LOGAN,[1]† E. DIANE WILLIAMSON,[1]* RICHARD W. TITBALL,[1] DAVID A. PERCIVAL,[1]‡ ANDREW D. SHUTTLEWORTH,[1] J. W. CONLAN,[2] AND DAVID C. KELLY[1]

Chemical and Biological Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ,[1] and Department of Microbiology, University of Southampton Medical School, Southampton General Hospital, Southampton,[2] United Kingdom

Received 29 April 1991/Accepted 7 September 1991

A panel of monoclonal antibodies specific for the *Clostridium perfringens* alpha-toxin was produced by the fusion of X63.Ag8-653 cells with splenocytes from mice immunized either intrasplenically or intraperitoneally with an alpha-toxoid. The toxin-binding activity of each monoclonal antibody was evaluated. The monoclonal antibodies were also screened for their toxin-neutralizing potential in vitro, as determined by the inhibition of phospholipase C and hemolytic activities. In vivo inhibition of toxicity was assessed by the survival of mice challenged with preincubated alpha-toxin–antibody mixtures. Only one monoclonal antibody (3A4D10) was protective in vivo and neutralizing in both in vitro assays. Since 3A4D10 could inhibit both activities, the evidence suggests that these are colocated in the same area of the toxin molecule. This paper identifies a significant continuous linear binding region for 3A4D10 at positions 193 to 198 in the primary amino acid sequence of alpha-toxin.

Clostridium perfringens type A is the most important causative agent of gas gangrene in man. *C. perfringens* is also the etiological agent for diseases such as equine grass sickness, necrotizing colitis, and enterotoxemia of horses. The organism produces a multiplicity of toxins of which the alpha-toxin is the most widely studied (13). It was the first bacterial toxin to be identified as an enzyme; as phospholipase C (PLC), it catalyzes the hydrolysis of phosphatidylcholine to phosphorylcholine and 1,2-diglyceride (12). It has also been shown to have lethal, necrotizing, hemolytic, and cytolytic activities (3).

Unlike many other bacterial PLCs, the toxin is readily able to interact with cell membrane phospholipids (7, 16) and is thought to play a key role in the pathogenesis of *C. perfringens*-mediated gas gangrene infections by promoting local cell membrane disruption (11, 22). In support of this, alpha-toxin antiserum has been shown to prevent the establishment of experimental gas gangrene in guinea pigs (3, 4). The effectiveness of alpha-toxoids in disease prevention has also been demonstrated (8, 9).

Biochemical studies have indicated that the toxin is a zinc metalloenzyme in which zinc plays an important role in the stability and activity of the enzyme (20, 21). Calcium ions have also been shown to be essential for enzymatic activity and are thought to be involved in the binding of the toxin to the lipid interface (17). The gene coding for the toxin has been cloned, and the nucleotide sequence has been determined (18, 25, 27). The toxin cloned by Titball et al. (25) was found to have a molecular mass of 42.5 kDa and appeared to be identical to the alpha-toxin produced by the donor strain. Molecular masses of between 30 and 90 kDa have been previously reported for alpha-toxin depending on the method of analysis used (14, 23). A truncated form of the alpha-toxin which shows similar phosphatidylcholine-hydrolyzing activity to the native toxin but does not possess sphingomyelinase or hemolytic activities has been recently produced by recombinant DNA technology (26). This supports the previous suggestion that both phosphatidylcholine and sphingomyelin hydrolyzing activities are necessary for a bacterial PLC to be hemolytic (15).

Despite extensive interest in the biochemistry of alpha-toxin, its structure-activity relationship has not been fully clarified. To further analyze this relationship, we have produced specific monoclonal antibodies (MAbs) and used them to map epitopes in the primary amino acid sequence of the toxin.

MATERIALS AND METHODS

Animals and reagents. Female 8- to 10-week-old BALB/c mice were obtained from Charles River Laboratories, Margate, Kent, United Kingdom. *C. perfringens* alpha-toxin was obtained from three sources: as PLC (type 14) from Sigma Chemical Co. (Poole, Dorset, United Kingdom), as purified from cultures of *C. perfringens* type A strain NCTC 8237 (25), and from *Escherichia coli* containing the cloned *C. perfringens* alpha-toxin gene (25). The truncated alpha-toxin (Cpa_{249}) was expressed from a recombinant plasmid encoding residues 1 to 249 of the alpha-toxin and purified before use (26). All other biochemicals were obtained from Sigma unless stated in the text.

Production of toxoid and immunization strategy. An immunogenic toxoid was produced by incubating the toxin with formaldehyde by the method of Ito (8). BALB/c mice were immunized by one of two regimes. (i) A conventional immunization schedule with an intraperitoneal (i.p.) priming dose of 0.3 μg of toxoid in 50 μl of phosphate-buffered saline (PBS) and then booster doses on days 7, 14, and 28 with 30 μg of toxoid in 50 μl of PBS was used. Spleens and sera were taken 3 days after the final boost to maximize yields of activated B lymphocytes and serum antibody levels. (ii) Alternatively, a single dose schedule, in which 20 μg of toxoid in PBS was injected directly into the spleens of

* Corresponding author.
† Present address: Pfizer Ltd., Sandwich, United Kingdom.
‡ Present address: Unipath Ltd., Bedford, United Kingdom.

anaesthetized mice was used (24). Spleens were removed for fusion on the third postoperative day.

Production of hybridoma lines. Standard techniques to prepare hybridoma cell lines and to maximize their yield of antibody by growth in ascitic mice (2) were used. Ascitic fluids were clarified by centrifugation at 1,000 × *g* (15 min at 4°C) prior to storage at −20°C. Ascites were purified by ammonium sulfate precipitation and then Sepharose-staphylococcal protein A adsorption (MAPs system; Bio-Rad, Hemel Hempstead, United Kingdom). The immunoglobulin concentration of the MAbs purified from ascites was calculated from the A_{280} of the samples. Isotyping of MAbs was performed by enzyme-linked immunosorbent assay (ELISA) with specific antimouse isotyping reagents (Bio-Rad).

ELISA. Hybridomas secreting toxin-specific MAbs were detected by using a standard ELISA format in which either alpha-toxin or Cpa_{249} was coated to the solid phase.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on gradient or homogenous gels. Gels were stained by using a silver staining technique (Bio-Rad), and immunoblotting was carried out by transferring proteins from the acrylamide gels to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Harrow, United Kingdom). The gel and membrane were placed in close contact within a gel holder cassette and immersed in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol) for 1 h at 37°C, with protein transfer occurring by diffusion. The blots were then probed with a 1:1,000 dilution of each purified MAb or with crude polyclonal antisera. Specific binding was visualized by using the Auroprobe BL^+ detection system according to the manufacturer's instructions (Cambio Ltd., Cambridge, United Kingdom).

Detection of alpha-toxin activity in vitro and in vivo. In vitro alpha-toxin activity was detected by using microtiter-plate-based assays as previously described (25). Briefly, PLC activity was determined by its effect on egg yolk lipoproteins. Dilutions of toxin in 0.9% saline were placed in 0.1-ml volumes in the wells of a microtiter plate. An egg yolk emulsion (Oxoid Ltd., Basingstoke, United Kingdom) was centrifuged before use (at 10,000 × *g* for 20 min at 4°C) and diluted 1:10 in 0.9% saline before 0.1 ml was added to each well of the plate. After 1 h at 37°C, the A_{510} of the samples was read. PLC activity was indicated by an increase in absorbance with the development of turbidity.

Hemolytic activity was determined by using freshly drawn citrated mouse erythrocytes suspended in borate-buffered saline at 5% (vol/vol). Volumes (0.1 ml) of erythrocyte suspension were added to equal volumes of toxin diluted in borate-buffered saline. After incubation for 1 h at 37°C, the transmission of light through sample wells was measured at 540 nm. Increased transmission indicated hemolysis.

In both assays, the neutralizing ability of the MAbs was assessed by preincubating cloned alpha-toxin with a 1.4-fold molar excess of purified antibody for 2 h at 37°C prior to addition of the egg yolk emulsion or erythrocyte suspension. Large molar excesses of antibody (70-fold) were also tested in the hemolysis assay.

To assess in vivo neutralization, groups of six BALB/c mice were given a single i.p. injection from mixtures of 1 μg of cloned alpha-toxin preincubated (2 h at 37°C) with a 70-fold molar excess (250 μg) of purified 3A4D10 or a representative MAb, 2D1D9, not found to be neutralizing in vitro. A control group received an equal volume of cloned alpha-toxin alone.

NTCB cleavage of alpha-toxin. NTCB (2-nitro-5-thiocyanobenzoic acid) fragments of alpha-toxin were prepared by using a modification of the methods described by Jacobson et al. (10) and Horiguchi et al. (6). Lyophilized alpha-toxin (Sigma type XIV) was dissolved in 0.05 M Tris Hcl, pH 8.0, containing 4 M guanidine hydrochloride and 0.2 mM dithiothreitol at a concentration of 1 to 5 mg of toxin per ml. The sample was incubated at room temperature for 30 min, after which time NTCB was added to a final concentration of 50 mM. Incubation was continued for 15 min more before the sample pH was adjusted to 9.0 by the dropwise addition of 0.1 M NaOH. After incubation at 37°C for 6 h, the sample was dialyzed for 18 h against 0.05 M Tris Hcl, pH 8.0. Following dialysis, insoluble aggregates were removed from the sample by centrifugation (at 2,000 × *g* for 1 h). Samples were stored at −20°C until required.

TABLE 1. In vitro inhibition of PLC and hemolytic activities

Treatment	Inhibition (mean ± SE)	
	PLC (A_{510})[a]	Hemolysis (A_{540})[b]
MAb		
2D1D9 (IgG1)	0.60 ± 0.01	1.17 ± 0.003
A6B7 IgG1	0.81 ± 0.02	1.16 ± 0.003
3A4F2 IgG1	0.60 ± 0.002	1.20 ± 0.01
3A4D10 IgG1	0.07 ± 0.001[c]	2.01 ± 0.01[d]
Polyclonal antiserum	0.14 ± 0.001[e]	1.92 ± 0.06[f]
Controls		
Toxin alone	0.48 ± 0.02	1.25 ± 0.06
No toxin	0.07 ± 0.01	2.18 ± 0.01

[a] Cloned alpha-toxin (1.56 μg/ml) was preincubated with a 1.4-fold molar excess of each MAb or a 5-fold excess (wt/wt) of polyclonal antiserum prior to the assay.
[b] Cloned alpha-toxin (1.56 μg/ml) was preincubated with a 70-fold molar excess of each MAb or a 100-fold excess (wt/wt) of polyclonal antiserum prior to the assay.
[c] Differs significantly from toxin control ($P > 0.1$).
[d] Differs significantly from toxin control ($P > 0.5$).
[e] Differs significantly from toxin control ($P > 0.5$).
[f] Differs significantly from toxin control ($P > 0.5$).

Fine epitope mapping. Overlapping hexapeptides were synthesized throughout the primary amino acid sequence of alpha-toxin by using a modification of the multiple-peptide synthesis technique originally described by Geysen et al. (5), supplied in kit form (Cambridge Research Biochemicals Ltd., Northwich, United Kingdom). The binding of 3A4D10, the other MAbs, and polyclonal antisera to the immobilized peptides was assayed by ELISA in accordance with the manufacturer's instructions.

The peptides corresponding to amino acid residues 193 to 198 (ARGFAK) and 196 to 200 (FAKTG) of alpha-toxin were synthesized on a Milligen/Biosearch 95100 synthesizer by using conventional tertiary butyl oxycarbonyl chemistry. Hydrogen fluoride cleavage was used to remove the peptide from the resin, with anisole and dimethyl sulfide as scavenging agents.

Inhibition assay. In a variation on the standard ELISA, concentrations of the hexapeptide ARGFAK and the pentapeptide FAKTG, ranging from 200 to 0.1 μg/ml, and 3A4D10 (1.5 μg/ml) were added simultaneously to plates coated with alpha-toxin (5 μg/ml). The plates were then incubated for 2 to 3 h at 37°C. After washing three times in PBS-Tween, the standard ELISA procedure (for conjugate and substrate addition) was followed.

Statistical methods. Paired *t* tests were used to determine the statistical significance of the results obtained.

TABLE 2. Survival times of BALB/c mice after i.p. injection with preincubated toxin-MAb mixtures

BALB/c mice	No. of mice surviving at time postinjection[a]			
	16 h	20 h	40 h	7 days
Toxin controls	0			
Toxin + 3A4D10[b]	5	4	3	3
Toxin + 2D1D9[b]	0			

[a] A total of six mice were used in each trial.
[b] Cloned alpha-toxin (1 μg) was preincubated with a 70-fold molar excess of each MAb prior to injection.

RESULTS AND DISCUSSION

Antibodies. Hybridoma cell clones were screened for MAb production, and only those positive after a third recloning were expanded in vitro and in vivo. Twelve cell lines, of which two (3A4D10 and 3A4F2) were derived from intrasplenically immunized animals, were produced.

MAbs from all clones and mouse polyclonal antisera, derived from spleen donors, were equally reactive with native toxin, formol toxoid, or cloned toxin when tested by ELISA, but they did not cross-react with the PLC produced by *Bacillus cereus*. Isotype analysis of the MAbs secreted by these cell lines indicated that 10 were immunoglobulin G1 (IgG1), 1 was IgG2a, and 1 was IgG2b. All clones contained the κ light chain.

In vitro and in vivo inhibition of toxicity. When first generated, all 12 MAbs were routinely screened for their neutralizing potential against alpha-toxin, but only 3A4D10 was found to be neutralizing in vitro and/or protective in vivo. Subsequently, three representative MAbs were reevaluated and compared with 3A4D10 (Table 1).

In vitro, 3A4D10 inhibited PLC activity when used at a 1.4-fold molar excess to toxin. None of the MAbs preincubated with the toxin at a molar ratio of 1.4 inhibited its hemolytic activity; but when the molar ratio of antibody to toxin was increased to 70:1, 3A4D10 neutralized this activity also. Polyclonal antisera also neutralized the PLC activity when used at approximately 5-fold excess (wt/wt); an approximate ratio of 100:1 (wt/wt) of polyclonal antiserum to toxin was required to inhibit hemolysis.

In vivo, mice challenged with toxin preincubated with 3A4D10 exhibited a significant increase in time-to-death (with five of six animals surviving for >16 h) when compared with control animals challenged with toxin alone or with a mixture of toxin and an equivalent concentration of the nonprotective MAb 2D1D9 (Table 2). The deaths observed after 16 h may be due to dissociation of the antigen-antibody complex.

Our observation that higher ratios of antibody to alpha-toxin were required for neutralization of hemolytic and lethal activities than were required for neutralization of PLC activity are consistent with our previous observations that the lethal activity of the toxin correlates with its hemolytic activity. The molecular basis of this difference in neutralizing ability is not clear. However, our observations coincide with those of other workers (19), who have reported the requirement of different stoichiometric ratios by any one antibody to inhibit, respectively, the PLC activity or in vivo effects of alpha-toxin.

Analysis of cleavage fragments with MAbs. Cleavage of the alpha-toxin at the single cysteine residue (169) by using NTCB (Fig. 1a) generated two fragments (NTCB1 [22.4 kDa] and NTCB2 [24.7 kDa]) with different mobilities in SDS-PAGE (Fig. 1b). On Western blotting (immunoblotting), the larger NTCB2 fragment was preferentially recognized by polyclonal antisera and was the only fragment recognized by 3A4D10. None of the other MAbs bound either fragment. Both the polyclonal sera and 3A4D10 recognized uncleaved alpha-toxin.

Reactivity of MAbs with Cpa_{249}. MAbs were screened for their binding capacity to Cpa_{249}. All recognized the toxin and Cpa_{249} equally, indicating that the N-terminal domain of the toxin which Cpa_{249} represents has a comparable tertiary structure to that of native alpha-toxin (26). Thus, the epitopes for these antibodies are located between residues 1 and 249 of the alpha-toxin, and the epitope for 3A4D10 lies in the region of 169 to 249. As 3A4D10 was the only MAb to show any neutralizing ability in vitro and in vivo, it was selected for use in fine epitope mapping studies.

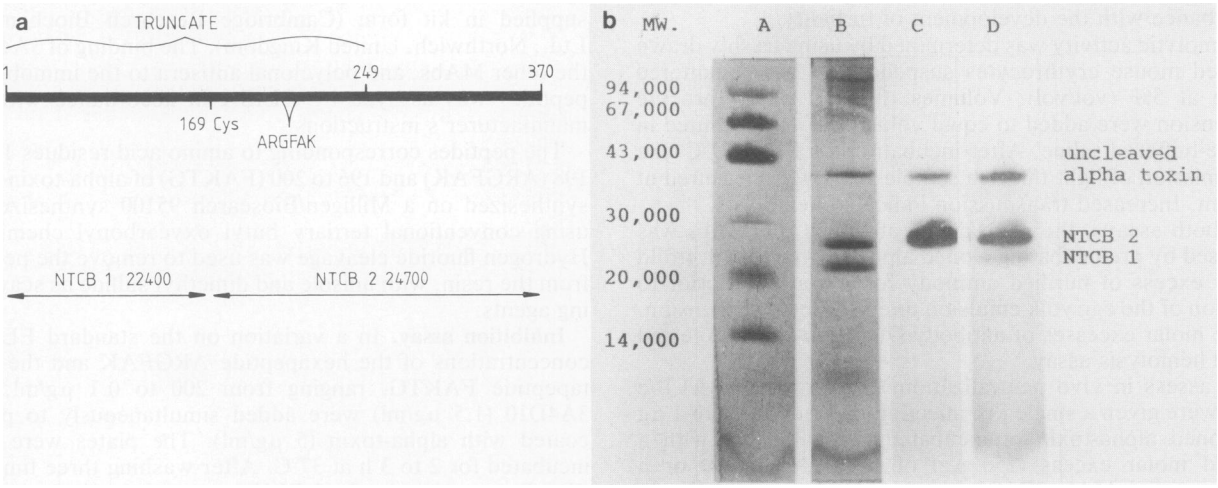

FIG. 1. (a) NCTB cleavage site at single cysteine residue in alpha-toxin; (b) SDS-PAGE and immunoblot analysis of NTCB-cleaved alpha-toxin. The cleavage mixture was electrophoresed and silver stained (lane B) or transferred to nitrocellulose and probed with 3A4D10 (lane C) or polyclonal antiserum (lane D). Molecular masses of protein standards (lane A) are shown in daltons.

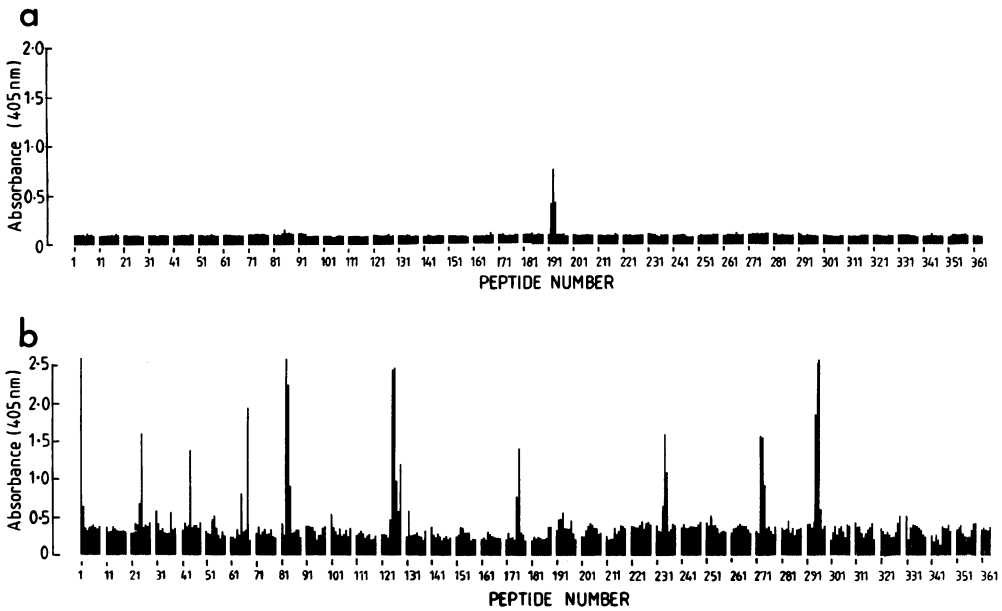

FIG. 2. Reactivity of MAb 3A4D10 (1:1,000 dilution) (a) or polyclonal antiserum (1:600 dilution) (b) against overlapping hexapeptides spanning the entire amino acid sequence of *C. perfringens* alpha-toxin. Peptide numbers correspond to the N-terminal amino acid of each peptide.

Fine epitope mapping. 3A4D10 reacted strongly and consistently with a single region, YARGFAKT, located at positions 192 to 199 in the primary amino acid sequence of the toxin molecule (Fig. 2a). The maximum signal was achieved with the hexapeptide ARGFAK.

The epitope profile achieved for the polyclonal antiserum is shown in Fig. 2b. Four dominant epitopes were identified in the sequence contained in the NTCB2 fragment. An additional six binding sites were found in the sequence contained in the NTCB1 fragment.

Inhibition assay. Free ARGFAK significantly reduced ($P > 0.5$) the binding of 3A4D10 to solid-phase toxin by threefold, although both 3A4D10 and toxin were in approximately 200-fold molar excess to ARGFAK. Thus, the peptide could substitute for the epitope recognized by 3A4D10 in the tertiary structure of the toxin molecule, indicating that ARGFAK is a significant linear binding sequence for 3A4D10. The peptide FAKTG did not influence the binding of 3A4D10 to plate-bound toxin, suggesting that either one or more of the amino acids A, R, or G is essential for antibody binding.

Of all the MAbs tested, only 3A4D10 identified the toxin after Western blotting of the denatured protein. This finding supports the concept that ARGFAK constitutes a short linear epitope for 3A4D10. The incidence of continuous epitopes on native proteins is low; the majority are discontinuous and therefore conformational (1). Peptide scan methodology is used only to identify minimal linear amino acid sequences recognized by MAbs. Therefore, the possibility that the ARGFAK sequence reported here is part of a conformational epitope exists.

The ARGFAK sequence was not a dominant B-cell epitope in the conventional immunization regime used to generate polyclonal antisera. It may be that the acute intrasplenic immunization procedure used to generate 3A4D10 promoted this region as an epitope.

Sato et al. (19) have claimed that the inhibition of different toxin-neutralizing activities is not linked, with one MAb able to neutralize lethality but not PLC activity. Consequently, they suggested that different sites in the toxin contribute to the PLC and lethal activities of the whole molecule. However, this hypothesis was eroded by their finding that any MAb which strongly inhibited PLC activity also afforded some protection against toxin in vivo. It is possible that phosphatidylcholine and sphingomyelin hydrolysis may occur at the same or overlapping site(s) in the alpha-toxin molecule. As both are thought to be required for the hemolytic activity of the toxin (15), our results suggest that 3A4D10 binds either directly to this site or to a site sterically related to it, preventing the ingress of enzyme substrates.

The results presented here support the model previously hypothesized (26) that the C-terminal region (250 to 370) confers sphingomyelinase, and therefore also hemolytic and lethal, activity on the N-terminal domain of the molecule. Thus, the MAb reported by Sato et al. to inhibit lethal activity without affecting PLC activity could map to the C-terminal region. We have not produced any MAbs to this region, although we have demonstrated that it contains epitopes.

3A4D10 neutralizes both PLC and lethal activities. That polyclonal antisera neutralize lethality without involvement of the linear ARGFAK sequence could be explained by a conformational change in the active site resulting from multiepitope attachment to the whole molecule.

In conclusion, we have demonstrated that 3A4D10 inhibits the PLC, hemolytic, and lethal activities of alpha-toxin and binds to a significant linear region at positions 193 to 198 in the primary amino acid sequence of the molecule. Strategies for the presentation of an immunizing peptide derived from this region of the protein are under investigation.

ACKNOWLEDGMENT

We thank T. Lewingdon for synthesizing ARGFAK and FAKTG.

REFERENCES

1. **Arnon, R.** 1983. Conformational antigenic determinants in proteins, p. 157–164. *In* F. Celeda, V. N. Schumaker, and E. E. Sercarz (ed.), Protein conformation as an immunological signal. Plenum Press, New York.
2. **Eshhar, Z.** 1985. Monoclonal antibody strategy and techniques, p. 1–41. *In* T. A. Springer (ed.), Hybridoma technology in the biosciences and medicine. Plenum Press, New York.
3. **Evans, D. G.** 1943. The protective properties of the Alpha antitoxin and the Theta haemolysin in Cl. welchii type A antiserum. Br. J. Exp. Pathol. **24**:81–88.
4. **Evans, D. G.** 1943. The protective properties of the Alpha antitoxin and antihyaluronidase occurring in Cl. welchii type A antiserum. J. Pathol. Bacteriol. **55**:427–434.
5. **Geysen, H. M., R. H. Meloen, and S. J. Barteling.** 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA **81**:3998–4002.
6. **Horiguchi, Y., T. Akai, and G. Sakaguchi.** 1987. Isolation and function of a *Clostridium perfringens* enterotoxin fragment. Infect. Immun. **55**:2912–2915.
7. **Ikezawa, H., R. Taguchi, Y. Asahi, and M. Tomita.** 1983. The physiological actions of bacterial phospholipases C on eukaryotic cells and their membranes. J. Toxicol. Toxin Rev. **1**:223–255.
8. **Ito, A.** 1968. Alpha toxoid of *Clostridium perfringens*. I. Purification and toxoiding of the alpha toxin of Clostridium perfringens. Jpn. J. Med. Sci. Biol. **21**:379–391.
9. **Ito, A.** 1970. Alpha toxoid of Clostridium perfringens. II. Immunogenicity of the toxoid. Jpn. J. Med. Sci. Biol. **23**:21–30.
10. **Jacobson, G., M. Schaffer, G. Stark, and T. Vanaman.** 1973. Specific chemical cleavage in high yield at the amino peptide bonds of cysteine and cystine residues. J. Biol. Chem. **248**: 6583–6591.
11. **Kameyama, S., H. Sato, and R. Murata.** 1975. The role of Alpha toxin of Clostridium perfringens in experimental gas gangrene in guinea pigs. Jpn. J. Med. Sci. Biol. **25**:200.
12. **MacFarlane, M. G., and B. C. J. G. Knight.** 1941. The biochemistry of bacterial toxins. I. The lecithinase activity of Cl. welchii toxins. Biochem. J. **35**:884–902.
13. **McDonel, J. L.** 1986. Toxins of Clostridium perfringens types A, B, C, D and E, p. 477–517. *In* F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, Oxford.
14. **Mitsui, K., N. Mitsui, and J. Hase.** 1973. Clostridium perfringens exotoxins. I. Purification and properties of alpha toxin. Jpn. J. Exp. Med. **43**:65–80.
15. **Mollby, R.** 1978. Bacterial phospholipases, p. 367–424. *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., London.
16. **Mollby, R., M. Thelestam, and T. Wadstrom.** 1974. Effect of Clostridium perfringens phospholipase C (alpha toxin) on the human diploid fibroblast membrane. J. Membr. Biol. **16**:313–330.
17. **Moreau, H., G. Pieroni, C. Jolivet-Reynaud, J. E. Alouf, and R. Verger.** 1988. A new kinetic approach for studying Phospholipase C (Clostridium perfringens alpha toxin) activity on phospholipid monolayers. Biochemistry **27**:2319–2323.
18. **Okabe, A., T. Shimizu, and H. Hayashi.** 1989. Cloning and sequencing of phospholipase C gene of Clostridium perfringens. Biochem. Biophys. Res. Commun. **150**:33–39.
19. **Sato, H., J. Chiba, and Y. Sato.** 1989. Monoclonal antibodies against alpha toxin of Clostridium perfringens. FEMS Microbiol. Lett. **59**:173–176.
20. **Sato, H., and R. Murata.** 1973. Role of zinc in the production of *Clostridium perfringens* alpha toxin. Infect. Immun. **8**:360–369.
21. **Sato, H., Y. Yamakawa, A. Ito, and R. Murata.** 1978. Effect of zinc and calcium ions on the production of alpha-toxin and proteases by *Clostridium perfringens*. Infect. Immun. **20**:325–333.
22. **Smith, L. D. S.** 1979. Virulence factors of Clostridium perfringens. Rev. Infect. Dis. **1**:254–262.
23. **Smyth, C., and J. Arbuthnott.** 1974. Properties of Clostridium perfringens (welchii) type-A alpha toxin (phospholipase C) purified by electrofocusing. J. Med. Microbiol. **7**:41–66.
24. **Spitz, M., L. Spitz, R. Thorpe, and E. Eugui.** 1984. Intrasplenic primary immunisation for the production of monoclonal antibodies. J. Immunol. Methods **70**:39–43.
25. **Titball, R. W., S. Hunter, K. Martin, B. Morris, A. Shuttleworth, T. Rubidge, D. Anderson, and D. Kelly.** 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun. **57**:367–376.
26. **Titball, R. W., D. L. Leslie, S. Harvey, and D. Kelly.** 1991. Hemolytic and sphingomyelinase activities of *Clostridium perfringens* alpha-toxin are dependent on a domain homologous to that of an enzyme from the human arachidonic acid pathway. Infect. Immun. **59**:[5].
27. **Tso, J. Y., and C. Siebel.** 1989. Cloning and expression of the phospholipase C gene from *Clostridium perfringens* and *Clostridium bifermentans*. Infect. Immun. **57**:468–476.