PURIFICATION AND CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* IOTA TOXIN

BY

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FOREWARD

This dissertation is written in the form of three published papers which are listed as follows:

Stiles, B. G., and T. D. Wilkins. 1986. Clostridium perfringens Iota Toxin: Synergism Between Two Proteins. Toxicon 24: 767-773.

Stiles B. G., and T. D. Wilkins. 1986. Purification and Characterization of Clostridium perfringens Iota Toxin: Dependence on Two Nonlinked Proteins for Biological Activity. Infect. Immun. 54: 683-688.

Simpson, L. L., B. G. Stiles, H. Zepeda, and T. D. Wilkins. 1987. Molecular Basis for the Pathological Actions of Clostridium perfringens Iota Toxin. Infect. Immun. 55: 118-122.

The last paper is the result of collaborative studies with laboratory at the Thomas Jefferson Medical College in Philadelphia, PA. After reading some of papers, of our laboratory noticed that enzymatic properties (ADP-ribosylating) of C. botulinum C_2 toxin were described using an in vitro assay. I was particularly interested in C_2 toxin because of its similarities with C. perfringens iota toxin. Both toxins kill mice, cause dermonecrosis in guinea pigs, and depend on two different, unlinked proteins for biological activity. I sent a letter to describing our findings with iota toxin and asked if he was willing to test the two purified components of iota toxin in his assay. My contributions to the subsequent paper include initial contact for collaborative work, providing purified material for the studies, and writing of pertinent sections of the Materials and Methods.

ACKNOWLEDGEMENTS

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SECTION I. PURPOSE OF DISSERTATION

Clostridium perfringens type E produces an iota toxin responsible for some fatal enterotoxemias of calves, lambs, and guinea pigs. Recently, rabbits used for research and as a protein source were found to be susceptible to a fatal "iota-like" enterotoxemia. A cross-reacting, iota-like toxin found in cecal contents of dead rabbits was neutralized by *C. perfringens* type E antiserum. *Clostridium spiroforme*, not *C. perfringens* type E, was found to be the causative agent of iota-like enterotoxemias in rabbits.

As with certain human gastrointestinal diseases like pseudomembranous colitis caused by *Clostridium difficile*, a disturbance of the normal flora in the intestinal tract must occur before colonization and subsequent toxin production by *C. spiroforme*. In the case of iota-like enterotoxemia of rabbits, antibiotics or weaning alter the normal flora and allow colonization by *C. spiroforme*.

Because of the importance of rabbits in research and as a protein source in developing countries, work was begun to purify, characterize, and study the mode of action of iota toxin. In vitro production of iota-like toxin by *C. spiroforme* was not dependable, making a sustained study difficult. Therefore, I chose to study the iota toxin of *C. perfringens* type E.

SECTION II. LITERATURE REVIEW

INTRODUCTION - THE TOXINS OF CLOSTRIDIUM PERFRINGENS

Clostridium perfringens is a Gram positive, sporeforming, bacillus found in soil, water, food, and gastrointestinal tracts of man and animals (124,142). This ubiquitous, anaerobic rod is responsible for many diseases of man and animals (88,124). The five toxin types (A, B, C, D, and E) produce a unique array of soluble, antigenic proteins (74). The typing scheme of C. perfringens is dependent on production of certain lethal and dermonecrotic protein toxins and neutralization by type specific antiserum in biological assays, such as mouse lethality or guinea pig dermonecrosis. There are at least 12 different extra-cellular toxins but only four "major" toxins (alpha, beta, epsilon, and iota) are used in the typing scheme (Table 1) (92). Biological properties of the "minor" toxins are listed in Table 2. Clostridium perfringens also produces a spore-related enterotoxin responsible for food poisoning in man and animals which will be discussed later.

ALPHA TOXIN

Alpha toxin, produced by all C. perfringens types, has phospholipase C activity which explains its hemolytic activity towards a wide range of erythrocytes (124). C. perfringens delta toxin and the oxygen-labile theta toxin are also hemolytic but have no phospholipase

Table 1. C. perfringens Toxin Typing Scheme

Toxin Type	Major Toxins alpha	beta	epsilon	iota	neutralizing type specific antiserum
A	+	-	-	-	A,B,C,D,or E
B	+	+	+	-	B only
C	+	+	-	-	B or C
D	+	-	+	-	B or D
E	+	-	-	+	E only

Table 2. Minor Toxins and Activities

	C. perfringens types					
Toxin	A	B	C	D	E	Activity
Delta	-	+	+	-	-	Hemolysin/cytotoxin
Eta	+	-	-	-	-	?
Gamma	-	+	+	-	-	?
Kappa	+	+	+	+	+	Collagenase
Lambda	-	+	-	+	+	Protease
Mu	+	+	+	+	+	Hyaluronidase
Nu	+	+	+	+	+	Deoxyribonuclease
Theta	+	+	+	+	+	O_2 Labile Hemolysin

C activity. Alpha toxin is a single polypeptide of 40,000 M_r with a pI of 5.5 (124,152). The toxin is stable at 100° C for 10 min but is inactivated by proteases (25,123).

Biologically active alpha toxin is dependent on zinc (116,116a). Inactive apotoxin produced in zinc deficient media is restored to a biologically active form by adding zinc. Calcium is necessary for phospholipase C activity by giving the substrate (choline or glycerophosphatides) a positive charge and enhancing attachment of negatively charged alpha toxin (61). Chelating agents like phosphate, citrate, and fluoride inhibit phospholipase C activity by binding calcium (124).

BETA TOXIN

Beta toxin, produced by C. perfringens types B and C, is responsible for necrotic enteritis of man and animals (65,74). The toxin is approximately 42,000 M_r and has a pI between 5 and 6 (147). It loses 75 % of activity after heating 50° C for 5 min and is inactivated by trypsin. The toxin kills mice (LD_{50} = 1.9 ug), and results in a purplish, necrotic lesion in the guinea pig skin assay (115).

Although beta toxin's mechanism of action is unknown, increased blood pressure and catecholamine release occurs in rats after intravenous injection of the toxin (115a). The authors suggest that hypertension is mediated by catecholamines released from peripheral sympathetic nerves and adrenal medulla. The rise in blood pressure is markedly decreased by inhibiting catecholamine release.

EPSILON TOXIN

C. perfringens types B and D produce epsilon toxin which is of veterinary importance. The toxin is not implicated in human diseases but is involved in fatal enterotoxemias of sheep (48). Epsilon toxin is produced as a biologically inactive protoxin, activated by endogenous proteases or added trypsin (124,149,150). The type of trypsin used for protoxin activation seems to be important (150). Protoxin treated with commercially available trypsin resulted in 16 fold more active toxin than N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin. TPCK inhibits chymotrypsin, a proteolytic enzyme which contaminates many trypsin preparations and will also maximally activate epsilon protoxin. Evidently, chymotrypsin preparations contain trace amounts of trypsin and the authors conclude that maximum activation of epsilon protoxin is dependent on both chymotrypsin and trypsin activity (150).

The protoxin and activated toxin have molecular weights of 32,700 and 31,200 (SDS PAGE), respectively (148). Isoelectric points of protoxin and active toxin are 8.0 and 5.4 to 5.7, respectively. The decrease in molecular weight and isoelectric point of the protoxin after proteolytic activation was consistent with the loss of a small basic peptide (44). Later work showed that epsilon protoxin is a single polypeptide chain of 311 amino acids and a basic 14 amino acid peptide is lost from the amino terminus after proteolytic activation (4,45).

Epsilon protoxin produced in the intestines is activated by

proteolytic enzymes of host or bacterial origin. The toxin increases intestinal permeability and causes swollen kidneys, lung edema, and fluid accumulation in the pericardial sac (10,55). Clinical symptoms are rarely seen, although affected sheep are markedly more nervous before death (12).

Besides causing edema in various organs, epsilon toxin damages the vascular endothelium of animal brain tissue. Brain damage is evidenced by leakage of horseradish peroxidase or radiolabeled (^{125}I) serum albumin into the brain of mice from the circulatory system (35,43,78,146). Epsilon toxin evidently binds to endothelial cells of various animal organs including the liver, lungs, heart, and cells lining the loops of Henle in kidneys (13,14). Interestingly, no binding was found with epithelial cells lining the large or small intestines.

Epsilon toxin indirectly increases levels of cyclic AMP in mice by causing brain edema and release of catecholamines which stimulate adenylate cyclase (14). Activated adenylate cyclase may explain the increased blood glucose levels of animals suffering from type D enterotoxemia since rises in intracellular cyclic AMP levels cause breakdown of glycogen to glucose (145).

IOTA TOXIN

Iota toxin was first described in 1943 by Bosworth (9). It is produced by C. perfringens type E and implicated in enterotoxemias of calves, lambs, rabbits, and guinea pigs (9,47,72,105,114). Besides being found in intestinal contents of diarrheic animals, C.

perfringens type E has also been isolated in pure culture from the kidneys of calves and blood of guinea pigs suffering from iota enterotoxemia (47,72).

Bosworth's new isolate grew in cooked meat medium and produced a unique extracellular toxin not neutralized by antisera against previously described C. perfringens types A, B, C, or D (9). The toxin killed mice and caused dermonecrosis in guinea pigs. Growth of the organism beyond 18 h up to 11 days in cooked meat medium did not decrease the biological activity of iota toxin. Storage of toxic supernatant on the lab bench for three weeks resulted in no loss of potency. Full activity was retained in supernatants stored at 4° C for 12 months or lyophilized for up to two years. Iota toxin from 18 h cultures was heat labile (65° C for 30 min) but resistant to trypsin treatment. When mice were intravenously injected with toxic supernatant, muscular spasms of the hind limbs occurred with eventual dragging of the limbs before death (1-24 h after injection).

Ross et al. reported in 1949 that active iota toxin resulted after proteolysis of an inactive protoxin (114). Activation occurred in young cultures (5 h at 37° C) only after trypsin treatment. Older cultures did not require trypsinization since proteases produced in late log and stationary phase grown cells activated the protoxin. The brief period in which iota protoxin is present differs markedly from the epsilon protoxin which is slowly activated by endogenous proteases but requires added trypsin and chymotrypsin for full activity (149,150). Differences in proteolytic activation may result from protoxin conformation and accessibility of the activation sites

or the types and amounts of proteases produced by C. perfringens types B, D, and E.

Craig and Miles were the first to partially purify iota toxin using ammonium sulfate precipitations and Berkfield candles (25). Their characterization data are similar to Bosworth's (9). The toxin was stable in cultures grown for 8 days and stored at 4° C for 3 months. Unlike iota toxin, they found that alpha toxin was inactivated in cultures older than 3 days. Lyophilized iota toxin lost approximately 30 % potency when held at room temperature for 30 days.

The medium used for toxin production affected protoxin activation by the organism. Papain digests of horsemeat sustained higher toxin production than cooked meat medium as determined by the guinea pig skin assay. Protoxin, but no active toxin, was found at 4 h in both media. After 6 h of growth, all protoxin in the papain digest medium had been activated yet protoxin remained in cooked meat medium up to 18 h.

Craig and Miles also determined the effects of pH and temperature on the toxin's biological activity. Samples were dialysed at the appropriate pH for 2 h (37° C) and adjusted to neutrality before guinea pig skin assays. No loss in activity was seen at pH 7, pH 6.2, or pH 5.2 but there was a 50 % decrease at pH 9.0, an 82 % decrease at pH 4.2, and no activity at pH 3.8. Heat stability studies showed a decrease in iota toxin activity after heating at 48° C for 15 min and loss of all activity after 53° C for 15 min.

Clostridium spiroforme - Its importance in iota-enterotoxemia

Work done in the past five years has shown that iota toxin is not unique to C. perfringens type E. Clostridium spiroforme produces an immunologically related, but not identical, toxin implicated in the fatal enterotoxemias of rabbits, guinea pigs, hamsters, rats, and mice (6,15,16,17,18,19,46). Because of a neutralizable "iota-like" toxin found in the cecal contents of diseased rabbits, C. perfringens type E was the suspected agent but never isolated (3,27,30,73). One investigator described the isolation of C. perfringens from cecal contents containing an iota or iota-like toxin yet none of the isolates produced the toxin in vitro (106,107). His isolates were later found to be the ubiquitous type A instead of type E (20).

Conditions predisposing rabbits to the disease include weaning and the use of antibiotics like lincomycin or clindamycin (17,63,73,112). In spontaneous and antibiotic associated diarrheas, the normal gastrointestinal flora is disturbed, allowing for colonization, growth, and toxin production by C. spiroforme (18). Antibiotics, weaning, nor the organism alone are sufficient to cause the disease (17,18). This suggests that C. spiroforme is not normal flora and is obtained from an outside source.

The iota-like toxin is similar to C. perfringens iota toxin since both are heat labile (56° C for 30 min), proteolytically activated, dermonecrotic in guinea pigs, mouse lethal, and neutralized by C. perfringens type E antiserum (7). No previous investigators have

described the bimolecular nature of C. perfringens iota toxin or C. spiroforme iota-like toxin (9,21,25,31,63,72,114,120,128,129).

Diseases Caused by Clostridium perfringens

The role of C. perfringens in infections and diseases of man and animals is well documented (88,124). C. perfringens is often involved in gas gangrene and various enterotoxemias. Gas gangrene results from deep, penetrating wounds soiled by a consortium of bacteria, including C. perfringens. Type A strains are most commonly found in nature and are most often seen in these infections (24). The traumatized and anoxic muscle tissue is digested by bacterial proteases with subsequent gas production. Treatment of this life-threatening infection includes antibiotics, hyperbaric oxygen, gas gangrene antiserum, or amputation.

All C. perfringens toxin types are associated with enterotoxemias of man or animals. C. perfringens type A is responsible for one of the most common forms of food poisoning in the United States (22). The organism grows in meats and gravies kept warm, but not hot enough to prevent cell multiplication. A large number of vegetative cells are ingested and sporulate in the small intestines. Upon sporangial lysis, excess spore coat protein is released. This spore coat protein is a potent enterotoxin causing nausea, abdominal cramps, diarrhea, and infrequent vomiting. The symptoms usually subside within 24 h after onset and death does not occur unless the victim is compromised by age and/or disease. Recently, C. perfringens enterotoxin has been

found in the stools of some patients suffering from antibiotic associated diarrhea (5,8).

Horses also suffer from the ill-effects of C. perfringens enterotoxin after ingesting grass contaminated by large numbers of type A vegetative cells. The disease, known as equine grass sickness, results from enterotoxin released from sporulating cells (93).

Much work has been done with C. perfringens enterotoxin produced by sporulating type A strains. A spore related enterotoxin is also produced by types C and D but has not been implicated in human or animal enterotoxemias (121,122,138). Type A enterotoxin is approximately 35,000 M_r (125), has a pI of 4.3 (127a), and is activated by trypsin with a resultant 3 fold increase in activity and loss of a 4000 dalton peptide (41,113).

A receptor for C. perfringens type A enterotoxin has been isolated from rabbit brush border membranes. The receptor is a 50,000 M_r protein and when mixed with enterotoxin, prevents the typical cytotoxic effects of the enterotoxin on Vero cells (143). It is unknown whether the receptor has lipid and/or carbohydrate moieties which are important in the binding of many bacterial toxins to target cells (28).

Other gastrointestinal ailments caused by C. perfringens, but not associated with spore-related enterotoxin, include enterotoxemias of lambs, calves, horses, sheep, goats, and man (48,124). Humans are particularly susceptible to a necrotic enteritis caused by type C strains. This sometimes fatal condition occurs when an unsuspecting victim, predisposed by a low protein diet, consumes large amounts of protein contaminated by type C organisms.

A necrotic enteritis called pig bel is endemic to the natives of Papua New Guinea and has a 40 % mortality rate among the young and old (124). Their diet consists of roots (like sweet potatoes), berries, and very little meat. Once a year they feast on whole pigs roasted in earthen pits. C. perfringens type C, present in large numbers in pig intestines and soil, contaminates the meat when the animal is gutted and roasted. Contamination of the meat, along with insufficient cooking time and temperature, set the stage for disease. After ingesting large amounts of contaminated meat, many individuals suffer from abdominal cramps and diarrhea with acute inflammation of the small intestines and localized areas of necrosis and gangrene. Sometimes the intestines are perforated, leading to an invariably fatal peritonitis unless surgically treated. Factors responsible for this disease include low pancreatic trypsin production, continuous consumption of trypsin inhibitors found in sweet potatoes, and sudden engorgement of a high protein meal contaminated with C. perfringens type C (131).

The beta toxin, which is readily inactivated by proteases, seems to play a role in C. perfringens type C necrotic enteritis of man. There is a rise and fall in beta antitoxin titers of patients suffering from the disease. The mortality rate decreases following the use of antitoxin specific for beta toxin (85,86). A vaccine, consisting of toxoided type C supernatant, has been successfully used to prevent pig bel (64). The vaccine is the first to combat clostridial enterotoxemias in man.

A similar necrotic enteritis of man, called Darmbrand or fire-belly, occurred in northern Germany after World War II. Individuals

on a low protein diet consumed canned meat contaminated with type C spores. As with pig bel, a sudden intake of meat with low trypsin production by the host contributed to the disease with symptoms similar to pig bel (153). Other cases of necrotic enteritis due to type C have been reported in Africa, South East Asia, and America (65).

C. perfringens type C is also associated with animal enterotoxemias, all of which involve necrosis and/or hemorrhaging of the small intestines with subsequent death (139). It is unknown whether beta toxin alone, or other toxins produced by the bacterium, are responsible for the pathology found in type C animal enterotoxemias.

A method developed for isolating C. perfringens type C from the feces of man and animals uses type specific antibody bound to silicate beads (66). Antibody coated beads were mixed with fecal sample, centrifuged, and washed extensively before plating. The significance of the work is not that it can be used for pig bel victims, from which type C is readily isolated. The method could be used in geographical areas where type C animal enterotoxemias are a problem and should be monitored. C. perfringens type C is very difficult to isolate from soil or fecal samples of healthy man or animals since type A strains are most commonly found in nature (142).

Besides man, animals also face diarrheic death from C. perfringens after sudden changes in their diet. Type D strains of C. perfringens, normally found in soil and gastrointestinal tracts of animals, can cause fatal enterotoxemias of sheep, lambs, and calves after a diet change (48,124). Introduction of a high starch diet

provides ample opportunity for proliferation and toxin production by opportunistic organisms like C. perfringens.

Structures of Bacterial Protein Toxins

Bacterial toxins exist in at least three different, biologically active forms including: 1) single polypeptide, 2) two biochemically different proteins bound noncovalently together (A-B model) or, 3) two or more different, unlinked proteins.

Single polypeptide toxins include E. coli heat stable enterotoxin (HST), closely related heat stable enterotoxins produced by other enteric organisms, and streptolysin S produced by streptococci. E. coli HST differs from the heat labile enterotoxin in several ways: HST contains no subunits, it activates eukaryotic guanylate cyclase instead of adenylate cyclase, and is nonantigenic (42). The lack of antigenicity of HST is probably due to its size, estimated at 4,400 M_r (2). Enterotoxins related to HST are produced by Yersinia enterocolitica (104), Klebsiella pneumonia (59), Enterobacter cloacae (60), and Aeromonas hydrophilia (11). Sharing of toxin genes, just like the sharing of resistance factors, may be a common occurrence within the Enterobacteriaceae.

Streptolysin S, a single polypeptide toxin, is low molecular weight (15,000 M_r), nonantigenic, and produced by groups A, C, and G streptococci (62). Streptolysin S is an oxygen stable cytolysin dependent on RNA core for activity. RNA core somehow stimulates production, release and/or stabilises the toxin and protects it from proteolytic degradation. Serum albumin, alpha-lipoprotein, Trypan

Blue dye, and non-ionic detergents may also aid in the release or activation of streptolysin S (39).

Sometimes single polypeptide toxins must be activated by proteases and/or reducing agents. Diphtheria toxin is produced as a single polypeptide (protoxin) and after trypsinization, an enzymatic (A fragment) and binding (B fragment) component are generated and held together by a single disulfide linkage (77). C. perfringens enterotoxin, epsilon toxin, and iota toxin are also proteolytically activated but the generation of binding and enzymatic fragments have not been described (4,41,114).

Diphtheria, cholera, and E. coli heat labile toxins require reduction of an internal disulfide linkage for activity. Cystine reduction releases an enzymatic fragment from the toxin complex into the cell. The released fragment modifies a target molecule like elongation factor 2 or adenylate cyclase, depending on the toxin (23,37,38,50,51,75,76,79).

In subunit toxins with the classic A-B form, like cholera or E. coli heat labile enterotoxins, neither enzymatic nor binding component alone has any effect on intact cells. When eucaryotic cell lysates and reduced A subunit are mixed in vitro, the adenylate cyclase complex is activated (38). The A subunit of cholera toxin contains two fragments which are held together by a disulfide linkage. Fragment A_1 has ADP-ribosylating activity while A_2 facilitates binding to the B subunits and entry of A_1 into the cell. The B subunit, which binds to a cell surface receptor, has no enzymatic activity and does not activate adenylate cyclase. Each B subunit binds to a receptor, G_{m1} ganglioside, inducing conformational change in the toxin and

insertion into the cell membrane (37). The B subunits form a hydrophilic tunnel in the cell membrane, allowing entry of the enzymatic A_1 fragment into the cell.

The A-B model is not unique to bacterial toxins. Thyrotropin, luteinizing hormone, human chorionic gonadotropin and follicle-stimulating hormone are glycoprotein hormones with analogous A and B subunits. Cholera toxin has amino acid sequence similarity with all of these hormones in both the A_1 fragment and B subunit (67).

The last group of bacterial toxins to be discussed require two or more unlinked proteins for activity. Toxin components do not associate in solution, therefore differing from the classic A-B model toxins. The toxins dependent on unlinked components include gamma hemolysin and leukocydin from S. aureus, B. cereus enterotoxin, anthrax toxin, and C. botulinum C_2 enterotoxin.

Bacillus anthracis Anthrax Toxin

Anthrax toxin is composed of three different, unlinked proteins called protective antigen, lethal factor, and edema factor (70). Protective antigen binds to cells, followed by lethal factor or edema factor binding. Both factors do not bind simultaneously to the same molecule of protective antigen (127). Although little is known about the mode of action of lethal factor, there is strong evidence that edema factor causes fluid efflux from cells by increasing intracellular cyclic AMP levels in target cells.

Edema factor is a soluble adenylate cyclase which gains entry into a cell via the protective antigen and is activated by a heat stable

host factor, calmodulin (69). Calmodulin is a calcium dependent protein regulating the activity of kinases within a eukaryotic cell. Edema factor differs from cholera toxin A subunit which activates eukaryotic adenylate cyclase by ADP-ribosylation of a regulatory component (38). Cells treated with protective antigen and edema factor show an immediate increase in intracellular cyclic AMP that can be stopped by washing intoxicated cells. The author suggests that the protective antigen and edema factor complex are not completely internalized and loosely associate on the cell's membrane (69). Levels of cyclic AMP increases in cells exposed to protective antigen and edema factor, but not in cells exposed to protective antigen and lethal factor or any individual component.

Staphylococcus aureus Leukocidin and Gamma Hemolysin

Staphylococcus aureus is a pathogen of man and animals which produces an array of toxins. Two of these toxins, a leukocidin and the gamma hemolysin, are dependent on two unlinked proteins for activity (133,144). Leukocidin is important in the pathogenicity of the organism because it induces cytotoxic changes in polymorphonuclear leukocytes and macrophages (90,91). The two components of leukocidin have been designated fast (F) and slow (S), based on their elution from a carboxymethyl cellulose column. S component binds first to a target cell followed by binding of the F component. Interestingly, binding of S component is competitively inhibited by the B subunit of cholera toxin. S component is also inactivated by the cholera toxin receptor, G_{m1} ganglioside (89). The S component

activates membrane associated phospholipase A_2 which catalyzes hydrolysis of certain fatty acids found on the cell membrane (91). The authors suggest that F component binds to the phospholipase A_2 product, but no evidence is provided.

Another synergistic staphylococcal toxin, gamma hemolysin, lyses human, rabbit, and sheep erythrocytes (133). Neutralizing antibody towards gamma hemolysin has been detected in human staphylococcal osteomyelitis cases, suggesting in vivo production of gamma hemolysin (133). The two proteins required for activity, designated components I and II, have molecular weights of 29,000 and 26,000 with very basic isoelectric points of 9.8 and 9.9, respectively (132). The mode of action is unknown, but sulfonated polymers like agar, heparin, and dextran sulfate inhibit biological activity of the combined components. Negatively charged, sulfonated compounds may bind to the positively charged, basic proteins of the hemolysin. The authors did not test whether one component is needed to bind or enzymatically act on erythrocytes before the other. After incubating cells with either component, erythrocytes could be gently washed with isotonic buffer, followed by the addition of complementary component.

Bacillus cereus enterotoxin

Bacillus cereus has been implicated in various infections like endocarditis, meningitis, and puerperal sepsis of man and mastitis of cattle (56,135). The organism produces an enterotoxin involved in food poisoning outbreaks (36,134). B. cereus enterotoxin depends on three different proteins for biological activity. Chromatography

fractions containing any two of the three proteins had no activity (134). Little is known about the toxin's mode of action.

Clostridium botulinum Neurotoxins and C_2 Enterotoxin

C. botulinum types A, B, C, D, E, F, and G produce seven antigenically distinct neurotoxins involved in food and wound-borne botulism of man and animals (117,124). The neurotoxins of types A-F are produced as protoxins of approximately 150,000 M_r and are proteolytically activated by trypsin (29,117). After activation, the neurotoxins of types A-F consist of a heavy chain (100,000 M_r) and a light chain (50,000 M_r) held together by a disulfide linkage(s). Although Type G neurotoxin has not been purified to homogeneity, trypsin activates type G protoxin (117).

Another botulinum toxin, called C_2 enterotoxin, is produced by types C and D and has a unique binary structure in which two different, unlinked proteins act synergistically (54,97,98,101,102,118). C_2 toxin is not a neurotoxin but causes fluid accumulation in mouse intestinal loops, increases vascular permeability in guinea pigs, and requires trypsin activation (96,97,98). The toxin is dependent on component I, a 55,000 M_r ADP-ribosylating enzyme, and component II, a 105,000 M_r binding protein, for biological activity (98,99,100). Proteolytic activation of C_2 toxin results in a 2000 fold increase in activity and involves nicking of the binding protein, component II. Unnicked component II will bind to target cells but not interact with enzymatic component I (97,100). Neither component alone has biological activity in animals.

Component I ADP-ribosylates poly-L-arginine homopolymers or nonmuscle actin from chicken tissue or human platelets using NAD as substrate (1,103,119). Component II has no such activity.

The C_2 toxin is produced during sporulation (87,151), like C. perfringens enterotoxin (33) or B. thuringiensis toxin (126), but is not produced during vegetative cell growth (151). Both components of C_2 toxin were extracted from the spore coat of type C strains (151). It would be interesting to determine whether immunological relatedness exists between C_2 toxin and other spore related toxins of C. perfringens and B. thuringiensis.

ADP-Ribosylation

Eukaryotes and procaryotes regulate many cellular processes by the enzymatic transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to an acceptor protein (34,49,137). O- or N- glycosidic linkages are formed between ADP-ribose and certain amino acids like arginine, asparagine, glutamic acid, lysine, or diphthamide, a post-translationally modified histidine. Single ADP-ribose groups (mono-ADP-ribosylation) can be added per site or a chain can be synthesized (poly-ADP-ribosylation), depending on the enzyme. All known bacterial ADP-ribosyl transferases are mono-ADP-ribosylators. These enzymes include bacterial toxins and an ADP-ribosyl transferase of Rhodospirillum rubrum which regulates the cell's nitrogenase complex (32,52,58,71,80,109,140,). Table 3 is a list of bacterial toxins that have known ADP-ribosylating activity.

Table 3. ADP-Ribosylating Bacterial Toxins

Toxin	ADP-ribose acceptor (amino acid)
Diphtheria	Eucaryotic or archaebacterial elongation factor II (diphthamide)
Pseudomonas exotoxin A	Same as Diphtheria toxin.
Cholera	Adenylate cyclase (arginine)
E. coli heat labile enterotoxin	Adenylate cyclase (arginine)
Pertussis	Adenylate cyclase (asparagine)
C. botulinum C_2 enterotoxin	Nonmuscle actin (arginine?)
C. perfringens iota	Nonmuscle actin (arginine?)

Some bacterial toxins with ADP-ribosylating activity exploit pre-existing systems found in eukaryotic cells. Lee and Iglewski found an ADP-ribosyl transferase in hamster kidney cells which modifies diphthamide on elongation factor 2 (68). This same site is modified by diphtheria toxin or Pseudomonas exotoxin A. Although the mode of action appears similar, no cross-reactivity exists between the eukaryotic enzyme and antisera developed against either bacterial toxin. Another example includes an ADP-ribosyl transferase from turkey erythrocytes which activates adenylate cyclase by specifically modifying an arginine residue also modified by cholera toxin (38,82, 83,84,141).

Viruses also use ADP-ribosylating enzymes to their advantage. The T4 phage of E. coli can modify RNA polymerase and modulate transcriptional specificity (40). Histones are ADP-ribosylated by a polyoma virus enzyme involved in viral transcription and replication (111).

Many eukaryotic processes are regulated by poly-ADP-ribosylation including the activation of DNA ligase involved in DNA excision repair (95,26), modification of histones (57,81,108,94,130,136), and intestinal or granulocyte cell differentiation (53,110).

As previously mentioned, the repair of eukaryotic DNA is associated with ADP-ribosylating activity. Damaged DNA results in increased excision repair, a rise in poly-ADP-ribosylating activity, and decreased levels of NAD within the cell. Inhibitors of ADP-ribosyl transferases retard the repair of damaged DNA and increased cytotoxicity of DNA-damaging agents. The mechanism of enhanced ligation by ADP-ribosylation is unknown but several possibilities exist. DNA ligase II may be directly ADP-ribosylated, thus increasing

ligase activity (26). Inhibitory effects of histones on DNA ligase may be reversed by neutralizing positively charged histones and relaxing DNA-histone interaction (95). Ligase activity in the presence of histones and DNA is approximately 1/2 of that seen with histone free DNA. Poly-ADP-ribose may alter the chromatin structure by cross-linking histones and disturbing nucleosomal complexes (130). The last possibility involves poly-ADP-ribosylation near DNA strand breaks, allowing DNA ligase, which has a high affinity for ADP-ribose, to "zero in" on the damaged site (95).

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Section III. *Clostridium perfringens* Iota Toxin: Synergism Between Two Proteins

ABSTRACT

The iota toxin of *Clostridium perfringens* type E is a guinea pig dermonecrotic, mouse lethal toxin which cross-reacts with the iota-like toxin of *Clostridium spiroforme*. Antiserum raised against *C. spiroforme* or *C. perfringens* type E neutralizes the toxin from both species. By using *C. spiroforme* antiserum and crossed immunoelectrophoresis, we have found that there are two cross-reacting proteins, designated iota a (i_a) and iota b (i_b) in the culture filtrate of *C. perfringens* type E. Both proteins of *C. perfringens* were separated by preparative isoelectric focusing and had very little toxic activity when tested alone. However, when they were recombined there was an 8 and 25 fold increase in bioactivity as determined by mouse lethal and guinea pig dermonecrotic assays, respectively. These results demonstrate that the iota toxin of *C. perfringens* requires two immunologically and biochemically different proteins for maximum activity.

INTRODUCTION

Clostridium perfringens types A, B, C, D, and E are the causal agents of many human and animal diseases (8,9). They are found in soil, air, water, and the intestinal tracts of man and animals and produce at least twelve extracellular protein toxins (8). One of

these toxins, the iota toxin, is produced by type E strains which have been implicated in calf and lamb enterotoxemias (3,17). Iota toxin is reportedly dependent upon proteolytic activation, either by proteases produced by the organism or by trypsin that is added to young cultures (17). The active toxin has a reported molecular weight of 70,000 (16), kills mice, and causes dermonecrosis in guinea pigs (3,11,17). The biological activity of the toxin is completely lost after heating at 53° C for 15 min or dialysing at a pH of 3.8 or below (4).

Borriello and Carman (1983) described an iota-like toxin found in the cecal contents of rabbits suffering from spontaneous and antibiotic-associated diarrhea (2). The toxin killed mice, caused dermonecrosis in guinea pigs, and was neutralized by C. perfringens type E antiserum. Clostridium spiroforme, and not C. perfringens, was isolated from the cecal contents and produced the iota-like toxin in vitro. In the present investigation, we show that the iota toxin of C. perfringens cross-reacts with C. spiroforme antiserum. By using preparative isoelectric focusing and C. spiroforme antiserum in different immunoelectrophoretic techniques, we show that C. perfringens iota toxin acts as a binary toxin, dependent on two distinct proteins for maximum biological activity.

MATERIALS AND METHODS

Bacterial strains and toxin production: The type strain of C. perfringens type E, NCIB 10748 (VPI 1589), and C. spiroforme NCTC 11493 were used for these studies. Culture filtrate containing C. perfringens iota toxin was produced in 2 liter brain heart infusion (BHI) dialysis flasks as previously described (19). Five ml of pre-reduced anaerobically sterilized (PRAS) BHI was inoculated with 0.5 ml of a chopped meat stock and incubated for 7 h at 37° C (5). One ml of this actively growing culture provided an inoculum for each flask. After incubating the flasks at 37° C for 72 h, we removed the cells and debris by centrifugation (8000 x g) for 15 min and filtered the supernatant through a 0.45 um membrane filter (Gelman Sciences, Ann Arbor, Michigan). The culture filtrate was brought to 70 % saturation with ammonium sulphate at 4° C. The precipitate was collected by centrifugation (7000 x g) for 20 min and dissolved in 1/12 the original volume using 0.05 M Tris-HCl buffer, pH 7.5. This supernatant concentrate was dialysed against 0.05 M Tris-HCl buffer to remove residual ammonium sulphate and stored at -20° C.

C. spiroforme NCTC 11493 was grown in dialysis flasks containing chopped meat broth and 0.5 % glucose. A chopped meat stock culture (0.5 ml) was used to inoculate each flask. After 96 h at 37° C, the contents of the dialysis sacks were harvested and stored at 4° C.

Preparative Isoelectric Focusing (IEF): Preparative IEF was performed in Sephadex G-75-40 (Sephadex IEF, Sigma Chemical Co.) on an LKB 2117

Multiphor using the flatbed isoelectric focusing kit (LKB, Application Note 198). A 4 % (w/v) gel slurry (100 ml) containing 2 % LKB ampholine (pH 4-6) was dried at room temperature. Two ml of supernatant concentrate (20 mg of protein) was dialysed in 0.005 M Tris-HCl buffer, pH 7.5 overnight before application to the bed in a trough 6 cm from the cathode. The sample was focused with constant power (8W) for 14 h at 4° C. The pH of each of the 30 fractions was determined with a glass electrode and the gel from each fraction mixed with 10 ml of 0.05 M Tris-HCl buffer, pH 7.5. Each fraction was assayed for the presence of i_a and i_b by fused rocket immunoelectrophoresis using C. spiroforme antiserum as described below.

Production of antiserum: Toxin and cells were produced as previously mentioned. For the preparation of vaccines, C. perfringens cells were diluted with culture supernatant fluid to a 1 $O.D._{660}$ suspension and C. spiroforme dialysis sack contents were diluted with normal saline to an equal turbidity. The 1 $O.D._{660}$ suspension was treated overnight at 37° C in a final 0.4 % formalin concentration. Each of the first six weekly vaccinations consisted of 1 ml of the formalized cell suspension mixed with 1 ml of Freund's incomplete adjuvant (Sigma Chemical Co.). A neutered, six month old alpine goat was injected subcutaneously at two different sites per weekly injection. For subsequent weekly vaccinations (six weeks), 1 ml of undiluted, unformalized whole culture was mixed with an equal volume of Freund's incomplete adjuvant.

Immunoelectrophoresis: Crossed immunoelectrophoresis (crossed IEP) and fused rocket immunoelectrophoresis (fused rocket IEP) were performed using the general methodology of Axelsen et al. (1). For crossed IEP, we used 5 ul of the supernatant concentrate of C. perfringens (50 ug of protein) or C. spiroforme (5 ug of protein) dialysis sack culture which had been diluted five fold with saline, centrifuged, and concentrated ten fold using a CS-15 Minicon concentrator (Amicon Corp., Danvers Ma.). C. perfringens and C. spiroforme antiserum concentrations were 5 ul/cm^2 and 1.5 ul/cm^2 respectively. Fused rocket IEP plates (8.2 by 8.2 cm) were coated with 15 ml of 1.2 % low EEO agarose (Sigma Chemical Co.) in 0.025 M Tris-Tricine buffer, pH 8.6. Samples of 5 ul were subjected to electrophoresis at 100 volts for 6 h into agarose containing C. spiroforme antiserum (3 ul/cm^2). Plates were washed, pressed, stained, and destained as previously described (7).

Biological assays: In the mouse lethality assay, we injected four to six week old Swiss-Webster mice (Dept. of Animal Science, V.P.I. and S.U.) each i.p. with 0.5 ml of test material and observed them over 48 h for death. A 1:30 dilution of C. perfringens supernatant (ammonium sulphate concentrate) was used for determining anti-iota titers of antisera. Dilutions of the concentrated supernatant and antiserum were made in 0.05 M Tris-HCl buffer, pH 7.5. Mouse lethal titers represent the reciprocal of the highest dilution that killed both mice.

For the guinea pig skin tests, exbreeding Dunkin-Hartley guinea pigs (Hazelton/Dutchland Laboratories, Denver, Pa.) were injected intradermally with 0.3 ml of test material and examined over 48 h for dermonecrotic lesions. Recorded titers are the reciprocal of the highest dilution that gave a dermonecrotic lesion. To neutralize the effects of any alpha toxin, 100 ul of C. perfringens type A antiserum (Burroughs Wellcome, Kansas City, Ka.) was added to 400 ul of C. perfringens supernatant concentrate and incubated at room temperature for 30 min before making dilutions (5). C. perfringens supernatant concentrate had a mouse lethal titer of 80 and a guinea pig skin titer of 640.

Effects of Trypsin: C. perfringens type E supernatant concentrate was treated with a 1 % trypsin solution (Difco 1:250 Trypsin) as described by Holdeman et al. (5). Nine parts of the supernatant concentrate were mixed with one part trypsin solution (trypsin dissolved in distilled water) and incubated at 37° C for 30 min. Guinea pigs were injected as described above using trypsin treated and untreated supernatant concentrate.

Protein Estimation: Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as the standard. Protein determinations of IEF fractions were made before the addition of C. perfringens type A antiserum.

RESULTS

Demonstration of immunological cross-reactivity between C. spiroforme and C. perfringens type E: Our initial studies showed that antiserum developed against a toxin producing strain of C. spiroforme reacted with only two antigens produced by C. perfringens type E (Fig. 1, plate 2). The cross-reacting antigens of C. perfringens type E and C. spiroforme were designated iota a (i_a) and iota b (i_b). We tested four strains of C. perfringens type E and all of them had the two cross-reacting antigens (data not shown). Mouse lethal titers were higher in strains with greater amounts of i_a and i_b (determined by crossed IEP). Two strains of each C. perfringens type A, B, C, and D, which do not produce iota toxin, did not show this cross-reactivity even after concentrating 12 h BHI supernatants 40 fold by CS-15 Minicon concentrators. Trypsinization of concentrated culture supernatants from C. perfringens type E (72 h growth) did not increase the biological activity of iota toxin as determined by the guinea pig skin assay (data not shown).

Our C. spiroforme antiserum had a titer to iota toxin of 32, similar to commercial type E antiserum (Burroughs Wellcome). Our C. perfringens type E antiserum, which had an anti-iota titer of 4, and Burroughs Wellcome antiserum to C. perfringens type E formed a precipitin arc to C. spiroforme i_b, but not i_a, in crossed IEP (Fig. 1, plates 5 and 6). The i_a and i_b components from one species are immunologically unrelated since the immunoprecipitin arcs cross (Fig. 1, plates 1 and 2). The two proteins also differ slightly

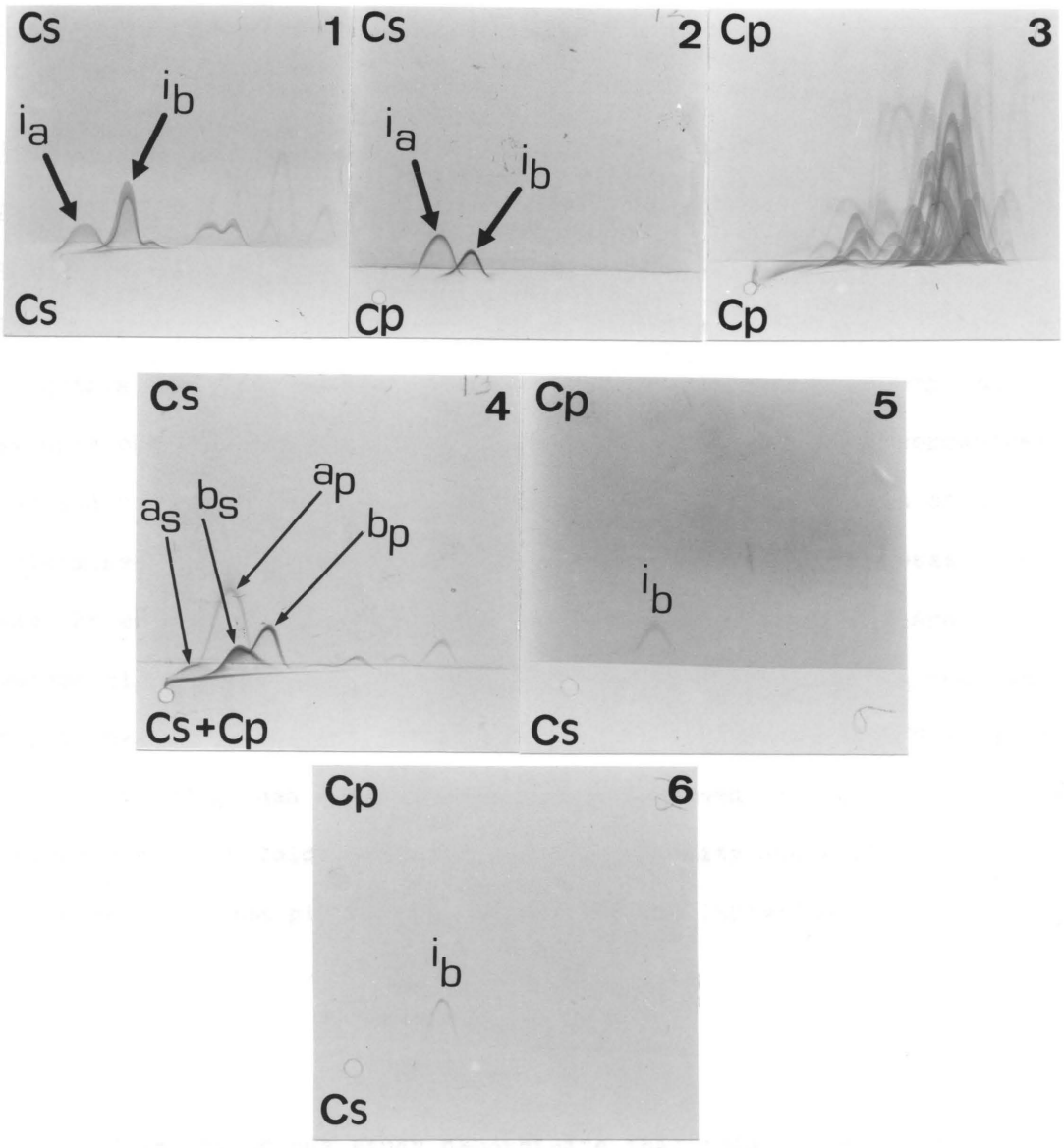

Fig. 1. Crossed IEP of iota and iota-like toxin preparations of C. perfringens type E (Cp) and C. spiroforme (Cs). Plates are labeled with the antigen on bottom and antiserum on top. Plate 1= 5.0 ug antigen and 1.5 ul/cm^2 antiserum, Plate 2 = 50 ug antigen and 5.0 ul/cm^2 antiserum, Plate 3 = 50 ug antigen and 5.0 ul/cm^2 antiserum, Plate 4 = mixture of C. spiroforme (i_a and i_b labeled a_s and b_s, respectively) and C. perfringens type E (i_a and i_b labeled a_p and b_p, respectively) antigens at 3 and 75 ug, respectively and 1.5 ul/cm^2 antiserum, Plates 5 and 6 = 50 ug antigen and 5 ul/cm^2 antiserum (Plate 5 contained our C. perfringens type E antiserum; plate 6 contained commercial type E antiserum)

between the species as shown by the faster relative migration of C. perfringens i_a and i_b (Fig. 1, plate 4).

Synergism between i_a and i_b: The i_a and i_b components of C. perfringens could be separated by isoelectric focusing in a pH 4-6 gradient (Fig. 2). The isoelectric points of i_a and i_b were 5.2 and 4.2, respectively. When each fraction was assayed for dermonecrosis of guinea pig skin, fractions 9-11 and 14-26 yielded only very small amounts of iota toxin activity. Table 1 gives the protein concentration and titers of reactive fractions. Fraction 10, the peak of i_b as determined by fused rocket IEP and relative migration on crossed IEP, was the most active fraction with a mouse lethal titer of 2 and a guinea pig skin titer of 20. When an equal volume of the i_b peak and the i_a peak (fraction 24) were combined, titers of 16 and 512 in mouse lethality and guinea pig skin tests were obtained, respectively. This represented an 8 fold increase in mouse lethality and a 25 fold increase in guinea pig dermonecrosis over the individual fractions.

DISCUSSION

The results of our study demonstrate that iota toxin is similar to other bacterial binary toxins, which require two separate proteins for maximum activity. C. perfringens type E iota toxin is dependent on i_a and i_b, which are immunologically and biochemically different proteins. Our crossed IEP results show that the electrophoretic mobility of C. perfringens i_a and i_b is relatively faster than i_a and i_b of C. spiroforme. This difference between the species indicates

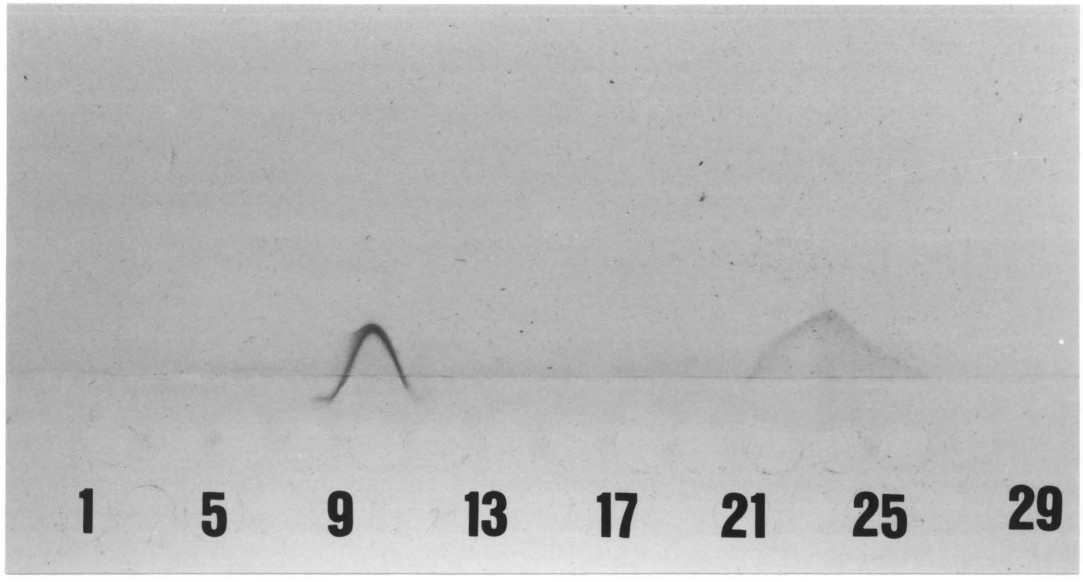

FIG. 2. Fused rocket IEP of isoelectric focusing fractions containing C. perfringens supernatant concentrate using a pH 4-6 gradient. Fractions 1-29 were subjected to electrophoresis into agarose containing 3 ul/cm^2 C. spiroforme antiserum. The i_b component focused in fraction 10 and i_a in fractions 22-26.

Table 1. Biological activity of culture filtrate concentrate after isoelectric focusing

Fraction(s)	Protein concentration (ug/ml)	Mouse lethal[a] titer	Guinea pig[b] skin titer
9	260	0	1
10 (i_b peak)	650	2	20
11	650	0	1
14	340	0	1
16	320	0	1
18	120	0	5
20	90	0	5
22	80	0	5
24 (i_a peak)	650	0	5
26	60	0	1
28	20	0	0
30	50	0	0
10+18[c]	390	4	64
10+20	370	8	64
10+22	370	16	256
10+24	650	16	512
10+26	360	16	256
10+28	340	4	64
10+30	350	0	32

[a] Titers represent the reciprocal of the highest dilution that killed both mice within 48 h.

[b]bTiters represent the reciprocal of the highest dilution that gave a dermonecrotic lesion within 48 h.

[c] 200 ul of each fraction was combined and 100 ul of C. perfringens type A antiserum added to the mixture. This mixture was further diluted in 0.05 M Tris-HCl buffer, pH 7.5.

variation in amino acid composition which could arise from differences in the DNA coding for the protein and/or protease activity.

Both components of C. perfringens iota toxin were detected by cross-reacting C. spiroforme antiserum in crossed IEP; however, Burroughs Wellcome antiserum or our own C. perfringens type E antiserum detected C. spiroforme i_b, but not i_a. Further work must be done to determine if C. perfringens type E antiserum contains antibody towards i_a. Purified i_a from both species and a more sensitive assay than crossed immunoelectrophoresis, such as Western blots, could be used to solve this problem. We expect some antibody towards i_a since we had used crude culture supernatant for antiserum production.

Previous investigators have described trypsin activation of C. perfringens iota toxin (4,17). Ross et al. (1949) found that supernatants of C. perfringens type E grown in a horse meat broth for 5 h at 37° C occasionally needed trypsinization for maximum biological activity (17). Cultures older than 5 h did not require activation. Our inability to increase the biological activity of a 72 h culture supernatant by trypsinization is probably due to the production of endogenous proteases which act upon the protoxin during growth.

The synergistic effect of i_a and i_b of iota toxin suggests that it may be a binary toxin, similar to Clostridium botulinum C_2 toxin (6,12,13,14,15) and Staphylococcus aureus leukocidin (10,20) which requires two nonlinked proteins for activity. Like iota toxin, C_2 toxin is reportedly enterotoxic, increases vascular permeability in guinea pig skin, and is lethal to mice. The C_2 toxin has a binding component and an ADP-ribosylating component which are not bound to each other in solution (18). Binding and enzymatic subunits of

bacterial toxins have been demonstrated with other toxins like cholera, diphtheria, exotoxin A of Pseudomonas, and tetanus. Work is continuing in our laboratory to determine the mechanism for the synergistic effect of i_a and i_b in C. perfringens iota toxin.

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Section IV. Purification and Characterization of *Clostridium perfringens* Iota Toxin: Dependence on Two Nonlinked Proteins for Biological Activity

ABSTRACT

Clostridium perfringens type E iota toxin, a dermonecrotic and lethal binary toxin, was purified to homogeneity. Each protein component of the toxin, iota a (i_a) or iota b (i_b), appeared as a single band by gradient or sodium dodecyl sulfate polyacrylamide gel electrophoresis and yielded a single immunoprecipitin arc by crossed immunoelectrophoresis using homologous antiserum. Individually, i_a (M_r 47,500) or i_b (M_r 71,500) had little biological activity. However, when combined in equal molar amounts there was a 64 fold increase in the guinea pig dermonecrotic titer. The biological activity of i_a was heat stable (85° C for 15 min) whereas i_b was inactivated at 55° C. Our results demonstrated that *C. perfringens* iota toxin required two different, nonlinked protein components for biological activity.

INTRODUCTION

Clostridium perfringens types A, B, C, D, and E produce at least twelve extracellular protein toxins (11). One of these toxins, iota toxin, is produced only by type E strains and has been implicated in fatal calf, lamb, and guinea pig enterotoxemias (2,10). An immunologically related iota-like toxin, produced by *Clostridium spiroforme*,

causes an acute and fatal enterotoxemia of rabbits (1,3,4,6). Antiserum developed against toxic supernatants of either species, neutralizes the iota toxin effects of the other (22). Crossed immunoelectrophoresis (crossed IEP) of crude C. perfringens type E supernatant using C. spiroforme antiserum results in two immunoprecipitin arcs which have been designated i_a and i_b. Strains of C. perfringens types A, B, C, or D, which do not produce iota toxin, do not produce either i_a or i_b (22).

We have previously shown that i_a and i_b of C. perfringens iota toxin can be separated by preparative isoelectric focusing (22). These two unlinked proteins which show no immunological relatedness by crossed IEP also differ in isoelectric points. Little activity is present in isoelectric focusing fractions containing either individual component; however, when combined there is a synergistic effect in biological assays. Our initial findings suggest that iota toxin is a binary toxin dependent on two nonlinked proteins for maximum activity.

In the following investigation, we describe the purification of i_a and i_b to homogeneity and provide further evidence that both are necessary for iota toxin activity.

MATERIALS AND METHODS

Bacterial strains, toxin, and antiserum production: A description of toxin and antiserum production has been previously presented using the type strain of C. perfringens type E (NCIB 10748) and C. spiroforme (NCTC 11493) (22). Briefly, C. perfringens supernatant was produced

in 2 l brain heart infusion dialysis flasks after 72 h of growth. A saturated solution of ammonium sulfate (4° C) was added to continuously stirred culture filtrate up to 70 % saturation. Precipitate was collected 4 h later by centrifugation (7000 x g for 20 min). The precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.5 and dialysed in the same buffer to remove residual ammonium sulfate.

Antiserum was produced in neutered, six month old alpine goats using cells and culture fluid from dialysis sack cultures. C. spiroforme was grown in chopped meat (0.5 % glucose) at 37° C for 96 h. Sack contents were diluted with normal saline to an optical density reading of 1 at 660 nm. C. perfringens type E was grown as described above and cells diluted with culture supernatant fluid to an optical density reading of 1 at 660 nm. Cell suspensions were treated overnight at 37° C with 0.4 % formalin (final concentration). The first six weekly vaccinations, which consisted of 1 ml of formalinized cell suspension and 1 ml of Freund's incomplete adjuvant (Sigma Chemical Co.), were injected subcutaneously. Subsequent weekly injections consisted of 1 ml of unformalinized whole culture mixed with an equal volume of Freund's incomplete adjuvant.

Immunoelectrophoresis (IEP): Fused rocket IEP and crossed IEP were done as previously described (22). Glass plates (100 x 100 mm) were used for quantitative rocket immunoelectrophoresis (quantitative rocket IEP). Plates were coated with 15 ml of 1.2 % low EEO agarose (Sigma) containing C. spiroforme antiserum (3.3 ul/cm^2). Antigen samples (15 ul) were subjected to electrophoresis at constant voltage

(30 volts) for 12 h. The plates were washed, pressed, stained, and destained as previously described (22).

DEAE Sepharose CL-6B ion exchange chromatography: Ammonium sulfate concentrated C. perfringens supernatant (800 mg in 80 ml) was applied to a column (1.5 x 13 cm) containing 12 ml of DEAE Sepharose CL-6B equilibrated with 15 bed volumes of 10 mM Tris-HCl buffer, pH 7.5. After the concentrate was applied, the gel was washed with 15 bed volumes of buffer. The i_a and i_b proteins were eluted using a 0.0-0.2 M NaCl linear gradient (140 ml total), in 10 mM Tris-HCl buffer, pH 7.5. Fractions were collected at a constant flow rate of 10 $ml.cm^{-2}.h^{-1}$. Both i_a and i_b were detected using fused rocket IEP and C. spiroforme antiserum as previously described. Fractions containing the peaks of i_a and i_b were combined, dialysed (12,000-14,000 molecular weight cutoff) overnight against 5 mM Tris-HCl buffer, pH 7.5, and concentrated to 2 ml using Aquacide II (Calbiochem).

Preparative Isoelectric Focusing: Preparative isoelectric focusing was performed in Sephadex G-75-40 (Sephadex IEF, Sigma Chemical Co.) on an LKB 2117 Multiphor using the flatbed isoelectric focusing kit (LKB, Application Note 198). We prepared a 4 % (w/v) gel slurry (100 ml) containing 2 % LKB ampholines (pH 4-6). A 2 ml sample containing i_a and i_b, partially purified by ion exchange chromatography, was applied in a trough 12 cm from the cathode and focused at constant power (8W) for 14 h at 4 ^{o} C. The bed was fractionated with a metal grid and each fraction mixed in 10 ml of 50 mM Tris-HCl

buffer, pH 7.5. Fractions containing the peaks of i_a and i_b were pooled and concentrated using Aquacide II.

Sephadex G-100 Gel Filtration: Samples of i_a and i_b partially purified by isoelectric focusing (1.5 ml) were applied separately to a column (2.5 x 100 cm) equilibrated in 50 mM Tris-HCl, pH 7.5. Dextrose (10 % w/v) was added to each sample to increase viscosity. Protein was eluted from the column at a flow rate of 3.7 $ml.cm^{-2}.h^{-1}$. Fractions were collected and assayed for i_a and i_b by fused rocket IEP, and the peaks of i_a and i_b were pooled and concentrated.

Flatbed Electrophoresis: Flatbed electrophoresis was performed in Sephadex G-75-40 using 25 mM Tris-Tricine buffer, pH 8.6, similar to the procedure outlined in LKB Application Note 198. A 4 % w/v gel slurry (100 ml buffer) was dried at room temperature and allowed to chill at 4° C for 5 h before sample application. The sample was applied to the bed in a trough 0.5 cm from the cathode and electrophoresed for 12 h at a constant power of 16 W. The bed was fractionated into 30 fractions using a slotted metal grid and mixed with 10 ml of 50 mM Tris-HCl buffer, pH 7.5. Fractions containing i_b were detected using fused rocket IEP. The i_a protein did not require flatbed electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE): PAGE was done in linear 4 -30 % gradient gels (Isolab Inc., Akron, Ohio) in 90 mM Tris-HCl/80 mM boric acid buffer, pH 8.3 at 150 volts for 20 h (3000 volt-h). Gels were stained overnight with 0.025 % Coomassie R-250 in methanol/

glacial acetic acid/water (5:1:4). Destaining consisted of repeated changes of a methanol/glacial acetic acid/water (7:5:88) mixture. Sodium dodecyl sulfate (SDS) gels (10 %) with a 3 % stacking gel were made and samples prepared in final concentrations of 2 % SDS, 5 % 2-mercaptoethanol, and 10 % glycerol as previously described (8). Samples were heated in boiling water for 2 min before being applied to the gel. We ran 10 ug of each molecular weight marker (Bio-Rad Laboratories, Richmond, California) at constant voltage (60 v) until the tracker dye was 1 cm from the bottom of the gel. Gels were stained and destained as described above.

Estimation of molecular weight by Sephadex G-100 Gel Filtration: A column (1.5 x 95 cm) containing 120 ml of Sephadex G-100 was equilibrated in 50 mM Tris-HCl buffer, pH 7.5. The void volume was determined by Blue Dextran 2000 (Pharmacia, Piscataway, N.J.) exclusion using a constant flow rate of 7.7 $ml.cm^{-2}.h^{-1}$. All samples applied to the column were mixed with dextrose (10 % w/v). Low molecular weight protein standards (Pharmacia, Piscataway, N.J.) were used at 5 mg/ml concentrations and chromatographed individually (1 ml) on the column. Protein peaks were detected using 280 nm absorbance. C. perfringens supernatant concentrate (1 ml) was applied to the column and fractions assayed for i_a and i_b by fused rocket IEP using C. spiroforme antiserum.

Biological Assays: Mouse lethal and guinea pig dermonecrotic assays were done as previously described (22).

Heat Stability of i_a and i_b: Separate aliquots (300 ul) of i_a (0.8 ug/ml) or i_b (1.2 ug/ml) in 50 mM Tris-HCl buffer, pH 7.5 were heated (15 min) in stoppered glass tubes using a circulating water bath at one temperature setting. A temperature range of 40° C to 100° C with 5 degree increments was used for the experiment. Heated components were allowed to cool to room temperature and 300 ul of the unheated complementary component added. The mixtures were tested for dermonecrotic activity as previously described (22). Positive controls (300 ul of a 1:1 mixture of each unheated component), and negative controls (300 ul of a 1:1 mixture of either component mixed with 300 ul of buffer) were included.

Protein Estimation: Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as the standard.

RESULTS

Purification of iota a (i_a) and iota b (i_b): The i_a and i_b proteins were concentrated from culture filtrate by ammonium sulfate precipitation. The i_a component precipitated at 70 % saturation and i_b precipitated at 55 % saturation. Concentrated filtrate was applied to an ion exchange column; i_a eluted with 0.05-0.07 M NaCl and i_b with 0.13-0.16 M NaCl. After ion exchange chromatography, fractions containing i_a and i_b were pooled, concentrated, and applied to a preparative isoelectric focusing bed. The i_a and i_b proteins differ

in isoelectric points (5.2 and 4.2, respectively). The i_a protein was purified to homogeneity by a final gel filtration step on Sephadex G-100; however, i_b required an additional step, flatbed electrophoresis, before it was homogeneous.

Both proteins were homogeneous by three different techniques: Gradient PAGE (Fig. 1), SDS PAGE (Fig. 2) and crossed IEP (Fig. 3). In crossed IEP, either purified component gave one immunoprecipitin arc using C. perfringens type E antiserum which yielded about 70 immunoprecipitin arcs with starting material. By using purified i_a and i_b with C. spiroforme antiserum in quantitative rocket IEP, we were able to generate linear standard curves for both components. Linearity was present upto 0.3 ug of i_a and 1.2 ug of i_b. Table 1 shows the percent recovery of i_a and i_b after each purification step, as determined by quantitative rocket IEP. Of the total protein in the supernatant concentrate, i_a and i_b represent 0.3 and 0.8 percent, respectively.

Molecular Weight Estimations: The estimated molecular weights of i_a, determined by SDS PAGE and gel filtration, are 47,500 and 48,000, respectively (Figs. 4 and 5). Estimated molecular weights of i_b using the same two methods were 71,500 and 67,000, respectively. SDS PAGE results showed that neither i_a or i_b dissociated into subunits but instead migrate as a single protein staining band (Fig. 2).

Biological Activity: The synergistic effect of i_a and i_b was shown using mouse lethality and guinea pig dermonecrotic assays. Results from these studies are shown in Tables 2 and 3. With equal molar

Fig. 1. 4 - 30 % Gradient polyacrylamide gel electrophoresis of purified iota a and iota b. Lanes contain the following: 1, supernatant concentrate (250 ug); 2, purified iota a (15 ug); 3, purified iota b (15 ug).

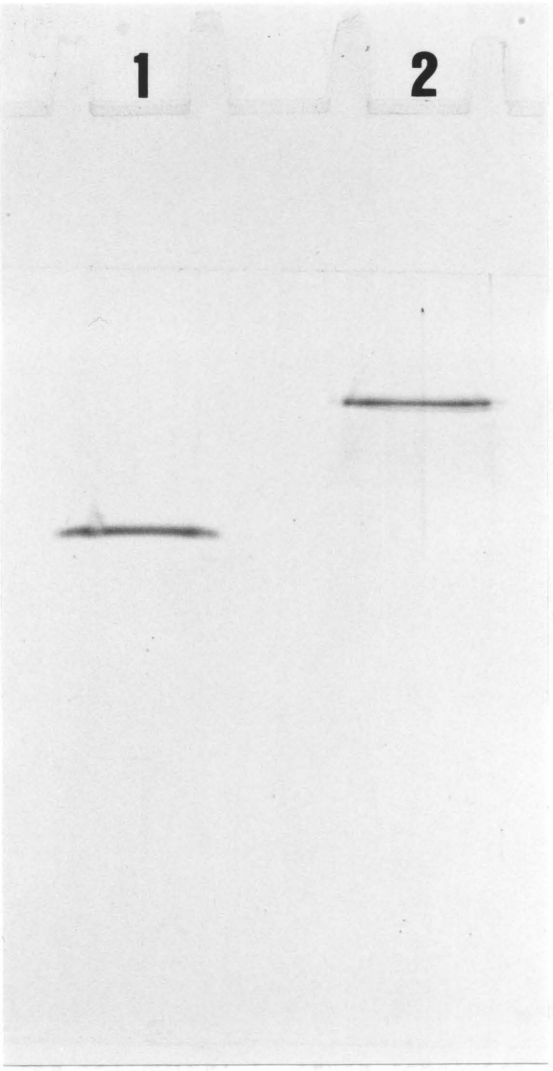

Fig. 2. Analysis of iota a and iota b by SDS PAGE. Lane 1 contains 25 ug of purified iota a and lane 2 contains 25 ug of purified iota b.

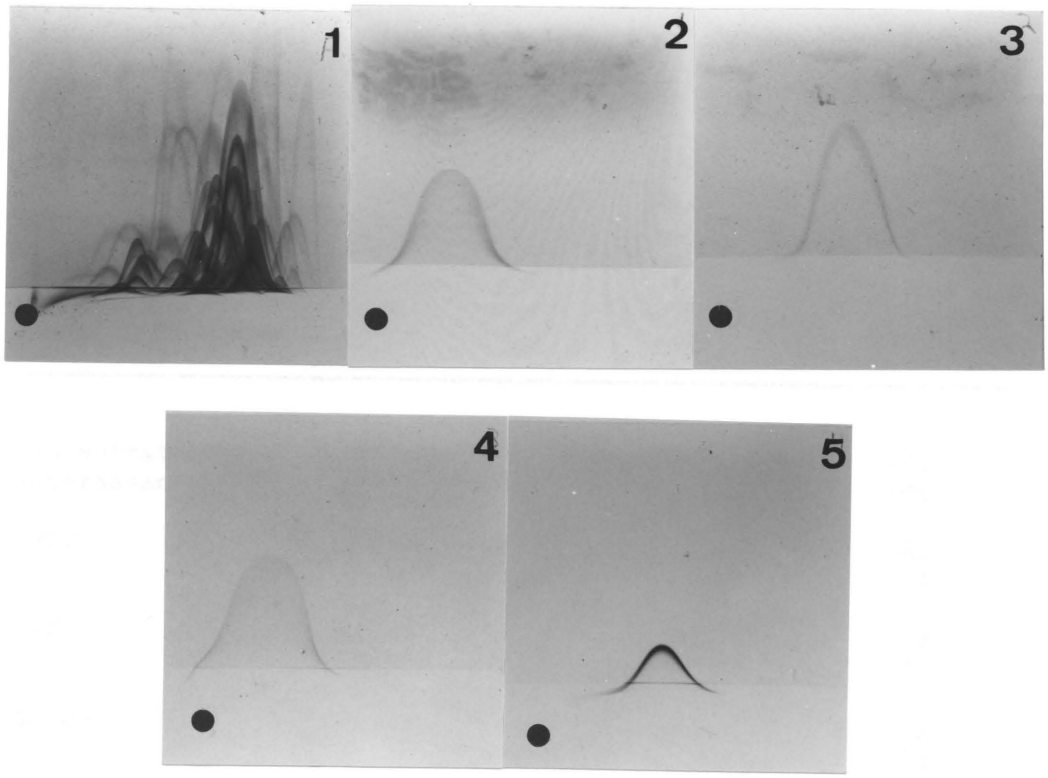

Fig. 3. Crossed immunoelectrophoresis of purified iota a and iota b. Plates contain the following: 1, crude concentrate (50 ug) + 5.0 ul/cm^2 of C. perfringens type E antiserum (CptE); 2, pure iota a (1.0 ug) + 7.5 ul/cm^2 CptE; 3, pure iota b (1.0 ug) + 7.5 ul/cm^2 CptE; 4, pure iota a (1.0 ug) + 3.0 ul/cm^2 of C. spiroforme antiserum (Cs); 5, pure iota b (1.0 ug) + 3.0 ul/cm^2 Cs.

Table 1. Purification of C. perfringens iota a and iota b

Purification Step	Component (mg)	Volume (ml)	Total Protein (mg)	% Recovery[a]
Concentrated Supernatant	ia (2.60)	80.0	800.00	100
	i_b (6.60)	80.0	800.00	100
DEAE	i_a (2.30)	22.5	16.80	88
	i_b (4.90)	27.0	92.80	74
IEF	i_a (0.95)	33.0	1.60	37
	i_b (2.30)	24.0	3.50	35
G-100	i_a (0.32)	18.0	0.32	12
	i_b (1.30)	22.5	1.80	20
Flatbed Electrophoresis[b]	i_b (0.33)	26.0	0.33	5

[a] Percent protein recovered from starting material found in supernatant concentrate.

[b] Flatbed electrophoresis not needed for i_a.

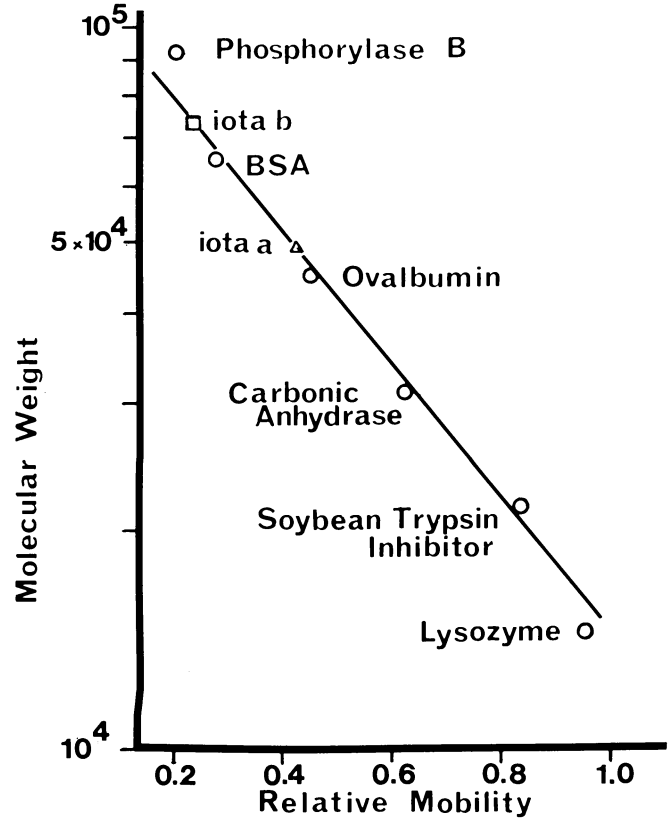

Fig. 4. Estimation of iota a and iota b molecular weights by SDS PAGE. Standards are phosphorylase B (M_r=92,500), bovine serum albumin (BSA) (M_r=66,200), ovalbumin (M_r=45,000), carbonic anhydrase (M_r=31,000), and soybean trypsin inhibitor (M_r=21,500).

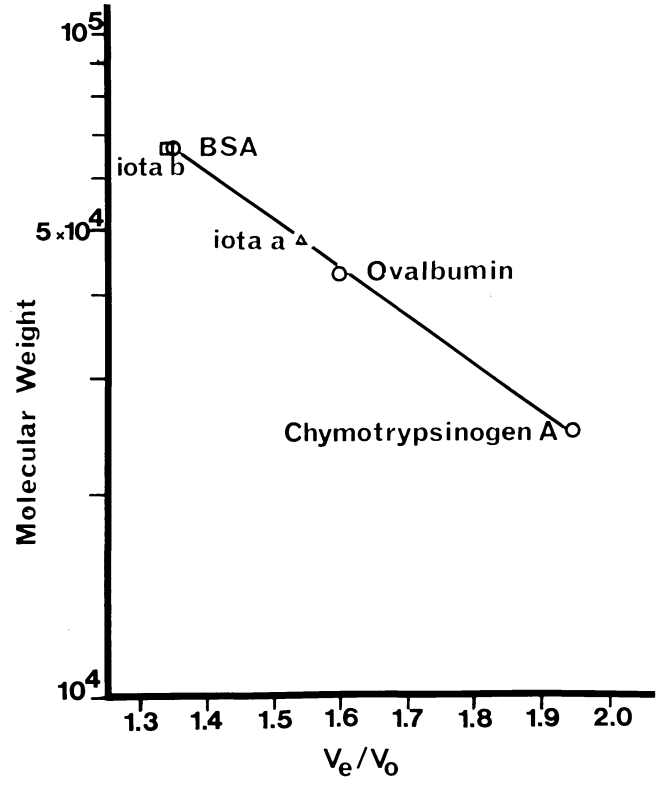

Fig. 5. Estimation of iota a and iota b molecular weights by Sephadex G-100 gel filtration. Standards are BSA (M_r=67,000), ovalbumin (M_r=43,000), and chymotrypsinogen A (M_r=25,000).

Table 2. Mouse lethal effects of iota a and iota b

TOXIN INJECTED			LETHAL RESPONSE
IOTA A (ug)	IOTA B (ug)	nMOLE/COMPONENT[a]	# DEAD/# MICE
5.0	0.0	0.1	0/4
0.0	7.5	0.1	0/4
5.0	7.5	0.1	4/4
2.5	3.75	0.05	4/4
1.25	1.88	0.025	4/4
0.62	0.94	0.012	2/4
0.31	0.47	0.006	0/4

[a] Molar amounts based on SDS PAGE results.

Table 3. Guinea pig dermonecrotic effects of iota a and iota b

Component(s)	picomoles[a]	Protein[b] (ng)	Dermonecrosis/ Lesion size(mm)[c]
iota a	63	3000	-/0
iota b	40	2800	-/0
iota a+iota b	2.5	120+180	+/10
iota a+iota b	1.25	60+90	+/6
iota a+iota b	0.62	30+45	+/6
iota a+iota b	0.31	15+22	-/0
iota a +	2.8	133	+/10
iota b	1.4	100	
iota a +	2.8	133	+/10
iota b	0.7	50	
iota a +	2.8	133	-/0
iota b	0.35	25	
iota b +	2.8	200	+/9
iota a	1.4	67	
iota b +	2.8	200	+/5
iota a	0.088	4	
iota b +	2.8	200	-/0
iota a	0.044	2	

a Molarity based on SDS PAGE results.

b Protein amount injected into the animal. Rows with two protein amounts represent iota a and iota b, respectively.

c Readings taken 48 h after the injection.

amounts of i_a and i_b (molecular weight estimates based on SDS PAGE results), the dermonecrotic assay was 20 fold more sensitive than the mouse lethal assay. When i_a and i_b were combined in equal molar amounts, there was at least a 4 and 64 fold increase in biological activity over individual components in the mouse lethal and guinea pig dermonecrotic assays, respectively. When one component was kept at a constant amount and the other diluted, we found that i_a could be diluted 8 fold more than i_b and still yield a dermonecrotic lesion.

Thermal Stability of i_a and i_b: Biological activity of the heated individual components showed that i_a retained the same dermonecrotic activity at 85° C (based on lesion diameter), as unheated i_a when combined with unheated i_b. A mottled pattern of hemorrhage, but no dermonecrosis, was detected after i_a was heated at 90° C or 95° C. No skin reaction was seen after boiling the i_a component. The i_b component retained full activity up to 45° C, when combined with unheated i_a, with slight hemorrhaging at 50° C and complete loss of activity at 55° C.

DISCUSSION

The biological activity of C. perfringens iota toxin results from the synergistic activity of two proteins, i_a and i_b. The proteins differ in molecular weight, isoelectric point, heat stability, and do not share common immunodeterminants (22). Each component of iota toxin appears to be a single polypeptide as determined by SDS PAGE. Amino terminus sequencing has been attempted but both molecules are

blocked by unknown functional groups (Stiles and Wilkins, unpublished data).

The synergy of i_a and i_b suggests that we are working with a binary toxin dependent on two different, nonlinked proteins for biological activity. Other bacterial binary toxins include Staphylococcus aureus leucocidin (12,13,24,25), Bacillus anthracis anthrax toxin (9), Bacillus cereus enterotoxin (23), and the C_2 enterotoxin of C. botulinum (7). C_2 toxin has biological activities similar to iota toxin, such as increased vascular permeability in guinea pigs, lethality in mice, and enterotoxic properties (2,5,14, 15,16,17,18,20). Component II of C_2 toxin acts as a binding subunit and component I possesses ADP-ribosylating activity specific for poly-L-arginine (18,21). Interestingly, i_a of iota toxin also ADP-ribosylates poly-L-arginine (21a).

A recent report shows that C_2 toxin ADP-ribosylates nonmuscle actin, a cytoskeleton component found in target cells (19). This novel acceptor for ADP-ribose differs from other ADP-ribosylating toxins like diphtheria and Pseudomonas exotoxin A which modify eukaryotic elongation factor 2 or cholera and E. coli heat labile toxin which modify the adenylate cyclase complex. We have not yet determined the physiological acceptor of ADP-ribose from iota toxin.

The biological activities of iota or C_2 toxins are reportedly dependent on proteolytic activation (14,18,20). Work done by Ohishi has shown that component II of C_2 binds to brush border preparations irregardless of trypsinization yet the enzymatic component I will not bind component II unless the later has been treated with trypsin (18).

We have been unable to show trypsin activation of our iota toxin preparations (22).

Thermal stability studies of i_a and i_b showed that i_a is heat stable. Heated i_a (85° C for 15 min), when combined with unheated i_b, gave a dermonecrotic lesion of the same size as unheated i_a and i_b. Previous investigators have studied the thermal stability of partially purified preparations of iota toxin and found that vascular permeability in guinea pigs decreases at 48° C and is abolished at 53° C (5). Our results indicate that i_b was inactivated in this temperature range, yet i_a remained active.

With the evidence presented in this paper, we believe that C. perfringens iota toxin is a binary toxin, dependent on two non-linked proteins for activity. The i_a component possesses in vitro ADP-ribosylating properties yet a physiological acceptor or its role in the biological activity of the toxin has not been discovered. With further work, we hope to determine how i_a and i_b interact to form biologically active iota toxin.

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Section V. Molecular Basis for the Pathological Actions of *Clostridium perfringens* Iota Toxin

ABSTRACT

Clostridium perfringens type E iota toxin is composed of two separate and independent polypeptide chains that act synergistically in mouse lethal assays. The light chain is an enzyme that mono-ADP-ribosylates certain amino acids. The enzyme displays substantial activity when homopoly-L-arginine is used as a substrate, but it shows little activity when poly-asparagine, poly-lysine, or poly-glutamic acid are used. In keeping with the properties of an ADP-ribosylating enzyme, the toxin possesses the following characteristics. It produces incorporation of radioactivity into poly-arginine when adenine-labeled NAD is used, but radioactivity is not incorporated when nicotinamide-labeled NAD is used. Irrespective of labeling, enzymatic activity is accompanied by the release of free nicotinamide. After incorporation of ADP-ribose groups into poly-arginine, enzymatic and chemical techniques can be used to release the incorporated material. Snake venom phosphodiesterase releases mainly AMP; hydroxylamine releases AMP and ADP-ribose. The heavy chain of iota toxin has little or no enzyme activity, and it does not substantially affect the enzyme activity of the light chain. The heavy chain may be a binding component that directs the toxin to vulnerable cells. The data suggest that iota toxin is a representative of a novel class of ADP-ribosylating toxins.

INTRODUCTION

Clostridium perfringens is a gram positive, anaerobic organism that is relatively ubiquitous in its distribution (7). There are five types of Clostridium perfringens, designated types A, B, C, D and E (15). Each of these organisms produces a semi-unique spectrum of protein toxins. Clostridium perfringens type E produces at least six soluble toxins, one of which is iota toxin (7,15).

Iota toxin was first described more than four decades ago (1), but the substance was not isolated and purified to homogeneity until recently (27a). When tested in vivo, the toxin produces death in mouse lethality assays (1,23); when tested in situ, it produces dermonecrosis in guinea pigs (1). Although the full structure-function relationships of the toxin have not been determined, one aspect of the structure of the substance is important here. Iota toxin is composed of two separate polypeptide chains (27a). The individual chains possess relatively little toxicity, but the combination of chains is very potent. The data suggest the iota toxin is a true binary toxin.

Binary toxins of microbial origin are not common substances. Only a small number have thus far been reported, including the components of anthrax toxin (26), leukocidin (18), and one of the botulinum toxins (10,21). The mechanism of action of two of the binary toxins has been partially determined. One of the components of anthrax toxin is an enzyme that possesses adenylate cyclase activity (14). One of the components of botulinum binary toxin is also an enzyme, possessing ADP-ribosylating activity (Leppla, personal communication).

Iota toxin appears to share certain properties with the botulinum binary toxin, including the ability to promote movement of fluids across membranes (5,11,19,20,24). There are also the obvious similarities that both toxins are clostridial in origin and both can be released into the gut of a host that has succumbed to an opportunistic infection. These similarities suggest that the two toxins should be compared in terms of cellular and molecular actions. The present study has focused on the molecular properties of Clostridium perfringens iota toxin. Data were obtained which show that one of the components of the toxin is an enzyme with mono-ADP-ribosylating activity.

MATERIALS AND METHODS

Growth of Bacteria. The type strain of C. perfringens type E NCIB 10748 (VPI 1589) was used in this study. The organism was grown in brain heart infusion dialysis flasks as previously described (27). Each flask was inoculated with cultured organisms and incubated at 37° C for 72 h. Cells and debris were removed by centrifugation (8,000 x g; 15 min) and subsequent filtration (0.45-um-pore-size membrane; Gelman Sciences, Inc., Ann Arbor, Mich.).

Toxin Purification. The full details on isolation and purification of the toxin have been described elsewhere (27a). For the purposes of this study, two polypeptides were used that migrated as single bands in polyacrylamide gel electrophoresis (13). These two components of iota toxin have previously been referred to as i_a and

i_b, but they are referred to here as light chain and heavy chain, respectively (see Discussion).

Radioisotope Assay for ADP-Ribosylation. The heavy and light chains of iota toxin were assayed for ADP-ribosylating activity according to techniques previously reported (4,16). Substrate (10 mg/ml) was suspended in dimethylglutaric acid buffer (100 mM, pH 7.0) that contained 5 uCi of [^{3}H]NAD plus other ingredients as indicated under Results (total volume, 100 ul). The solution was incubated at 33° C for various times. When the substrate in question was homo-poly-L-arginine, the ADP-ribosylated product was precipitated by adding phosphate buffer (1 ml, 100 mM, pH 7.0). The mixture was centrifuged (4,500 x g), and the pellet was washed three times. The final pellet was dissolved in 0.5 ml of 1.0 mM HCl, followed by 1.0 ml of 100 mM dimethylglutaric acid buffer (pH 7.0). A sample of the solution was added to a scintillation cocktail, and radioactivity was quantified by liquid scintillation spectrometry. Experimental values were corrected by substracting background activity in solutions without enzyme.

When the substrate in question was a homo-poly-L-amino acid other than poly-arginine, the reaction mixture and incubation procedures were identical to those described above. At the end of experiments, [^{3}H]ADP-ribosylated homopolymer was separated from unreacted [^{3}H]NAD by fractionation on Sephadex G-25 (12 x 270 mm). The column was eluted with unbuffered saline (1 ml per minute), and fractions of eluate were measured for radioactivity as described above.

Irrespective of the substrate used or the separation procedures, each assay was performed in duplicate, and each experiment was done at least twice. Thus, each result represents the mean of at least four observations.

HPLC Assay for ADP-ribosylation. The methods used for high-performance liquid chromatography (HPLC) were basically similar to those of Brown et al. (2). The technique allowed for resolution of NAD, nicotinamide, AMP, and ADP-ribose.

The reaction conditions were the same as described above for the radioisotope assay. At the end of incubation, samples were injected into a Waters Associates HPLC system with a gradient controller. The gradient was created with 20 mM Tris hydrochloride (pH 8.0) and 50 mM Tris-hydrochloride (pH 8.0) containing 1 M NaCl. The conditions for elution of the compounds are given in Table 1. The elution times were: NAD, ~ 6 min; nicotinamide, ~ 10 min; AMP, ~ 20.5 min; and ADP-ribose, ~ 21.5 min.

Compounds were separated on an anionic-exchange column (Mono-Q; Pharmacia Fine Chemicals, Piscataway, N.J.). The A_{254} was monitored and plotted on a strip-chart recorder. Fractions were collected at 1-min intervals. Results with unlabeled compounds were quantified by measuring the areas under the curve; results with labeled compounds were quantified by liquid scintillation spectrometry.

Cleavage of ADP-Ribosylated Homo-poly-L-arginine. Two techniques were used to release incorporated products from homopolymer. In the first, ADP-ribosylated poly-arginine was incubated with snake venom

TABLE 1. Gradient formation for HPLC separations [a]

Condition[b]	Time (min)	Flow rate (ml/min)	% Buffer A[c]	% Buffer B[d]
A	0	0.5	100	0
B	9.99	0.5	100	0
C	10.00	1.0	100	0
D	20.00	1.0	90	10
E	22.00	1.0	90	10
F	25.00	1.0	0	100

[a] An extensive presentation of chromatograms can be found in reference 2.

[b] Isocratic conditions were used from A to C and from D to E. A linear gradient was used between C and D and between E and F.

[c] 20 mM Tris hydrochloride (pH 8.0)

[d] 50 mM Tris hydrochloride (pH 8.0) - 1 M NaCl.

phosphodiesterase as described under Results. The products were characterized by HPLC (details are given below). In the second, ADP-ribosylated poly-arginine was reacted with hydroxylamine by the technique of Wielckens et al. (30). The procedure was as follows. At the end of incubation, the washed and precipitated polyarginine was dissolved in 150 ul of 5 mM HCl. A sample (50 ul) of 2 M NH_2OH was added, and the solution was adjusted to pH 7.4 with concentrated NaOH. The material was heated at 56° C for 15 min, and it was maintained at 37° C for an additional 60 min. Samples of the reaction mixture were injected into the HPLC.

The hydroxylamine method of Wielckens et al. (30) releases the ADP-ribose moiety incorporated into protein, either as the ADP-ribose molecule or as AMP. The amount of radioactive ADP-ribose and AMP released from poly-arginine was determined after the homopolymer was incubated with toxin and tritiated NAD. The results of exposure to hydroxylamine were compared with those of exposure to water. The data are expressed as the percentage of radioactivity lost from poly-arginine and as the percentage of radioactivity recovered in ADP-ribose and AMP.

Animals and Toxicity Testing. A limited number of experiments were done on mice (male Swiss-Webster, 20 to 30 g). The sole purpose of the experiments was to determine whether the individual chains from the botulinum toxin and from the perfringens toxin would combine to form a hybrid binary toxin. The various components were added together and administered intravenously in a volume of 0.1 ml (0.154 M NaCl). Because of the nature of the experiment and the data being

obtained, it was deemed inappropriate to let the animals die. When mice became visibly ill, they were sacrificed painlessly with a volatile anesthetic (ethyl ether).

Reagents. Unlabeled NAD and NADase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tritiated NAD labeled in the adenine moiety (nicotinamide [4-^{3}H]adenine dinucleotide; 30 Ci/mmole) was obtained from ICN Biomedicals, Inc. (Irvine, CA). Tritiated material labeled in the nicotinamide moiety ([2,4-^{3}H]nicotinamide adenine dinucleotide; 3.2 Ci/mmol was obtained from Amersham Corp. (Arlington Heights, Ill.). Homo-poly-L-amino acids (arginine, asparagine, glutamic acid and lysine) and snake venom phosphodiesterase were purchased from Sigma Chemical Co. (St. Louis, MO). The heavy and light chains of botulinum toxin were generously provided by Dr. I. Ohishi (University of Osaka Prefecture, Osaka, Japan).

RESULTS

Enzyme activity. The light and heavy chains of iota toxin were tested for ADP-ribosylating activity. In the initial experiments, toxin was mixed with homo-poly-L-arginine and adenine-labeled NAD. The light chain of iota toxin produced time- and concentration-dependent incorporation of radioactivity into the protein fraction (Fig. 1). This result was obtained at toxin concentrations of 10^{-9} to 10^{-7} M, and at elapsed times of 1 to 4 h. When the heavy chain was tested at 10^{-9} M for an elapsed time of 1 h, it possessed only 0.7 % of the activity of the light chain.

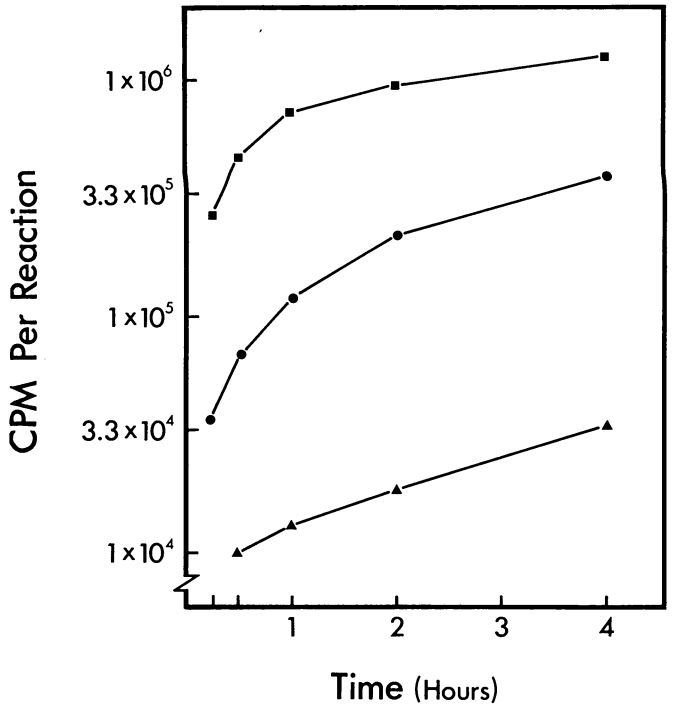

FIG. 1. Counts per minute of radioactivity incorporated into homopoly-L-arginine. The reaction was carried out as described in Materials and Methods. Three concentrations of the light chain of iota toxin were tested (▲ , 10^{-9} M; ● , 10^{-8}; ■ , 10^{-7}).

Various mixtures of heavy and light chains were tested for enzyme activity (ratios tested were 4:1, 2:1, 1:1, 0.5:1, 0.25:1). Irrespective of the ratio tested, there was no substantial effect of the heavy chain on light chain activity. Enzyme activity was equal to, or only slightly greater than (15-20 %), that which could have been accounted for on the basis of additivity. The data indicate that interaction between the two chains in terms of toxicity (see below) cannot be explained in terms of synergistic enzyme activity.

Additional experiments were done to determine whether poly-amino acids subject to mono-ADP-ribosylation (i.e., asparagine and lysine; [29]) or poly-ADP-ribosylation (i.e., glutamic acid and lysine; [28]) were substrates for the light chain of iota toxin. Unlike poly-arginine, these other poly-amino acids were not effective as substrates for the toxin (Table 2).

The experiments just described suggest that the light chain of iota toxin is an ADP-ribosylating enzyme. An additional series of experiments was done to confirm the nature of the enzymatic activity. This work involved the use tritiated NAD labeled in the nicotinamide group, unlabeled NAD, and NADase.

In the presence of nicotinamide-labeled NAD, the light chain of iota toxin (10^{-9} to 10^{-7} M) failed to produce significant incorporation of radioactivity into poly-arginine. Furthermore, when a 50-fold molar excess of the nicotinamide-labeled NAD was used in the presence of adenine-labeled NAD, there was a predictable decrease (>90 %) in the amount of radioactivity incorporated into poly-arginine. In the latter experiment, the nicotinamide-labeled material presumably acted as a competitive substrate and displaced the adenine-labeled material.

Table 2. Homo-poly-L-amino acids as substrates for iota toxin[a]

	CPM with:			
Chain	Arginine	Asparagine	Glutamic acid	Lysine
Heavy	<1000	<1000	<1000	<1000
Light	92,764	<5000	<5000	<5000

[a] The reaction (1h, 33° C) was carried out in the presence of 10^{-8} M heavy or light chain, 5 uCi of adenine-labeled NAD, and 100 ug of poly-amino acid in a volume of 100 ul. The results represent the ADP-ribosylated protein collected after chromatography on Sephadex G-25.

In the next series of experiments, the light chain of iota toxin (10^{-9} M) was incubated with a large molar excess of unlabeled NAD (10^{-4} M) and with poly-arginine. At the end of various incubation times, the reaction mixture was injected into the HPLC. The results showed that the toxin produced time-dependent loss of NAD and time-dependent appearance of nicotinamide. For example, when the mixture was incubated for 24 h, approximately 82 % of the NAD was lost. Within the limits of resolution of the HPLC, there appeared to be nearly a stoichiometric appearance of nicotinamide. In keeping with the expected outcome, there was virtually no appearance of free ADP-ribose.

In the final experiments, NADase (0.01 U per reaction tube) was incubated (1 to 4 h, 35° C) with adenine-labeled NAD before enzyme assay. At the end of the assay, aliquots of the reaction mixture were injected into the HPLC. The identity of labeled compounds (poly-arginine, NAD, nicotinamide) was determined on the basis of coelution with larger amounts of unlabeled compounds (see Materials and Methods) (Table 1).

NADase expressed time-dependent glycohydrolase activity, and this was associated with a decrease in the subsequent incorporation of radioactivity into poly-arginine. For example, incubation of NADase with NAD for 4 h produced almost complete loss of NAD and a simultaneous appearance of its products (i.e., nicotinamide). In the subsequent ADP-ribosylation assay, the loss of NAD was associated with a marked reduction (>90 %) in the amount of radioactivity found in poly-arginine.

Phosphodiesterase experiments. Poly-arginine (100 ug) was incubated (4 h, 33° C) with adenine-labeled NAD (5 uCi) and the light chain of iota toxin (10^{-8} M). At the end of incubation, the poly-amino acid was precipitated and washed as described under Materials and Methods. The pellet was resuspended and divided into two aliquots. Half of the material was incubated (33° C) for an additional 4 h without modification; the balance of the material was similarly incubated, but in the presence of snake venom phosphodiesterase (100 ug). Both solutions were injected into the HPLC.

The reaction mixture that had not been exposed to phosphodiesterase had one major peak, which was radioactive polyarginine. There was a small peak that represented unreacted [^{3}H]NAD. The reaction mixture that was exposed to phosphodiesterase lost most (~ 80%) of its radioactive poly-arginine. Radioactivity was recovered predominantly, but not exclusively, in AMP. There were other radioactive products that were not characterized (see Discussion).

Hydroxylamine Experiments. ADP-ribosylation assays were performed as described above, and the individual reaction tubes were treated with water (control) or hydroxylamine (experimental) as described under Materials and Methods.

When reacted poly-arginine was exposed to hydroxylamine, the presence or absence of cleavage products varied with treatment conditions. When incubation was at 37° C, there was almost no difference between control and experimental preparations. However, when the reaction tubes were warmed to 56° C for 15 min and then

incubated at 37° C, the incorporated radioactivity was significantly hydroxylamine sensitive. For example, after 1 h of incubation the radioactivity in experimental tubes was approximately 40 % less than that in the control tubes, and after 4 h the difference was approximately 60 %. Virtually all the hydroxylamine-sensitive material was recovered as ADP-ribose and AMP.

Nicotinamide experiments. Nicotinamide produced concentration-dependent inhibition of the ADP-ribosylation reaction (Figure 2). When the enzyme concentration was 10^{-9} M and when incubation was for 1 h, nicotinamide (0.1 M) diminished by approximately 90 % the amount of radioactivity incorporated into protein. The inhibition was sustained over time.

Binary toxin experiments. Mice (n = 5) were injected with 2.0 ug of the light chain of iota toxin, either alone or in combination with the heavy chain of iota toxin or the heavy chain of botulinum toxin (each at 2.0 ug). The light chain by itself did not produce symptoms of illness within 250 min. When the light chain was combined with heavy chain of iota toxin, onset of symptoms (respiratory distress) occurred within 100 minutes. By contrast, when the light chain of iota toxin was combined with the heavy chain of botulinum toxin, no symptoms developed within 20 hours. The data indicate that the components of iota toxin act like a binary toxin, but heterologous components of the C. perfringens and botulinum toxins do not interact to form a hybrid binary toxin.

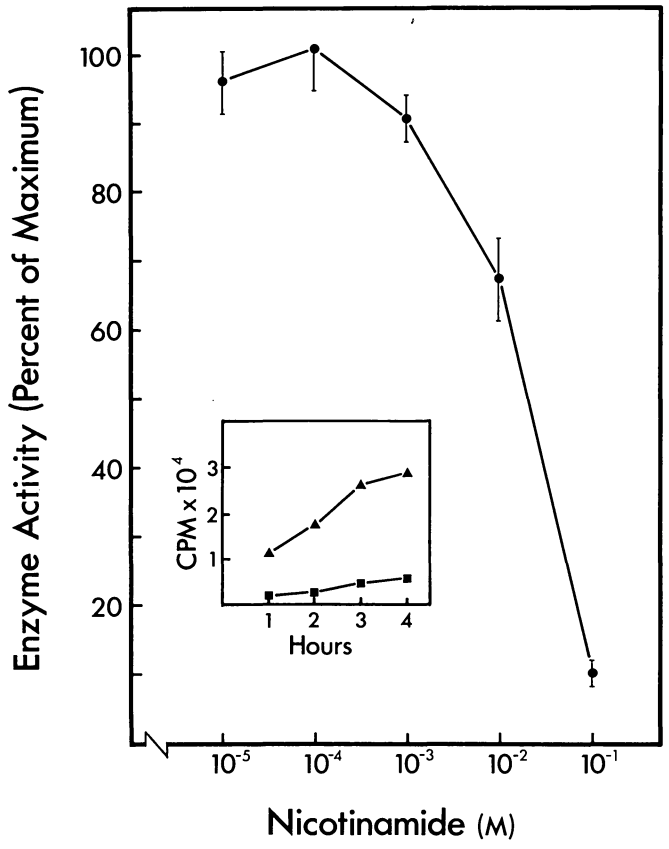

FIG. 2. Effect of various concentrations of nicotinamide on enzyme activity. The reaction (1 h, 33° C) was carried out in the presence of 10^{-9} M light chain, 5 uCi of adenine-labeled NAD, 100 ug of polyarginine and the indicated concentrations of nicotinamide in 100 ul. The inset represents the data from experiments in which incubation time was varied. Nicotinamide was not added to control tubes (▲), but it was added to experimental tubes (■, 10^{-1} M).

DISCUSSION

Two major classes of ADP-ribosylating toxins have been described in the literature. Diphtheria toxin (8) and Pseudomonas aeruginosa exotoxin (9) act on elongation factor 2 in eucaryotic cells. By virtue of catalytically modifying the translocase, they inhibit protein synthesis and ultimately cause cell death. Cholera toxin (3,6), E. coli enterotoxin (17) and pertussis toxin (12) act on regulatory proteins that govern adenylate cyclase activity. By modifying nucleotide-binding proteins, they disrupt the ability of eucaryotic cells to control cytoplasmic levels of cyclic AMP.

More recently, one of the botulinum toxins (type C_2) has been found to possess ADP-ribosylating activity (S. Leppla, personal communication). This toxin was at one time thought to be part of a homologous series of neurotoxins, but it is now known to be a unique substance. One of its distinctive characteristics is that it is a binary toxin. Although the botulinum binary toxin possesses ADP-ribosylating activity, it appears to be different from other ADP-ribosylating toxins. Unlike diphtheria toxin and Pseudomonas aeruginosa exotoxin, it does not typically cause cell death. The botulinum toxin superficially mimics cholera toxin and related substances, because it does promote the movement of fluid across membranes (11,19,20.25), but an action of botulinum toxin on nucleotide-binding proteins has not been reported.

There is evidence for the existence of at least one other toxin that may be similar to the botulinum binary toxin. C. perfringens

produces an iota toxin that is composed of two independent polypeptide chains. The individual chains have relatively little activity, but the combination of chains is highly active. In addition, some of the systemic actions of iota toxin are the same as those of the botulinum binary toxin. These findings suggest that the underlying structure-function relationships of the two toxins are the same.

In the case of botulinum binary toxin, certain structure-function relationships have already been determined. The heavy chain mediates cell surface binding (22,24) and the light chain is an enzyme with ADP-ribosylating activity (25). The present study was done to determine whether the structure-function relationships of the C. perfringens binary toxin mimic those of the botulinum binary toxin.

The light chain of iota toxin was found to possess ADP-ribosylating activity. When tested in a simple assay system that contained only adenine-labeled NAD and a substrate, the light chain caused radioactivity to accumulate in the homopolymer fraction. As predicted with an ADP-ribosylation reaction, nicotinamide was an effective antagonist.

A number of experiments were done to confirm that ADP-ribosylation was involved. The results can be summarized as follows. In the presence of unlabeled NAD and poly-arginine, iota toxin produced disappearance of the nucleotide and a concomitant appearance of free nicotinamide. The toxin did not produce free ADP-ribose. This is the expected outcome, because ADP-ribose moieties should be incorporated into poly-arginine. The results with NAD supported those with unlabeled material. The toxin produced incorporation of radioactivity

into poly-arginine when adenine-labeled NAD was present but not when nicotinamide-labeled material was used.

When adenine-labeled NAD was used as a source for the radioactivity incorporated into polyarginine, both enzymatic and chemical means could be used to release the incorporated substance. Phosphodiesterase releases AMP from mono-ADP-ribosylated protein (28). When the enzyme was added to poly-arginine that had been exposed to iota toxin and adenine-labeled NAD, it released labeled AMP. There was labeled material in addition to AMP, but this was almost certainly due to the fact that the snake venom enzyme was not homogeneous. It probably contained trace enzymes other than phosphodiesterase. Therefore, a companion experiment was done with hydroxylamine, which releases ADP-ribose and AMP from mono-ADP-ribosylated protein (30). In this case, virtually all the radioactivity that was cleaved from poly-arginine was recovered as ADP-ribose and AMP.

The cleavage experiments suggest that mono- rather than poly-ADP-ribosylation was involved. The products of the cleavage experiments indicate this, as do two other observations. First, microbial toxins with enzyme activity (e.g., diphtheria toxin, cholera toxin) usually transfer only one ADP-ribose group per substrate group (29). Second, arginine is typically a substrate for mono- rather than poly-ADP-ribosylation reactions (29). These several clues suggest that the toxin is responsible for mono incorporation of ADP-ribose groups.

The discovery that the light chain of iota toxin has ADP-ribosylating activity is interesting, but nevertheless there are substantive questions that remain to be answered. In keeping with the structure-function relationships of other ADP-ribosylating toxins and

of the botulinum binary toxin in particular, one would expect that the heavy chain of iota toxin plays a role in cell surface binding. The data reported in this study make it clear that the heavy chain possesses little if any enzyme activity and that this chain does not substantially enhance the enzyme activity of the light chain. The role of the heavy chain is more probably that of tissue targeting the binary toxin, but further work is needed to explore this possibility.

It would be desirable to determine the intracellular substrate for iota toxin. There is the possibility that the botulinum binary toxin and the iota toxin act on the same intracellular proteins. If this were true, it would suggest that the toxins are the first known representatives of a novel class of ADP-ribosylation enzymes.

ADDENDUM IN PROOF

During the preparation of this manuscript, two studies were published showing that the C. botulinum C_2 binary toxin acts on eucaryotic actin as substrate (K. Aktories, M. Barmann, I. Ohishi, S. Tsuyama, K. H. Jacobs, and E. Habermann, Nature [London] 322: 390 - 392, 1986; I. Ohishi and S. Tsuyama, Biochem. Biophys. Res. Commun. 136: 802-806, 1986). We (L.L.S. and H.H.Z.) have confirmed their findings and have found that the C. perfringens iota toxin also ADP-ribosylates actin.

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SECTION VI. SUMMARY AND DISCUSSION

Before our laboratory began to work on iota toxin in 1983, much was known about its biological properties but very little about its physical characteristics (3,6,22). The toxin kills mice, causes dermonecrosis in guinea pigs, and is produced as a protoxin requiring proteolytic activation. Effects of pH and temperature on the biological activity of iota toxin have also been studied (6). Our temperature studies using purified i_a and i_b agree with the results of Craig and Miles (6,26). They found complete loss of biological activity after heating partially purified iota toxin at 53° C for 15 min. In our studies, pure i_b was inactivated at 55° C/15 min but i_a remained active even after heating at 85° C/15 min. These results suggest that Craig and Miles inactivated i_b, yet i_a remained active. Since iota toxin depends on both proteins for activity, inactivation of one component would result in loss of biological activity.

No one has previously described the two different proteins necessary for biological activity of iota toxin or the iota-like toxin of C. spiroforme (6,7,12,14). We found that both toxins require two biochemically and immunologically different proteins, unlinked in solution, for biological activity (4,25,26). Other bacterial toxins depending on two or more unlinked proteins for activity include the gamma hemolysin and leukocydin of S. aureus, B. cereus enterotoxin, B. anthracis anthrax toxin, and C. botulinum C_2 toxin.

ENZYMATIC PROPERTIES OF IOTA A

An A-B model toxin consists of an enzymatic (A) subunit and binding (B) subunit(s) linked by covalent (disulfide linkages) or noncovalent bonds. Cholera toxin is the classic A-B model toxin. The cholera A subunit enters a target cell after the B subunits bind to the cell surface receptor, G_{m1} ganglioside (9). After entry, the A subunit mono-ADP-ribosylates the adenylate cyclase complex. The modified complex increases levels of intracellular cyclic AMP and loss of ions and fluid from the cell.

Binary toxins, like botulinum C_2 toxin, differ from cholera toxin because the components are not linked in solution (11,19). More experiments must be done before the synergy of i_a and i_b of iota toxin is understood. Through collaborative work with Dr. L. L. Simpson's laboratory, we know that i_a is an ADP-ribosyltransferase and i_b has very little ADP-ribosylating activity (0.7 % of the activity of i_a) (24). The small amount of ADP-ribosylating activity found in the i_b preparation may be contaminating i_a not detected by SDS PAGE or crossed immunoelectrophoresis. There is no synergistic effect between i_a and i_b in the enzyme assay, unlike that seen in biological assays.

The mono-ADP-ribosylating activity of C. perfringens iota toxin and C. botulinum C_2 toxin is specific for poly-L-arginine (23,24). Many bacterial toxins have mono-ADP-ribosylating activity including cholera, E. coli heat labile toxin, diphtheria, pertussis, and Pseudomonas exotoxin A (10,16). The physiological acceptor of ADP-ribose from the enzymatic component of C_2 or iota toxin may be

nonmuscle actin (1,21,24). This form of actin is an essential building block of microfilaments necessary for the cytoskeletal structure of eukaryotic cells. ADP-ribosylation of actin by C_2 toxin prevents in vitro polymerization (1). The effects of ADP-ribosylated actin on target cells are unknown, but it is possible that cytoskeletal structure and intracellular transport of vesicles may be disrupted.

Another bacterial toxin, Clostridium difficile toxin B, reportedly ADP-ribosylates actin and disrupts polymerization (28). This same toxin may also directly or indirectly ADP-ribosylate a 90,000 M_r protein, possibly elongation factor 2, of fibroblast cells (8). Since work was done with an impure toxin preparation, there is uncertainty whether toxin B or a contaminant was responsible for the ADP-ribosylating activity.

WHAT IS THE ROLE OF IOTA B ?

The role of i_a in the synergistic mechanism of iota toxin may be ADP-ribosylation of actin, but this has not been proven in vivo. Presently, the role of i_b in the synergy is unknown. I suspect i_b to be a binding component based on preliminary evidence. It is common for bacterial toxins, dependent on two different proteins for biological activity, to have enzymatic and binding components (16). With a sensitive ELISA, I tried to detect binding of i_a and i_b to rabbit brush border membranes (BBMs) (unpublished data). This was done by incubating BBMs (37° C) with toxin for 1 h, pelleting the BBMs by centrifugation, and assaying the supernatant for i_a or i_b. Preliminary studies showed that approximately 40 % of i_b was bound to

crude BBMs; no effect was seen with i_a. A subsequent experiment using a highly purified preparation of BBMs showed no binding of i_b. Maybe specific or nonspecific binding had occurred with something other than BBMs in the crude preparation. Further work must be done to clarify this point.

No binding experiments were done with radiolabeled i_a or i_b. I felt that a sensitive ELISA, with a detection limit of a few nanograms, may be sufficient to detect binding. Binding experiments using radiolabeled i_a and i_b may be the best choice for future studies but there can be problems with retention of biological activity after radiolabeling.

Although I have tried to detect binding of i_b to BBMs, maybe there is another role for i_b in the synergy. Conceivably, i_b may have undiscovered enzymatic properties. It may have lipase activity, like the leukocydin binary toxin of S. aureus, or some other membrane disruptive activity allowing i_a to act on or within a target cell (17).

Large amounts of purified i_b (10 ug) causes guinea pig dermonecrosis, similar to the effects of crude iota toxin (unpublished data). The lesion progresses much slower than when both components are added before injecting. There may be contaminating i_a, although i_a was not detected in i_b preparations using SDS PAGE or crossed immunoelectrophoresis. Small amounts of i_a in the i_b preparation are strongly suspected since biological activity can be neutralized by monospecific i_a antiserum (unpublished data).

Work done by Dr. L. L. Simpson's laboratory also suggests that

purified i_b preparations have small amounts of contaminating i_a. They found slight ADP-ribosylating activity (0.7 % compared to purified i_a) in purified i_b preparations (24). By using monospecific i_a antiserum, they attempted unsuccessfully to neutralize the ADP-ribosylating activity of i_b preparations (personal communication, L. Simpson). Problems with ADP-ribosylation of serum components may be avoided by purifying the IgG fraction using a DEAE Blue column or 33.3 % saturated ammonium sulphate solution.

COMPARISONS OF IOTA AND IOTA-LIKE TOXINS

It should be obvious, and maybe to some painfully obvious, that my efforts have concentrated on the purification and characterization of C. perfringens iota toxin instead of C. spiroforme iota-like toxin. Toxin production by C. spiroforme was inconsistent and the thought of working with a toxin which is "here today and gone tomorrow" was not very comforting. I had no problems producing C. perfringens iota toxin. Robert Carman and Roger Van Tassel of our laboratory have subsequently found that divalent cations, like zinc, calcium, and cobalt, increase production and/or release of iota-like toxin from C. spiroforme (5). This same phenomenon was not seen with C. perfringens type E (unpublished data). R. Carman has found that a synergy exists between two protein components, also designated i_a and i_b, with the iota-like toxin of C. spiroforme (4). Comparisons between C. perfringens iota toxin and C. spiroforme iota-like toxin are given in Table 1.

Slight physical differences between both toxins suggest a

Table 1. Comparisons between C. perfringens Iota Toxin and C. spiroforme Iota-Like Toxin

Characteristic	Iota Toxin		Iota-Like Toxin[a]	
	i_a	i_b	i_a	i_b
Molecular Weight (Gel Filtration)	48,000	67,000	45,000	68,000
Isoelectric Point	5.2	4.2	5.3	4.6
Heat Stability[b]	85° C/15min	45° C/15min	ND	ND
ADP-Ribosylating Activity	YES	NO	YES?[c]	NO

[a] The molecular weight and isoelectric point data for C. spiroforme iota-like toxin were kindly provided by R. Carman.

[b] Study was done heating one component, allowing it to cool to room temperature, and adding the unheated complementary component. Biological activity was tested using guinea pig skin dermonecrosis. Values given are where biological activity was the same as unheated component. An increase in 5° C resulted in at least partial loss of biological activity. Studies were not done (ND) with C. spiroforme i_a and i_b.

[c] C. spiroforme iota-like toxin studies were done with partially purified material free of any detectable complementary component as determined by crossed immunoelectrophoresis.

variation in amino acid composition. The two toxins may differ because of mutations in the toxin gene and/or different proteases active in culture supernatants. The amino acid composition of purified C. perfringens i_a and i_b is given in Table 2. Composition of C. spiroforme i_a and i_b has not been determined.

The i_a component of iota toxin and C. spiroforme iota-like toxin show only partial immunological relatedness (25). The i_b components also show partial immunological relatedness. This data, along with toxin neutralization by heterologous antiserum, and slight differences in molecular weights and isoelectric points, suggest that similar components between the species are closely related but not identical. No immunological relatedness exists between i_a and i_b of the same toxin, as shown by crossing-over of the immunoprecipitin arcs (25). Monospecific antisera developed against purified i_a or i_b of C. perfringens iota toxin neutralize the biological activity of either toxin in culture supernatants and show one immunoprecipitin arc in crossed immunoelectrophoresis (Fig 1).

THE FORMATION OF HYBRID IOTA TOXINS

As described earlier, C. perfringens iota toxin and the C. spiroforme iota-like toxin are closely related. Since the toxins are very similar, we have successfully produced "hybrid iota toxins" using complementary components between both species. The hybrid toxins kill mice and are dermonecrotic in guinea pigs (Table 3). Even if changes have occurred in the toxin genes, the essential working domains of

Table 2. AMINO ACID ANALYSIS OF C. PERFRINGENS IOTA A AND IOTA B

Amino Acid	Mole % in Iota a [a]	Mole % in Iota b [a]
Asx	17.0	20.0
Thr	3.7	6.8
Ser	4.3	4.8
Glx	11.1	11.6
Pro	5.5	4.6
Gly	6.0	8.3
Ala	5.8	4.5
Val	5.5	17.0
Met	Not Detected	Not Detected
Ile	10.0	5.4
Leu	10.4	5.7
Tyr	5.1	3.6
Phe	5.0	4.8
His	Not Detected	Not Detected
Lys	7.8	4.6
Arg	2.8	1.5

[a] Results given are the average of 3 runs done with 24 h hydrolysis. Tryptophan or half cystine was not estimated.

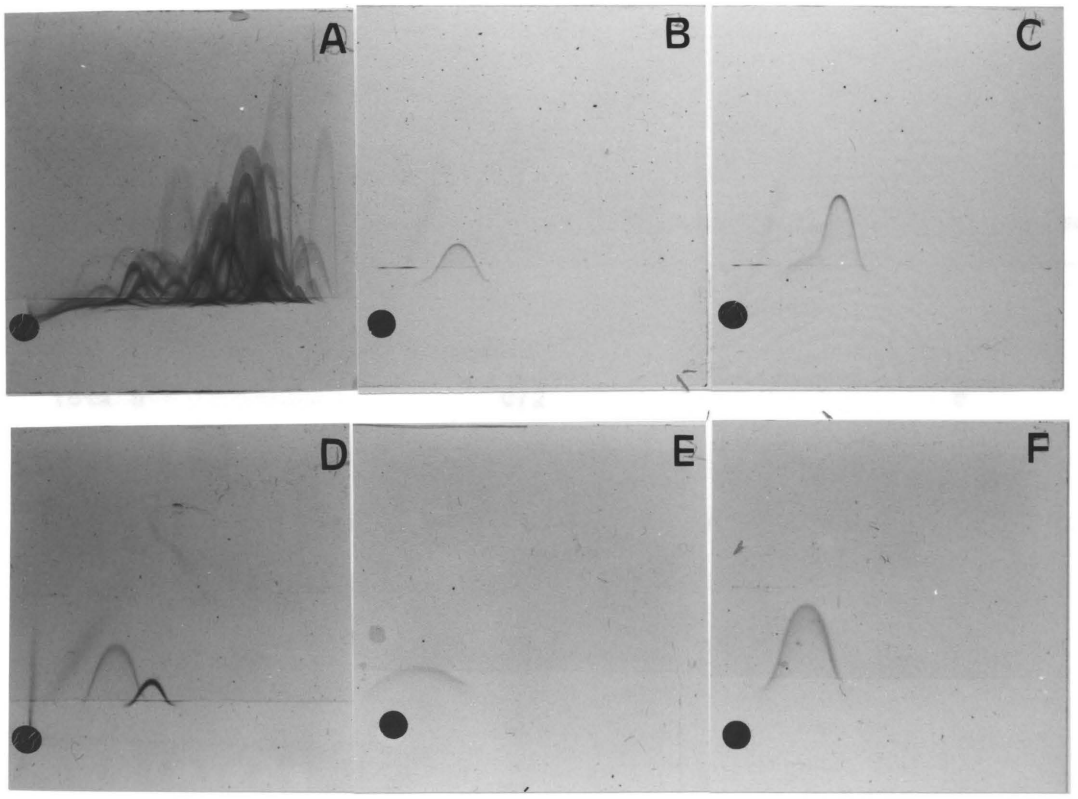

Fig. 1. CROSSED IMMUNOELECTROPHORESIS USING C. perfringens TYPE E/ C. spiroforme ANTIGENS AND ANTISERA

PLATE	ANTIGEN/ANTISERUM
A.	50 ug C. perfringens typeE supernatant/100ul C. perfringens type E
B.	60 ug C. perfringens type E supernatant/10 ul monospecific iota a
C.	60 ug C. perfringens type E supernatant/10 ul monospecific iota b
D.	50 ug C. perfringens type E supernatant/30 ul C. spiroforme
E.	5 ug C. spiroforme supernatant/40 ul monospecific iota a
F.	5 ug C. spiroforme supernatant/40 ul monospecific iota b

TABLE 3. HYBRID IOTA TOXIN ACTIVITIES

Toxin	Mouse Lethality[a] (# killed/# injected)	Guinea pig dermonecrosis [b] (mm)
C. perfringens (Cp)		
iota a	0/2	0
iota b	0/2	0
iota a + iota b	2/2	15
C. spiroforme (Cs)[c]		
iota a	0/2	0
iota b	0/2	5
iota a + iota b	2/2	12
Hybrid Mixes		
Cp iota a + Cs iota b	3/4	20
Cp iota b + Cs iota a	3/4	22

[a] When tested individually, 5 ug of *C. perfringens* iota a or iota b were injected into each mouse. Mixtures of *C. perfringens* iota a and iota b contained 2.5 ug per component. Individual components of *C. spiroforme* were injected at 20 ug per mouse. Mixtures of *C. spiroforme* iota a and iota b contained 10 ug per component. Hybrid mixes contained 2.5 ug of *C. perfringens* component and 10 ug of *C. spiroforme* component.

[b] Individual components of *C. perfringens* were tested with 0.6 ug per injection site. Mixtures contained 0.3 ug of each component. *C. spiroforme* individual components were tested at 3.0 ug per injection site. Mixtures contained 1.5 ug per component. Hybrid mixtures contained 0.3 ug of *C. perfringens* component and 1.5 ug per *C. spiroforme* component.

[c] *C. spiroforme* preparations of iota a or iota b were not homogeneous. Both components were separated from each other by DEAE ion exchange chromatography and not contaminated by the complementary component as determined by crossed immunoelectrophoresis.

both toxins still remain compatible enough to allow for hybrid activity.

To my knowledge, there is only one other example of hybrid toxin formation using bacterial toxins. That example involves the immunologically related cholera toxin and heat labile enterotoxin of E. coli (27). These toxins differ from iota or the iota-like toxins since they have binding and enzymatic subunits linked in solution by non-covalent linkages.

Dr. L. L. Simpson's laboratory tried unsuccessfully to form hybrid toxins using components of C_2 toxin and iota toxin (24). By using heterologous antiserum and crossed immunoelectrophoresis, no immunological cross-reactivity was found between C_2 toxin and the iota or iota-like toxins (personal communication, R. Carman). Differences in molecular weight are also evident (enzymatic component of C_2 and iota toxins = 55,000 and 48,000 M_r and non-enzymatic component of C_2 and iota toxins = 105,000 and 72,000 M_r, respectively) (19,26). Although physically and immunologically different, the toxins may have similar mechanisms of action (19,23,24).

HOW DO IOTA A AND IOTA B RELATE TO THE PROTOXIN ?

Previous literature describes the appearance of protoxin in young cultures of C. perfringens and proteolytic activation by trypsin (22). As the cultures aged, amounts of protoxin decreased and biologically active iota toxin increased because of endogenous proteases produced by C. perfringens (6,22).

My initial attempts at determining the relationship of i_a and i_b

to protoxin were not very encouraging. No effects of trypsin on early culture supernatants were seen using mouse lethal experiments or crossed IEP. Experiments were also done using benzamidine, an inhibitor of serine-type proteases like trypsin. By growing the organism in the presence of benzamidine concentrations (5 mg/ml) that did not inhibit growth (determined by turbidometric readings), I thought that large amounts of inactive protoxin may be produced. Since the inhibitor is low molecular weight (less than 500 daltons), I could dialyse it from the supernatant, add trypsin, and subsequently activate the protoxin. After growing C. perfringens type E at 37° C for 24 h, i_a and i_b were present in the control tube containing no benzamidine yet neither of the components was present in the inhibitor treated tube (components were detected by crossed immunoelectrophoresis). After extensive dialysis, trypsin was added with much anticipation but i_a and i_b were not generated. Extracellular protein production between inhibitor treated and inhibitor-free supernatants was not compared. I do not know whether benzamidine affects production of iota toxin specifically or whether other proteins are also affected.

The relationship of i_a and i_b to protoxin was further studied using more sensitive assays like the ELISA and guinea pig skin. Trypsin activation of protoxin was seen in early culture using guinea pig skin assays. Interestingly, I saw increased ELISA absorbance readings for i_b (up to 70% more absorbance compared to water treated control) after trypsinizing early culture supernatant. No effect was seen with i_a.

The increased ELISA readings of trypsinized i_b suggests that i_b

changes conformation after proteolysis. Conformational change may result in more antibody binding sites and increased absorbance readings. Antisera used in the ELISA were developed against 72 h (C. perfringens type E) and 96 h (C. spiroforme) supernatants already proteolytically activated. Presumably, endogenous proteases produced by older cultures activate the protoxin. The ELISA results also suggest that i_a and i_b are not generated from a common protoxin molecule since i_a absorbance readings remained constant after trypsin or water treatment.

To test the hypothesis that i_b is produced as the protoxin described by other investigators, I wanted to separate both components found in early cultures. DEAE ion exchange chromatography and a linear gradient (0.0-0.3 M NaCl in 0.01 M Tris-HCl, pH 7.5) separated i_a and i_b. After detecting both components by ELISA, aliquots of fractions containing i_a or i_b were treated with trypsin or water, purified complementary component was added, and the biological activity was tested in guinea pigs. Water or trypsin treated i_a was active whereas i_b was active only after trypsinization. Based on these results, I think i_a is produced in an active form and i_b requires proteolytic activation.

Besides already asking "What is the role of i_b in the synergy?", we must also ask "What happens to i_b after trypsinization?". There are at least two possibilities. After nicking of the i_b protoxin molecule, i_b may be able to bind to a target cell and/or interact with i_a better.

Another binary toxin, dependent on two unlinked proteins for activity, is also dependent on proteolytic activation for biological

activity. Both components of C. botulinum C_2 toxin are not generated from a common protoxin by trypsinization, but biological activity is dependent on nicking of the binding component (20). The binding component binds to target cells regardless of trypsinization, but the enzymatic component only interacts with nicked, and not unnicked, binding component. Besides having ADP-ribosylating properties, proteolytic activation of C. botulinum C_2 toxin and iota toxin may also be similar.

METHODS OF QUANTITATING IOTA TOXIN

Estimating the amount of iota toxin in culture supernatant fluid can be done using animals or immunological methods. Before developing C. spiroforme antiserum in our laboratory, amounts of C. perfringens iota toxin were determined by titrations in mice or guinea pigs (18). Animal assays can be stressful, expensive, less sensitive than some immunological methods, and a potential source of allergens for those of us blessed (cursed?) with a hyperactive immune system. After purifying i_a and i_b of C. perfringens iota toxin, I determined the detection limits of four different assays (Table 4). The most sensitive method is a sandwich ELISA using C. spiroforme antiserum and monospecific antisera, which recognizes i_a or i_b of C. perfringens iota toxin or C. spiroforme iota-like toxin. The ELISA is specific (Table 5) and approximately 30 times more sensitive than the next best assay, guinea pig dermonecrosis. Mouse lethality and quantitative rocket immunoelectrophoresis are approximately 1000 times less sensitive than the ELISA. The efficacy of using the ELISA to detect

Table 4. DETECTION OF IOTA A AND IOTA B

Method	Iota a (ug/ml)	Iota b (ug/ml)
Quantitative Rocket IEP [a]	5.3	4.9
Mouse Lethality (LD_{100}) (equal molar amounts[b])	2.5	3.8
Guinea Pig Dermonecrosis (equal molar amounts[b])	0.10	0.15
ELISA (linear portion of curve)	0.003-0.20	0.003-0.20

[a] IEP = Immunoelectrophoresis

[b] Equal molar amounts of iota a and iota b were determined by molecular weight estimates from SDS PAGE.

Table 5. SPECIFICITY OF THE IOTA TOXIN ELISA

Organism			Absorbance (405 nm) Iota a antiserum	Iota b antiserum
C. perfringens type				
VPI	6748	A	0.09	0.09
VPI	6079	A	0.10	0.12
VPI	2528	B	0.06	0.09
VPI	10793	B	0.07	0.09
VPI	7387	C	0.04	0.05
VPI	10209	C	0.07	0.08
VPI	9312	D	0.12	0.12
VPI	6442	D	0.02	0.04
NCIB	10748	E	2.33	2.47
VPI	10339	E	2.95	2.59
VPI	1590	E	1.93	2.61
C. spiroforme RPO-980 [a]			1.74	1.18
C. spiroforme B-61 [a]			1.43	0.89
C. spiroforme BA-1 [a]			1.13	0.83
C. spiroforme NCTC 11493			1.30	1.07
C. difficile VPI 10463			0.11	0.10
C. sporogenes VPI 5037B			0.11	0.09
C. irregularis VPI 13758			0.12	0.10
C. bifermentans VPI 5690			0.12	0.13
C. sordellii VPI 8827			0.08	0.10
B. vulgatus VPI 4245			0.06	0.09
B. fragilis VPI 2553			0.07	0.10
B. thetaiotaomicron VPI 5482			0.07	0.10
BHI Control			0.08	0.12

[a] Dr. Robert Carman's numbers for these strains.

iota toxin in diarrheic stools or cecal contents has not been tested, but others have found this technique to be sensitive and specific for the detection of other bacterial toxins in stool specimens (13,15).

FUTURE AREAS OF INTEREST

The project described in this dissertation has answered some, but obviously not all, of the many questions about C. perfringens iota toxin. I have made some progress by answering questions like "What is iota toxin?", "What are its physical characteristics?", and "How similar are C. perfringens iota toxin and C. spiroforme iota-like toxin?". Some of the many questions that remain unanswered have been previously discussed, like the role of i_b in the synergy and what happens to early culture i_b after trypsinization.

An intriguing question I have asked myself, but have not answered, is the origin of iota toxin. Is it plasmid or chromosomally coded? Are i_a and i_b on the same segment of DNA and controlled by the same promotor as one might expect from a toxin dependent on two different proteins for activity? In preparation for DNA probe synthesis and cloning experiments, I tried unsuccessfully to get an amino terminus sequence for i_a and i_b of C. perfringens iota toxin. I could not get a sequence since both components have a blocked amino terminus (unpublished data).

Instead of using a DNA probe to detect the i_a and i_b gene(s), polyclonal, monospecific antisera could be used to screen colonies resulting from shotgun cloning. After cloning both components of iota toxin, comparisons could also be made with cloned C. spiroforme iota-

like toxin. I know that similar toxin components differ slightly in molecular weight, isoelectric point, and immunological relatedness, but analysis of toxin genes would give more definitive information. The two toxins may be identical at the gene level but differences in the types and amounts of bacterial proteases produced during growth may result in slightly different final products.

Cloned i_a and i_b could provide a specific means of studying the protoxin phenomenon. After trypsin activation, I do not know if the i_b protoxin loses a peptide fragment or is simply nicked, resulting protein conformational changes. If the latter were true, we should see at least two different bands on an SDS gel after treating active i_b with SDS and 2-mercaptoethanol, but I do not. Specific site(s) of proteolytic activation may be determined using cloned i_b.

Besides cloning the toxin components, another area of interest is the comparison of C. perfringens iota toxin and C. spiroforme iota-like toxin mechanisms of action. Through collaborative work with Dr. L. L. Simpson's laboratory, C. perfringens i_a was shown to have ADP-ribosylating activity specific for arginine and nonmuscle actin (24). Enzymatic properties of C. spiroforme iota-like toxin are currently being studied by his group. I provided them with partially purified i_a and i_b of C. spiroforme iota-like toxin, free from detectable complementary component. His laboratory has found ADP-ribosylating activity, specific for arginine and actin, present in the i_a preparation, but not the i_b preparation (personal communication, L.L. Simpson). The next question they plan to answer is whether enzymatic activity is due to i_a or a contaminant in the preparation. Collaboration with Dr. L. L. Simpson has undoubtedly given us some clues

about the mode of action of iota toxin and a continued interest in future collaborations will provide more pieces to the puzzle.

In summary, this dissertation covers many areas concerning bacterial toxins and for those specifically interested in iota toxin, I offer new data about its physical and biological properties. I hope the information contained in this dissertation will be useful to future investigators.

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Purification and Characterization of *Clostridium perfringens* Type E Iota Toxin

by

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Anaerobic Microbiology

(ABSTRACT)

Clostridium perfringens type E iota toxin is implicated in some cases of fatal diarrhea in calves, lambs, and guinea pigs. A cross-reacting "iota-like" toxin, produced by *Clostridium spiroforme*, is responsible for antibiotic-associated and weaning related enterotoxemias of rabbits. Antisera developed against culture supernatant of either organism neutralized the biological activity of iota or iota-like toxin. By using *C. spiroforme* antiserum and crossed immunoelectrophoresis (crossed IEP), we found two cross-reacting antigens in *C. perfringens* type E supernatants. *C. perfringens* types A, B, C, and D, which do not produce iota toxin, did not cross-react with *C. spiroforme* antiserum.

To determine if either antigen had iota toxin activity, we separated the cross-reacting antigens of *C. perfringens* by preparative isoelectric focusing (IEF) and tested all IEF fractions for biological activity in guinea pigs and mice. The fraction containing the faster migrating antigen seen in crossed IEP, designated iota b (i_b), had some guinea pig dermonecrotic and mouse lethal activity. Other fractions, including the one containing the slower migrating iota a (i_a) antigen, had little to no biological activity. When fractions containing i_a and i_b were mixed, there was an 8 and 25 fold increase

in mouse lethal and dermonecrotic titers, respectively. Activity was neutralized by C. perfringens type E or C. spiroforme antisera and other fractions, when mixed with i_a or i_b, did not have a synergistic effect.

Both components of C. perfringens iota toxin were purified using ammonium sulfate precipitation, DEAE anion exchange chromatography, preparative IEF, Sephadex G-100 gel filtration, and flatbed electrophoresis to yield a 12 and 5 % final recovery of i_a and i_b, respectively. Each protein was homogeneous by SDS PAGE, gradient PAGE, and crossed IEP using homologous antiserum. There was at least an 8 fold increase in mouse lethal titer and 64 fold increase in dermonecrotic titer when equimolar amounts of i_a and i_b were mixed. Monospecific antisera against purified i_a and i_b neutralizd the iota or iota-like activity of crude supernatants. A sensitive and specific ELISA was developed using monospecific and C. spiroforme antisera.

The i_a and i_b proteins have a pI of 5.2 and 4.2 and molecular weights of 48,000 and 71,000 (SDS PAGE), respectively. The i_a protein is heat stable (85° C/15 min) while i_b lost its activity at 55° C. Amino terminus sequencing revealed that both proteins were blocked by an unknown functional group(s). Purified i_a, but not i_b, has ADP-ribosylating activity specific poly-L-arginine in vitro. Recent evidence suggests that nonmuscle actin, involved in the cytoskeletal structure of eucaryotic cells, may act as the in situ acceptor.