INFECTION AND IMMUNITY, Feb. 1981, p. 536-546
0019-9567/81/020536-11$02.00/0

Vol. 31, No. 2

Purification and Characterization of *Clostridium perfringens* Delta-Toxin

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Delta-toxin, an extracellular hemolysin released by *Clostridium perfringens* type C, was purified from culture supernatant fluid by sequential ammonium sulfate precipitation, thiol-Sepharose gel chromatography, isoelectric focusing, and Sephadex G-75 gel filtration. The purified preparation had a specific activity of 320,000 hemolytic units per mg of protein and was homogeneous, as determined by immunochemical and electrophoretic tests. This toxin was characterized as a single polypeptide chain composed of 391 amino acid residues, 30% of which were hydrophobic. The molecular weight was found to be 42,000, and the isoelectric point was pH 9.1. Delta-toxin appeared to be amphiphilic by charge shift electrophoresis in a three-detergent system. It was immunogenic in rabbits and lethal to mice at a dose of 0.12 μg. The lytic activity of delta-toxin was restricted to erythrocytes of even-toed ungulates (sheep, goats, and pigs). This activity was inhibited by G_{M2} ganglioside but not by other gangliosides, cholesterol, lecithin, or sphingomyelin.

Clostridium perfringens delta-toxin is one of the three extracellular hemolytic toxins released, along with many other exotoxins and exoenzymes, by a number of type C strains and also possibly by type B strains (7, 14, 30, 32, 40, 43). In contrast to alpha-toxin and theta-toxin (hemolysins), which have been purified and studied widely (28, 41), delta-toxin was little studied until our first attempt to purify and characterize it (46). Previous investigators established that this toxin was immunogenic and lytic for the erythrocytes of even-toed ungulates (sheep, cattle, goats, and swine) but not for the erythrocytes of other species such as humans, rabbits, and horses (7, 30, 49). The effects of delta-toxin on other cells are not known, and the possible lethal action of this toxin has not been demonstrated with certainty (15). Optimal production of delta-toxin was shown to occur within a narrow pH range (pH 7.5 to 8.0) (33).

We report here an improved method for purifying delta-toxin, which resulted in relatively good yields from culture supernatant fluids obtained in pH-controlled fermentors. The study of this toxin may be of interest for a better evaluation of the pathological role of clostridial toxins and virulence factors (40). Delta-toxin may also prove to be useful as a membrane probe similar to other cytolytic toxins (13), especially because of its restricted hemolytic spectrum.

MATERIALS AND METHODS

Bacterial strain. *C. perfringens* type C strain CW 8 was kindly provided by A. H. W. Hauschild, Bureau of Microbial Hazards, Ottawa, Canada. This strain produces large amounts of delta-toxin (33).

Culture medium. The medium used consisted of a diffusate of 25 g of proteose peptone 3 (Difco Laboratories, Detroit, Mich.) and 6 g of yeast extract (Difco) which were dissolved in 150 ml of distilled water and dialyzed for 3 days in the cold against 1 liter of water. The diffusate (1,000 ml) was supplemented with 10 g of sodium bicarbonate, 2 g of cysteine hydrochloride, 9.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.4 g of KH_2PO_4, and 20 g of dextrin (Difco) and adjusted to pH 8.0 with 3 N NaOH. It was sterilized at 115°C for 15 min in a 1.2-liter fermentor (Biolafitte, Poissy, France). The fermentor was allowed to cool and then maintained until inoculation under a stream of nitrogen.

Growth and toxin production. The lyophilized bacterial strain was inoculated anaerobically into 20 ml of TGY medium (21) and then incubated for 16 h at 37°C (end of logarithmic growth phase). This culture was transferred to the fermentor and allowed to grow at 37°C with a continuous nitrogen flow and moderate stirring. The pH was strictly maintained throughout growth between pH 8 and 8.3 by using an automatic pH control device connected to an automatically operated valve which admitted 3 N NaOH. This pH is critical for the production of delta-toxin (33). No antifoam agent was added. After 6 h of growth, the culture was removed and centrifuged at 10,000 × *g* and 4°C for 20 min. The supernatant (crude toxin) was separated and kept frozen until fractionation. The titer of delta-toxin ranged from 8,000 to

12,000 hemolytic units (HU) per ml.

Equine antisera. Diagnostic standard sera against *C. perfringens* type A (800 IU/ml) and type C (700 IU/ml), which were obtained from Institut Pasteur Production, Paris, France, were from horses that had received several courses of injection of type A and C culture supernatant fluids.

Immunization of rabbits with delta-toxin. Antisera against purified delta-toxin (step 5 of the purification scheme) were prepared in three white Bouscat rabbits (Institut Pasteur Production, Garches, France) weighing 2 to 2.3 kg. Each rabbit received three subcutaneous injections at 3-week intervals of 1 mg of toxin emulsified with 2 mg of Freund complete adjuvant (Difco). The rabbits were bled aseptically 7 days after the last injection. After clotting, the sera were clarified by centrifugation at 2,500 × *g* for 10 min, and the supernatant was stored in portions at −20°C.

Assay of delta-toxin. The lytic effect of delta-toxin on sheep erythrocytes (SRBC) at pH 8.0 (optimal for lysis) was used for an assay of this toxin. Sheep blood (Institut Pasteur Production, Garches) was centrifuged, and the erythrocytes were collected after centrifugation at 1,500 × *g* for 20 min and removal of the plasma and buffy layer by aspiration. The cell pellet was suspended in 20 times its volume of pH 8.0 isotonic borate-buffered saline (BBS) ($Na_2B_4O_7 \cdot 10H_2O$, 2.59 g; H_3BO_4, 4.5 g; NaCl, 9 g; and distilled water to make 1 liter) and centrifuged again at 1,500 × *g* for 20 min. The washing process was repeated twice. Finally, the SRBC were suspended (~5%, vol/vol) in BBS such that 0.5 ml of the suspension after lysis plus 14.5 ml of a 0.1% (wt/vol) Na_2CO_3 solution gave an absorbance at 541 nm of 0.200. This standard SRBC suspension (~6×10^8 cells per ml) was kept at 4°C and used within 4 days. Test solutions of delta-toxin were diluted in BBS containing 0.1% bovine serum albumin (Serva, Heidelberg, Germany). Volumes of appropriately diluted toxin decreasing in 0.1-ml amounts from 1 to 0.1 ml were placed into tubes (12 by 75 mm) along with 25 μl of *C. perfringens* type A antiserum in order to inhibit the possible hemolytic effects of alpha-toxin and perfringolysin O (theta-toxin). The volume in all tubes was brought to 1 ml by adding BBS. The tubes were maintained for 10 min at room temperature, and then 0.5 ml of the SRBC suspension was added to each tube. The tubes were incubated at 37°C for 45 min and then centrifuged briefly. The percentage of hemolysis was estimated by the optical absorbance of the hemoglobin in the supernatant fluid at 541 nm compared with the absorbance of standards; 1 HU was defined as the amount of test material needed to release the hemoglobin from 50% of the cells. Values were estimated graphically by plotting on a log-probit graph (2) the percentage of lysis versus the volume of the sample of toxin preparation used in the assay.

Assays of perfringolysin O and alpha-toxin. Determinations of perfringolysin O (4) and alpha-toxin (phospholipase C) in delta-toxin preparations were performed by hemolytic tests. The lytic activity of perfringolysin O was determined with a rabbit erythrocyte suspension (3 × 10^8 cells per ml) in 0.158 M NaCl (pH 6.8) after activation with 40 mM cysteine, as described previously for streptolysin O (2). Interference by alpha-toxin was prevented by using 4% sodium citrate in 0.158 M NaCl (pH 6.8) for dilution of test solutions; 1 HU of perfringolysin O activity was defined as the dilution of toxin solution that caused 50% hemolysis. Toxin titers were calculated as described above for delta-toxin.

The lytic activity of alpha-toxin was determined with the same rabbit erythrocyte suspension (final concentration 0.5% vol/vol); test samples were incubated previously with a dispersion of 500 μg of cholesterol in 0.158 M NaCl for 20 min to inhibit perfringolysin O. Hemolysis was determined on twofold dilutions of test solution in 0.158 M NaCl buffered with 0.02 M tris(hydroxymethyl)aminomethane (Tris) hydrochloride (pH 7.0) containing 1 mM $CaCl_2$ after 1 h of incubation at 37°C, followed by standing for 2 h at 4°C (hot-cold hemolysis); 1 HU was defined as the amount of toxin causing 50% hemolysis under these conditions (29).

API-ZYM tests. The API-ZYM system (Société Analytab Products, Inc., Montalieu, France), which allows detection of 19 different enzymatic reactions (see Table 1), is a standard commercial device ready for use (20). Cupules of API-ZYM strips containing the different substrates were inoculated with 65 μl of test solution and incubated for 4 h at 37°C. The reactions were read after adding 1 drop each of API reagents A and B. The intensities of the reactions which developed were graded from 0 to 5 by using the API-ZYM color reaction chart. By using this chart the reactions obtained could be divided into negative (grade 0), weak positive (grades 1 and 2), and strong positive (grades 3 to 5).

Protein determination. Protein content was measured by the method of Lowry et al. (27), using crude and purified fractions after extensive dialysis overnight against quartz-distilled water. Bovine serum albumin was used as a standard.

IEF. Preparative isoelectric focusing (IEF) was performed with a 110-ml column (LKB Produkter, Stockholm, Sweden), as directed in the LKB manual; we used a final concentration of 1% carrier ampholytes (LKB Ampholines) which had a pH range of 3.5 to 11 (pH 3 to 10 Ampholines, 0.5 ml; pH 4 to 6 Ampholines, 1 ml; pH 9 to 11 Ampholines, 1 ml) in a sucrose density gradient. The less dense solution contained the sample and 0.8 ml of a 40% Ampholines mixture. The more dense sucrose solution contained 1.7 ml of the Ampholines mixture. The central electrode compartment (anode) contained 1% sulfuric acid in 60% sucrose. The cathode, which consisted of 0.2 ml of ethylenediamine in 10 ml of distilled water, was layered on the top of the gradient. A final potential of 1,600 V was applied for 16 h; the gradient was cooled by circulating water at 2°C. At the end of the run, 2-ml fractions were collected by draining dropwise into a fraction collector. The pH (at 4°C), optical density at 280 nm, and hemolytic activity of each fraction were determined. The Ampholines-sucrose gradient was not itself hemolytic.

Thiopropyl-Sepharose 6B column. A total of 8 g of freeze-dried thiopropyl-Sepharose 6B (Pharmacia, Uppsala, Sweden) was washed twice with 100 ml of 0.05 M Tris–formic acid buffer (pH 8.0) containing 158 mM NaCl. The wet powder was allowed to swell in

this buffer for 2 h. The slurry was poured into a column (2.5 by 30 cm; Pharmacia), and this was equilibrated by washing with 100 ml of buffer. Elution was performed with buffer at a flow rate of 5 ml/h.

Disc SDS-PAGE. Using disc sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we determined the molecular weight of delta-toxin (see Fig. 5) by the method of Swank and Munkres (45); the gel contained 12.5% (wt/vol) acrylamide, 1.25% bisacrylamide, and 8 M urea. Test samples (250 to 500 μg of protein per ml) and standard proteins (2 mg/ml) of known molecular weights (Pharmacia) were dissolved in a solution containing 1% SDS, 8 M urea, 1% 2-mercaptoethanol, and 0.01 M H_3PO_4, which was adjusted to pH 6.8 with Tris. All SDS-PAGE reagents were from Serva. Protein samples were heated for 90 s in boiling water and layered (50 μl) on the tops of gels. Bromothymol blue was used as tracking dye. Electrophoresis was performed at pH 6.8 toward the anode in a disc gel apparatus (model 1270; Isco, Lincoln, Neb.) at 5 mA/tube. Gels were stained with 0.25% Coomassie brilliant blue R-250 in a solution containing acetic acid, methanol, and water (8:45:47; vol/vol). Excess dye was removed by using the same solvent.

Slab SDS-PAGE. Toxin preparations (1 to 5 mg of protein per ml) were analyzed by slab SDS-PAGE as described by Laemmli (25), using 0.025 M Tris–0.2 M glycine buffer (pH 8.4) containing 0.1% SDS. Toxin samples (20 to 50 μl) containing 1.5% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromothymol blue (tracking dye) in Tris-glycine buffer were boiled for 90 s in a water bath before being loaded onto the gel. Electrophoresis was on a 7.5 to 20% gradient slab polyacrylamide gel (10 by 14 cm; 2 cm thick) at 7 mA for 24 h until the dye reached the bottom of the gel. Sample wells were made from 3% polyacrylamide containing 0.1% SDS. After electrophoresis, the gels were stained and destained as described above for disc SDS-PAGE.

Charge shift electrophoresis. The technique of Helenius and Simons (16) was used for testing the amphiphilic properties of purified toxin. Electrophoresis was performed with an LKB Multiphor apparatus at room temperature in 1% agarose (Indubiose A 37; Industrie Biologique Française, Gennevilliers, France) at pH 9 on glass plates (11 by 20.5 cm) in the presence of the nonionic detergent Triton X-100 (E. Merck AG, Darmstadt, Germany), a mixture of Triton X-100 and the cationic detergent cetyltrimethylammonium bromide (Serva), and a mixture of Triton X-100 and the anionic detergent sodium deoxycholate (Merck). Toxin migration after electrophoresis was detected by immunodiffusion against anti-delta-toxin antiserum (see Fig. 4). Diphtheria toxin, which was kindly provided by P. Boquet from this laboratory, was used as a hydrophilic control protein (5).

Amino acid analyses. Amino acid analyses were performed by the method of Spackman et al. (42) with a Beckman Multichrom B analyzer, using the monocolumn procedure of Devenyi (10). Samples (1 mg) of the purified toxin were dialyzed against water and then hydrolyzed for 24, 48, and 72 h at 110°C with 6 N HCl in sealed Pyrex tubes after evacuation and degassing. The hydrolysates were dried under air before analysis. The tryptophan content was estimated by the method of Liu and Chang (26). The samples were hydrolyzed in 4 N methane sulfonic acid. The half-cystine content was determined as cysteic acid by the performic acid oxidation method described by Hirs (17).

Hemolytic activity of delta-toxin in the presence of various lipids. Cholesterol (5-cholesten-3β-ol), bovine sphingomyelin, egg lecithin, and mixed brain gangliosides were obtained from Sigma; G_{M1} ganglioside [galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide], G_{M2} ganglioside [*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide], G_{D1a} ganglioside [*N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide], and G_{T1} ganglioside [*N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl-*N*-acetylneuraminyl)-galactosylglucosylceramide] were purchased from Supelco, Bellefonte, Pa. All lipids were dissolved (1 mg/ml) in chloroform-methanol (2:1), except cholesterol, which was dissolved in 99% (vol/vol) ethanol.

The effects of lipids on delta-toxin activity were determined by placing varying amounts of test lipid (1 to 1,000 μg) into small test tubes (12 by 75 mm). The solvent was removed by bubbling nitrogen into the tubes. The lipids were then dispersed by blending with a Vortex mixer for 2 min with 700 μl of BBS (pH 8.0) containing 0.1% bovine serum albumin. A toxin solution in the same buffer (300 μl, containing 1 HU; equivalent to 3 ng of protein) was added, and the mixture was incubated for 10 min at 37°C. The SRBC suspension (0.5 ml) was then added with stirring to all tubes, and incubation was continued for 45 min at 37°C. Control tubes contained BBS instead of lipid for each series tested. Hemolysis was measured by absorbance at 541 nm after removal of the cells by centrifugation. Percent inhibition was expressed relative to the hemolysis observed with the controls. The 50% inhibitory concentration (the concentration required to inhibit 50% of the activity of delta-toxin) was estimated from a plot of hemolysis versus concentration of test component.

Proteolytic activity. Proteolytic activity was assayed by a modification of the Azocoll substrate method described by Jackson and Matsueda (22). The protease assay reaction mixture, which contained 1 mg of insoluble Azocoll (Calbiochem, Los Angeles, Calif.) in 1 ml of 0.1 M phosphate buffer (pH 7.0), 10 to 500 μl of test solution, and buffer to a final volume of 2 ml, was incubated for 15 min at 37°C. The reaction was stopped by immersion in an ice bath, and the mixture was centrifuged at 0°C. The absorbance of the dye released from the insoluble carrier was measured in the supernatant fluid at 520 nm relative to a blank to which no toxin fraction was added. A linear rate of increase was obtained up to an absorbance at 520 nm of approximately 0.35; 1 U of activity was defined as the amount of material required to increase the absorbance at 520 nm by 0.001 absorbance unit. Trypsin (Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

Carbohydrate analyses. Carbohydrate analyses were performed on 100- and 200-μg samples of purified

toxin by the anthrone method (37).

Determination of the neutralizing titer of immune sera. The neutralization of the hemolytic activity of delta-toxin by antisera was measured by incubation (37°C, 10 min) of a series of tubes containing 25 HU of purified toxin in 0.2 ml of BBS and increasing volumes (0.1 to 1 ml) of an appropriate dilution of test serum; total volumes were adjusted to 1 ml with BBS. SRBC (0.5 ml) were added to each tube, the contents were blended with a Vortex mixer, and the mixture was maintained at 37°C for 45 min. The percentage of hemolysis was determined as described above for the toxin assay. The neutralizing titer of a serum was defined as the reciprocal of the highest serum dilution for which 50% lysis was observed.

RESULTS

Characteristics of the crude toxin. The supernatant fluid used for toxin purification contained 10,000 HU of delta-toxin per ml, 2,750 HU of perfringolysin O per ml, and 940 HU of alpha-toxin per ml. Testing by the API-ZYM system (Table 1) revealed the presence of the following enzymatic activities: alkaline phosphatase, esterase, esterase-lipase, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and α-mannosidase. No significant proteolytic activity was found by the Azocoll test. Slab SDS-PAGE of a culture supernatant concentrated 25-fold in a stirred cell equipped with a PM-10 membrane (Amicon) revealed about 20 extracellular products which were stained by Coomassie brilliant blue (Fig. 1).

Purification of delta-toxin. All steps of the purified scheme (Table 2) were carried out at 4°C unless otherwise stated.

(i) Ammonium sulfate precipitation. The culture supernatant fluid (1,200 ml) was adjusted to 60% saturation with $(NH_4)_2SO_4$, adjusted to pH 8.0 with ammonia, and allowed to precipitate overnight before centrifugation at 16,000 × *g* for 10 min. The precipitate was dissolved in about 75 ml of 0.05 M Tris–formic acid buffer (pH 8.0) containing 158 mM NaCl. The protein solution obtained was centrifuged at 16,000 × *g* for 10 min to eliminate a small insoluble residue. The resulting supernatant liquid was designated fraction F1.

(ii) Thiol-Sepharose gel chromatography. Fraction F1 was reduced at room temperature by adding 50 mM dithiothreitol and then applied in three successive fractions to a Biogel P4 (Bio-Rad Laboratories, Richmond, Calif.) column (2.6 by 38 cm) equilibrated with Tris-formic acid buffer containing NaCl. The column was eluted with this buffer. The protein eluate in the void volume was then applied to a thiopropyl-Sepharose 6B column at a flow rate of 5 ml/h. This column was washed at the same flow rate with 100 ml of buffer. Delta-toxin and other proteins were not retained by the column, whereas some SH-containing proteins, including

TABLE 1. *Testing by the API-ZYM system for 19 enzymatic activities in crude and purified toxin preparations*

Test reaction	Reaction grade[a]				
	Crude toxin	Fraction F1[b]	Fraction F2[b]	Fraction F3[b]	Fraction F4[c]
Alkaline phosphatase	1	2	3	3	0
Esterase	1	3	2	2	0
Esterase lipase	1	2	2	2	0
Lipase	0	0	0	0	0
Leucine aminopeptidase	0	0	0	0	0
Valine aminopeptidase	0	0	0	0	0
Cystine aminopeptidase	0	0	0	0	0
Trypsin	0	0	0	0	0
Chymotrypsin	0	0	0	0	0
Acid phosphatase	0	3	5	4	0
Phosphoamidase	1	1	4	1	0
α-Galactosidase	2	3	3	0	0
β-Galactosidase	1	5	5	5	0
β-Glucuronidase	0	0	0	0	0
α-Glucosidase	0	3	1	0	0
β-Glucosidase	0	1	0	0	0
β-*N*-acetylglucosaminidase	0	1	0	0	0
α-Mannosidase	2	5	5	5	0
α-Fucosidase	0	0	0	0	0

[a] Numerical values from 1 to 5 indicate increasing color intensity.
[b] See Table 2.
[c] Purified delta-toxin.

perfringolysin O, remained fixed through a thiol-disulfide interchange reaction, as reported for streptolysin O (34) and many SH-containing enzymes (6). The entire effluent (220 ml) from the column was concentrated to about 15 ml by ultrafiltration with a PM-10 membrane (fraction F2).

(iii) IEF. Fraction F2 was submitted to preparative IEF in a pH 3.5 to 11 gradient. As Fig. 2 shows, the hemolytic material focused around a pI of 9. This material was collected and concentrated by ultrafiltration (fraction F3).

(iv) Gel filtration. Fraction F3 was applied to a Sephadex G-75 column (2.5 by 100 cm) equilibrated with Tris-formic acid buffer containing NaCl. The column was eluted with this buffer. The toxin emerged as a symmetrical peak (Fig. 3), which was collected and concentrated by ultrafiltration (fraction F4). This fraction constituted the purified toxin. The overall recovery of the purification process was 16%.

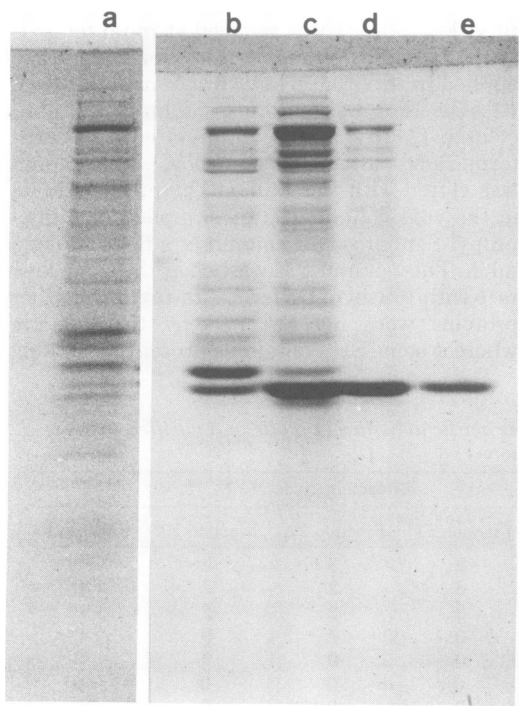

FIG. 1. *Slab SDS-PAGE of purification fractions (Table 2). The anode was at the bottom of the gels. Lane a, 20-fold concentrate of culture fluid; lane b, fraction F1; lane c, fraction F2; lane d, Fraction F3; lane e, fraction F4 (purified toxin).*

Characteristics of the purified toxin. Fraction F4 was homogeneous on the basis of electrophoretic and immunochemical tests. A single band was revealed by slab SDS-PAGE (Fig. 1). A single immunodiffusion band was observed with the two rabbit antisera raised against this fraction (Fig. 4), which also neutralized toxin activity (75 and 160 U of antitoxin per ml). No immunoprecipitation was found with a commercial anti-*C. perfringens* type C equine serum. This serum exhibited a high neutralizing titer (1,000 U/ml).

The apparent specific activity of fraction F4 was 320,000 HU/mg of protein. However, the actual specific activity must be higher for the native toxin, as some denaturation occurred in step 4 (Table 2), which was reflected by the decrease in the relative activity of the preparation.

The maximum absorbance of the purified toxin was at 280 nm. The $E^{1\%}_{280\ nm,\ 1\ cm}$ was 9.6. The toxin preparation contained no hemolytic activity attributable to perfringolysin O and alpha-toxin. None of the enzymatic activities revealed by the API-ZYM test in the crude toxin and intermediate fractions (Table 1) was detected at a level of 20 μg (64,000 HU) of purified toxin. No proteolytic activity was found for the same amount with Azocoll reagent. No carbohydrates were detected in toxin samples containing 100 μg of protein.

Molecular weight and isoelectric point. Disc SDS-PAGE of fraction F4 in the presence of urea and mercaptoethanol yielded a single sharp band, indicating that the toxin is a single polypeptide of 42,000 daltons (Fig. 5). As inferred from IEF (Fig. 2), delta-toxin appeared to be a basic protein with a pI of 9.1. The two forms (pI ~8.8 and 9.4) reported previously (46) were not observed in the present work.

Amphiphilic character of delta-toxin. Fraction F4 was submitted to charge shift electrophoresis in the three-detergent system (16) described above. Figure 6 shows the patterns

TABLE 2. *Purification of C. perfringens delta-toxin*

Step	Purification stage	Fraction	Total vol (ml)	Total protein (mg)	Total activity (HU)	Sp. act (HU/mg)	Relative activity	% Yield
1	Culture supernatant		1,200	7,800	12,000,000	1,538	1	100
2	Ammonium sulfate (60%)	F1	83	305	11,400,000	37,377	24.3	95
3	Thiopropyl-Sepharose	F2	15.2	65	10,860,000	167,077	109	90.5
4	IEF	F3	9.5	23	2,400,000	104,347	67.8	20
5	Sephadex G-75	F4	22	6	1,920,000	320,000	208	16

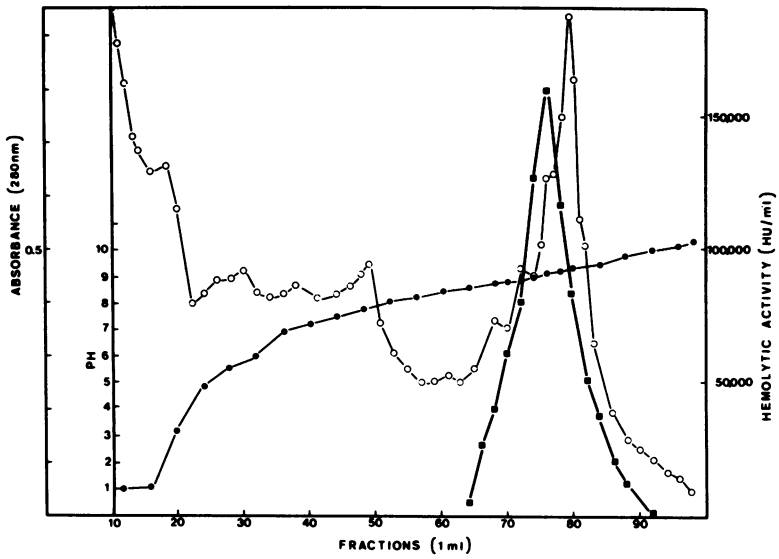

FIG. 2. *IEF of fraction F2 (Table 2). Symbols:* ○, *optical absorbance;* ●, *pH at 4°C;* ■, *hemolytic activity.*

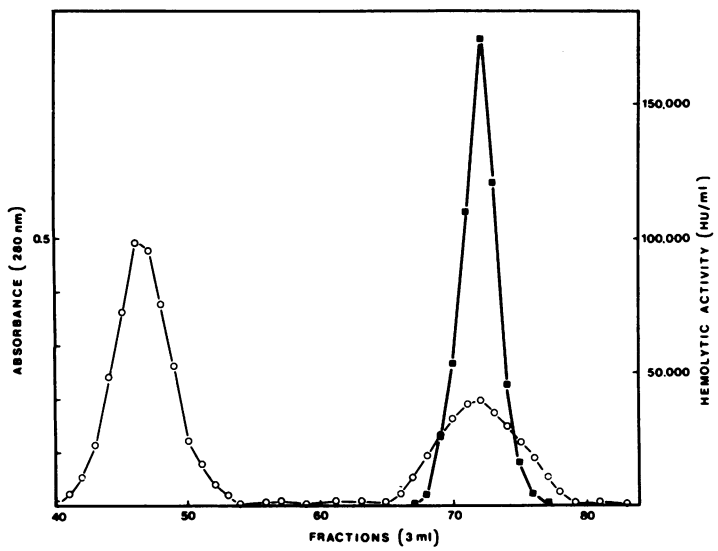

FIG. 3. *Gel filtration on Sephadex G-75 column of fraction F3 (Table 2). Symbols:* ○, *optical absorbance;* ■, *hemolytic activity.*

obtained for delta-toxin and the hydrophilic protein diphtheria toxin (5), which was used as a control. The electrophoretic mobility of the latter was not affected, whereas delta-toxin was amphiphilic. It shifted anodally (24 mm) in the Triton X-100–deoxycholate system and cathodally (4 mm) in the Triton X-100–cetyltrimethylammonium bromide system compared with its mobility in Triton X-100 alone.

Amino acid composition. The amino acid analysis of delta-toxin is given in Table 3. The values for serine content and threonine content were determined by extrapolating to zero hydrolysis time. The values for valine and isoleucine are from the 72-h hydrolysis. No detectable carbohydrates were found when the anthrone method was used. Calculations from the amino acid composition showed that the toxin contained a total of 391 amino acid residues.

The basic amino acid residues (arginine, lysine, histidine) and the acidic residues (aspartic acid, glutamic acid) accounted for 11 and 23%, respectively, of the total residues. However, as the pI of the toxin is high (pH 9.1), a substantial

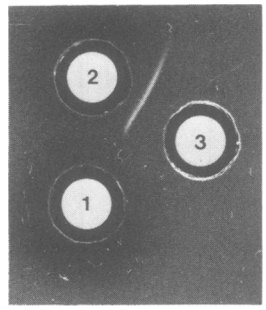

FIG. 4. *Double immunodiffusion analysis of purified delta-toxin. Well 1, Diagnostic standard equine serum against C. perfringens type A; well 2, rabbit serum raised against purified delta-toxin; well 3, purified delta-toxin (40 μg/ml). Each well contained 25 μl of reagent. The sera were undiluted.*

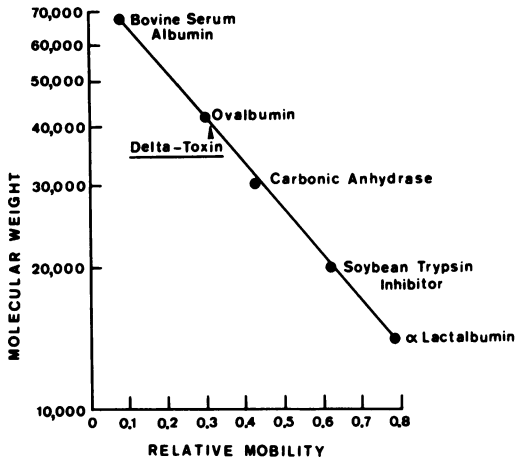

FIG. 5. *Estimation of molecular weight of purified delta-toxin (step 5 of Table 2) by SDS-PAGE. The molecular weights of the marker proteins were 68,000, 43,000, 30,000, 20,100, and 14,400 for bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin, respectively.*

number of the aspartic acid and glutamic acid residues must occur as uncharged asparagine and glutamine. The hydrophobic nonpolar amino acid residues (alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, methionine) accounted for 29% of the total residues (30% on a weight basis).

Sensitivity of erythrocytes to lysis. The purified delta-toxin solution (50,000 HU/ml) was titrated with suspensions of erythrocytes from various species, which were made up to contain the same concentration of hemoglobin as the standard SRBC suspension. The variations in the sensitivities of erythrocytes to toxin were very great (Table 4), in agreement with previous reports (see above). Only sheep, goat, and, to a lesser extent, pig erythrocytes were lysed whereas human erythrocytes and erythrocytes from various other species were practically in-

TABLE 3. *Amino acid composition of delta-toxin*

Amino acid	No. of residues per molecule of toxin[a]
Aspartic acid	52
Threonine	29
Serine	48
Glutamic acid	39
Proline	12
Glycine	52
Alanine	17
Valine	20
Methionine	7
Isoleucine	30
Leucine	17
Tyrosine	10
Phenylalanine	11
Lysine	25
Histidine	8
Arginine	11
Half-cystine	2
Tryptophane	6

[a] Based on a molecular weight of 42,000.

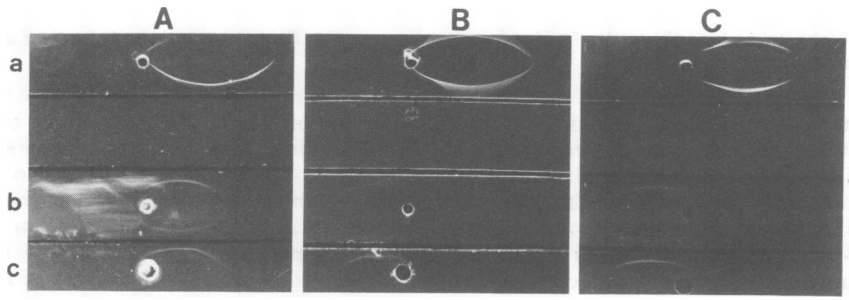

FIG. 6. *Charge shift electrophoresis of purified delta-toxin in the presence of detergents. Wells b and c contained 2 and 5 μg of delta-toxin, respectively, and well a contained 25 μg of purified diphtheria toxin, which was used as a hydrophilic protein control. The anode was at the right. (A) Migration in Triton X-100–sodium deoxycholate. (B) Migration in Triton X-100 alone. (C) Migration in Triton X-100–cetyltrimethylammonium bromide. The sera used were undiluted.*

TABLE 4. *Sensitivity of erythrocytes of different animal species to the lytic activity of a solution of purified delta-toxin*[a]

Species	Lytic activity (HU/ml)	Amt (ng) of toxin required to cause 50% lysis	Relative sensitivity (%)[b]
Sheep	50,000	3	100
Goat	12,500	12	25
Pig	4,160	36	8
Frog	1,000	150	2
Chicken	100	1,500	0.2
Human	20	7,500	0.04
Rabbit	10	15,000	0.02
Horse	8	18,000	0.015
Mouse	8	18,000	0.015
Guinea pig	5	30,000	0.01
Rat	5	30,000	0.01
Monkey	<1	>10^5	<0.002

[a] The density of each erythrocyte suspension was adjusted so that it contained the same hemoglobin content as the standard SRBC suspension (~6 × 10^8 cells per ml). By definition, 1 HU was that amount of toxin needed to release the hemoglobin from 50% of SRBC under the conditions specified. Frog (*Xenopus laevis*) cells were kindly provided by P. Boquet. Human cells were from blood banks, and the cells of other species were supplied by Pasteur Institute Facilities, Garches, France.

[b] Compared with SRBC sensitivity.

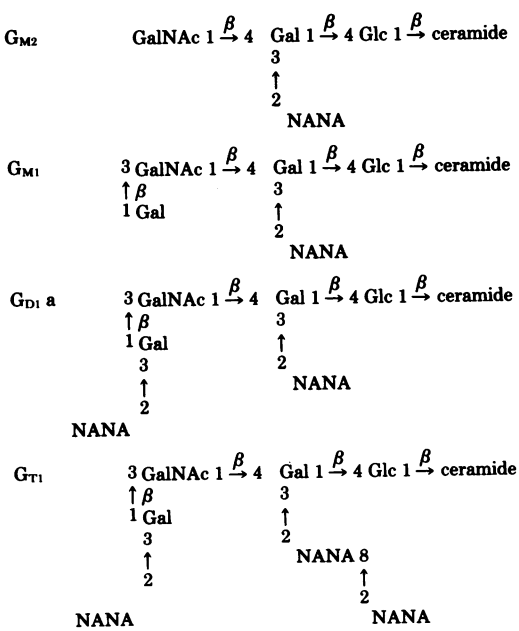

FIG. 7. *Code names and structures of the gangliosides tested. Code names are from Svennerholm (44). Abbreviations: NANA, N-acetylneuraminic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; ceramide, N-acylsphingosine.*

sensitive. However, lysis of the latter cells was observed with amounts of toxin which were 1,000- to 10,000-fold larger than the amount required for lysis of SRBC. Lysis under these conditions was not attributable to traces of alpha-toxin or perfringolysin, as testing was undertaken after prior incubation of delta-toxin with 750 IU of *C. perfingens* type A antitoxin.

Lethal effects. Delta-toxin was injected intravenously (0.1 ml) into Swiss mice weighing 20 to 25 g at doses between 4,000 and 10 HU (12 to 0.03 μg of protein). The toxin was lethal, and in terms of lytic activity, the minimum lethal dose (all four mice tested died) was equivalent to 40 HU (0.12 μg of protein) per mouse. Death occurred within 1 to 2 min. The symptoms produced included respiratory distress with the head in extreme extension. The animals receiving sublethal doses were sick for about 1 h and then recovered. Prior incubation of the toxin for 10 min with rabbit or equine antiserum prevented the lethal effects.

Dermonecrotic tests. Dermonecrotic tests were performed on shaved Hartley guinea pigs (200 to 300 g); 0.1 ml of delta-toxin (300 to 3,000 HU) or control buffer was injected intradermally into the dorsal skin. No apparent lesions were observed up to 96 h after injection.

Effects of various lipids on hemolytic activity. The lytic effect of delta-toxin (1 HU, equivalent to 3 ng in the test system) on SRBC was measured after incubation with a variety of lipid suspensions. No inhibition of lysis was observed with cholesterol (1 mg), egg lecithin (1 mg), and sphingomyelin (200 μg). In contrast, inhibition of toxin activity was observed with G_{M2}, G_{M1}, G_{D1a}, and G_{T1} gangliosides (Fig. 7) and mixed brain gangliosides. However, we found marked differences in 50% inhibitory concentrations between G_{M2} (6 μg/ml, equivalent to 4.5 μmol/liter) on the one hand and mixed brain gangliosides (250 μg/ml), G_{D1a} (60 μg/ml), G_{M1} (70 μg/ml), and G_{T1} (180 μg/ml) on the other.

Effect of metallic ions and other agents on hemolytic activity. We used the procedure described above for the study of lipid effects on the lytic activity of delta-toxin against SRBC. Fe^{2+}, Cu^{2+}, Mg^{2+}, and p-chloromercuribenzoate at concentrations as high as 10^{-3} M had no effect on activity. At the same concentrations inhibition was partial with Zn^{2+} and total with Ca^{2+}. ethylenediaminetetraacetate, citrate, and tartrate ions. Toxin activity was not affected by cysteine or dithiothreitol at concentrations of up to 5 × 10^{-2} M.

DISCUSSION

Although *C. perfringens* delta-toxin was recognized 47 years ago (14) as a distinct hemolysin

among the extracellular products of type C strains, this toxin remained practically unknown until our previous report (see above). This paper provides for the first time a detailed biochemical and biological characterization of delta-toxin.

The culture conditions described above allowed us to obtain large amounts of toxin in the extracellular fluid (~10,000 HU/ml, equivalent to 31 mg of protein per liter). A 200-fold purification was achieved by using a four-step process (Table 2). Some denaturation occurred during the IEF step. Experiments are now in progress to overcome this drawback.

The toxin was obtained with a reasonable yield (5 mg from 1 liter of crude material) in an apparently homogeneous state, as assessed by immunological and electrophoretic tests. The molecular weight of 42,000 determined by SDS-PAGE was substantially higher than the molecular weight (~25,000) estimated from the elution volume of the toxin on a Sephadex G-75 column (Fig. 3) calibrated with several marker proteins. As the toxin is amphiphilic and has a tendency to adsorb non-specifically on surfaces (46), it is likely that the apparent low molecular weight found by gel filtration is incorrect and may have been due to retarded elution on dextran gels. Since artifacts of this type are rare in disc SDS-PAGE, it is probable that the 42,000-dalton value is more accurate.

Delta-toxin is a basic protein (pI ~9.1); this is in contrast to most bacterial cytolysins (2), as well as noncytolytic protein toxins, which are rather acidic or neutral (pI ~4.8 to 7.6) (Alouf, manuscript in preparation). The other basic protein toxins with pI values of >9 known to date are staphylococcal leucocidin F and alpha-, beta-, gamma-, and delta-toxins (36, 48), *Corynebacterium ovis* exotoxin (31), and streptococcal pyrogenic exotoxin B (3).

Charge shift electrophoresis of the toxin in the three-detergent system of Helenius and Simons (16) showed important anodal and moderate cathodal shifts (Fig. 6), indicating that delta-toxin is an amphiphilic protein; this was also shown for diphtheria toxin fragment B by the same technique (5). The amphiphilic character of delta-toxin confirms the tendency of this protein to adsorb strongly on plastic surfaces (46). Using hydrophobic alkylagarose gels (Sepharose C-9) (39), we have observed the total retention of the toxin and its desorption by 0.5 M NaCl (G. Tixier and J. E. Alouf, unpublished data). The amino acid composition of delta-toxin (Table 3) shows that hydrophobic amino acid residues account for 30% of the total residues. This figure is not unusually high, since many hydrophilic proteins have similar or greater proportions of hydrophobic residues (for example, diphtheria toxin fragment A [34%] [9]). However, it has been stated that amphiphilic proteins (which by definition contain defined polar and nonpolar regions [16]) have polypeptide chains made up of long hydrophobic sequences or regions (19). In most cases the hydrophobic regions are thought to serve as membrane anchors (19).

We tested the effects of various lipids on the lytic activity of delta-toxin against SRBC since membrane lipids are known or thought to be involved in the action of a number of bacterial cytolytic toxins (1, 4, 13). Cholesterol, lecithin, and sphingomyelin, which are the receptors or substrates of SH-activated toxins, clostridial alpha-toxin, and staphylococcal beta-toxin, respectively (1), did not inhibit delta-toxin at concentrations as high as 100 μg to 1 mg of lipid per HU (equivalent to 3.1 ng or 7.2×10^{-5} nmol of protein). Under the same conditions, G_{M2} ganglioside, but none of the other gangliosides tested (Fig. 7), appeared to be a significant and possibly specific inhibitor. The inhibition by large amounts of mixed brain and gangliosides and by G_{M1}, G_{1a}, and G_{T1} gangliosides suggests either a nonspecific effect, as described by Cowell and Bernheimer for cereolysin (8), structural similarities to G_{M2} ganglioside, or possible contamination by G_{M2} ganglioside. It should be noted that the 50% inhibitory concentrations of the gangliosides tested do not reflect the actual concentrations of gangliosides required for inhibition. These values might be much lower for the following two reasons: (i) the critical micelle concentrations are very low (10^{-8} to 10^{-10} M) (12) and ganglioside molecules are micellar at the 50% inhibitory concentrations observed, and (ii) serum albumin, which was present in the test system, is reported to interact with gangliosides (12), whereas gelatin does not. However, gelatin could not be used in our work since it showed some inactivation of the toxin. For these reasons, any calculation of the apparent molar ratio of G_{M2} ganglioside to toxin producing inhibition is meaningless. If G_{M2} ganglioside is a specific inhibitor of delta-toxin, we may infer that the molecular basis of this specificity is due to an oligosaccharide sequence terminating in a free *N*-acetylgalactosaminyl residue (Fig. 7).

Various gangliosides have been implicated as the receptors of ligands of many hormones, interferon, and wheat germ agglutinin (12), as well as of several bacterial protein toxins (e.g., cholera toxin and *Escherichia coli* thermolabile enterotoxin [35], tetanus toxin [47], botulinum toxin [24], staphylococcal alpha-toxin [23], group A streptococcal pyrogenic toxin [38], and

Vibrio cholerae hemolysin [18]. A G_{M2} ganglioside-like glycoconjugate has been reported as receptor for the CFA/I and K 99 hemagglutinin of *E. coli* (11).

As Table 4 shows, there are large differences in the sensitivity of the erythrocytes of different animal species to the lytic action of delta-toxin. The most sensitive species were sheep and, to a lesser extent, other even-toed ungulates (goats and pigs). Erythrocytes from many other species were practically insensitive. A level of 1 HU of toxin, which is capable of lysing 50% of 3×10^8 SRBC (280 molecules on the average lyse one cell), lysed less than 0.1% of similar populations of human, rabbit, and horse erythrocytes. Cells with very poor sensitivity might lack specific receptors (possibly G_{M2} ganglioside) for delta-toxin, or the receptors may be very low in number or hidden, as suggested by the correlation between binding of radiolabeled toxin onto cells and their degree of sensitivity (manuscript in preparation). The cells might also differ in their relative distributions of membrane lipids and proteins compared with sensitive erythrocytes. Work is now in progress on delta-toxin interactions with sensitive and insensitive erythrocytes.

ACKNOWLEDGMENTS

This investigation was supported by grant 77.7.126 4 from the Délégation Générale à la Recherche Scientifique et Technique.

We thank Patrice Boquet and Mary K. Johnson for their valuable advice and comments and Borivoj Keil and Nguyen Thanh Tong for amino acid analyses. The technical assistance of Danielle Ahu was appreciated.

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