INFECTION AND IMMUNITY, Apr. 1988, p. 926–929
0019-9567/88/040926-04$02.00/0
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Vol. 56, No. 4

Comparison of Toxins of *Clostridium butyricum* and *Clostridium botulinum* Type E

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Received 16 November 1987/Accepted 8 January 1988

The toxin of *Clostridium butyricum* strains isolated from two infants with botulism is neutralized by antitoxin for type E botulinum toxin. This toxin and that of a *C. botulinum* type E strain were purified by the same protocol. Both toxins were M_r 145,000 proteins which, when activated with trypsin, were composed of an H subunit of M_r 105,000 and an L subunit of M_r 50,000. The activated specific toxicity of purified butyricum toxin based on an intravenous assay was 2×10^8 mouse 50% lethal doses (LD_{50}s)/mg of protein, but that based on an intraperitoneal assay was 7×10^7 LD_{50}s/mg, compared with 6×10^7 LD_{50}s/mg for type E toxin as determined by both methods. Immunodiffusion tests with antitoxin raised with type E toxin indicated that the two toxins were serologically very similar except for a spur formed by type E toxin. The close similarities of the two toxins suggest that toxigenic *C. butyricum* could arise when a wild-type strain, which is normally nontoxigenic, acquires the toxin gene of a *C. botulinum* type E strain.

Although they differ in other important properties, all cultures that produce a neurotoxic protein that causes the flaccid paralysis of botulism are placed in the species *Clostridium botulinum*. The toxin itself differs antigenically and is classified as types A, B, C_1, C_2, D, E, F, and G. Recent findings indicate that toxin type C_2 (11) is cytotoxic instead of neurotoxic, so it more properly belongs in a different toxin category. Since most strains produce one toxin type, the cultures are typed by toxin. Some cultures produce pairs of toxin types and are identified as subtypes, such as Af (5), Ba (4), and Bf (9).

The isolates from three infants with clinically diagnosed botulism add another dimension to the classification of *C. botulinum*. The pathogen of one of these cases produced a toxin that is neutralized by antitoxin raised against type F botulinum toxin, but its other phenotypic properties are more like those of *C. barati* (7). The other two illnesses occurred at different times in Rome, Italy, and were caused by organisms which could be classified as *C. butyricum*, except for the production of a toxin that is neutralized by antitoxin for type E botulinum toxin (1, 8). The *C. butyricum* and *C. barati* toxins are, therefore, antigenically related to the botulinum toxin type used to raise the antiserum that renders them innocuous. However, the degree of similarity in the primary molecular properties of the serologically cross-reactive toxins remains uncertain.

This communication reports the purification of the toxin of the two toxigenic *C. butyricum* isolates. The butyricum toxin was purified by the protocol recently developed for *C. botulinum* type E toxin (6) so that the butyricum and botulinum toxins could be compared without the possibility of the toxin molecules being changed in different ways during purification. The two purified toxins were compared as to (i) sizes of the intact molecules and the disulfide-linked H and L subunits formed when the toxin is trypsinized to activate toxicity and (ii) antigenicities. The nontoxic proteins with which the two toxins naturally form bimolecular complexes were also purified and compared.

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MATERIALS AND METHODS

Cultures. Toxigenic *C. butyricum* 5839 and 5521, wild-type nontoxigenic *C. butyricum* ATCC 19398, and *C. botulinum* type E strain 5545 were kindly provided by C. L. Hatheway, Centers for Disease Control, Atlanta, Ga. They were grown for 5 days at 30°C in cooked meat medium (Difco Laboratories, Detroit, Mich.) and then stored at −20°C as stock cultures.

Purification. Toxins were produced and purified by the method previously described for *C. botulinum* type E toxin (6). The production medium was 12 liters of Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-peptone glucose–yeast extract–sodium thioglycolate, adjusted to pH 6.5. Cells obtained during 55 h of incubation at 30°C were collected and washed with 0.05 M acetate buffer, pH 5.0, both by centrifugation. Toxin in the washed cells was then extracted with 0.2 M phosphate buffer, pH 6.0.

Purification consisted of (i) chromatographing the extracted toxin on DEAE–Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N.J. [Div. Pharmacia, Inc.]) to remove nucleic acid, (ii) treating the pool of toxic percolate fractions on CM–Sepharose CL-6B (Pharmacia) to recover the toxin that is noncovalently associated with a similar size nontoxic protein (toxic complex; reference 12), and (iii) chromatographing the pooled toxic percolate fractions of the previous step on DEAE-Sephacel (Pharmacia) at alkaline pH to separate toxic from nontoxic proteins with an NaCl gradient (6).

Polyacrylamide gel electrophoresis. The M_rs of proteins were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (6). When needed, disulfides were reduced by heating the sample in a boiling water bath for 5.0 min in the presence of 4.0% 2-mercaptoethanol.

Protein and toxicity assays. Protein was determined spectrophotometrically by using an A_{278} of 1.63 for 1 mg of sample per ml in 0.03 M phosphate buffer, pH 6.0 (3). It was also assayed by the Bio-Rad method (Bio-Rad Laboratories, Richmond, Calif.) with standards made with serum albumin.

Specific toxicities were based on protein assayed by spectrophotometry unless otherwise indicated.

Toxicity was assayed in mice of 23 to 25 g by the intravenous (i.v.) time to death method (2). Samples were also titrated by the quantal method of injecting serial twofold dilutions intraperitoneally and obtaining mean 50% lethal doses (LD_{50}s) with the Reed and Muench calculation (10).

Toxin was activated with trypsin (type XIII [tolylsulfonyl phenylalanyl chloromethyl ketone treated]; Sigma Chemical Co., St. Louis, Mo.). Toxin in 0.03 M phosphate buffer, pH 6.0, and trypsin were mixed at a ratio of 2:1 (wt/wt) and incubated at 35°C. Samples of butyricum toxin were incubated for 3 min, and type E toxin was incubated for 30 min. The reported LD_{50}s are for trypsinized samples.

Immunodiffusion test. The gel was 3.5 ml of melted 1.0% agar (certified; Difco) in 0.01 M phosphate buffer, pH 7.4, spread evenly on a glass slide (25 by 76 mm). Wells were 4 mm in diameter, 4 mm from each other, and charged with 20-μl samples.

The antiserum was type E botulinum antitoxin raised with an immunogen made with a relatively impure type E toxin preparation (Centers for Disease Control). The center well was charged with antitoxin, and the outer wells were charged with toxin samples. After 48 h of incubation in a moist chamber at room temperature, the gels were stained for 5 h with 0.05% Coomassie brilliant blue R 250 (Sigma) and then destained with several changes of methanol-water-acetic acid (5:4:1).

RESULTS AND DISCUSSION

Purification. Since the toxins of the two *C. butyricum* strains behaved identically in all chromatographic steps, only the results given by strain 5839 are shown. The chromatographic patterns of the butyricum toxin on DEAE-Sephadex, CM-Sepharose, and DEAE-Sephacel are shown in Fig. 1, 2, and 3, respectively. These patterns and recoveries (Table 1) were comparable to those previously obtained with type E toxin (6).

Polyacrylamide gel electrophoresis. The results of electrophoresing butyricum and type E botulinum toxins at different stages of purification are shown in Fig. 4A. The two DEAE-Sephadex toxins showed two prominent and several light bands (Fig. 4A, lanes 1 and 2). Since CM-Sepharose toxins showed essentially only the two prominent bands (lanes 3 and 4) and had almost all of the toxicity applied to the column, the toxins consisted of bimolecular complexes made of an M_r 145,000 toxin and an M_r 127,000 nontoxic protein. The final chromatography on DEAE-Sephacel (lanes 5 and 6) separated the two proteins of the complex, with the NaCl gradient eluting pure toxin in the first protein peak and the nontoxic protein in the second peak (Fig. 3). When activated with trypsin and treated with 2-mercaptoethanol, the purified toxin samples showed only M_r 105,000 H subunits and M_r 50,000 L subunits (Fig. 4A, lanes 7 and 8).

The M_r of intact butyricum toxin and its H and L subunits therefore appeared comparable to those of type E toxin. Furthermore, when mixtures of the two purified toxins were electrophoresed, the mobilities of the intact molecules and the subunits of the two toxins were indistinguishable (Fig. 4B, lanes 1 and 2).

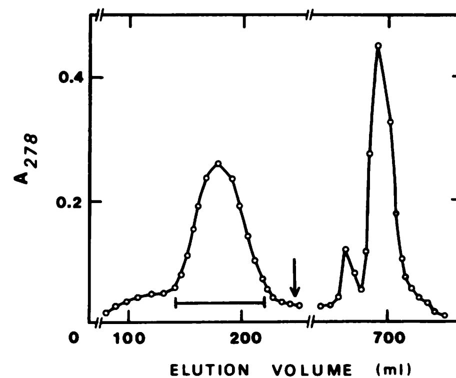

FIG. 2. CM-Sepharose CL-6B chromatography of DEAE-Sephadex toxin. Conditions: column, 3.1 by 43 cm; 0.025 M sodium citrate buffer, pH 6.0; flow rate, 30 ml/h; 6-ml fractions; 20°C. Adsorbed protein was eluted with citrate buffer containing 2.0 M NaCl (arrow). The sample was 60 ml containing 13.0 mg of protein and 2.0×10^9 LD_{50}s. The pool of fractions of the first protein peak (bar) is CM-Sepharose toxin.

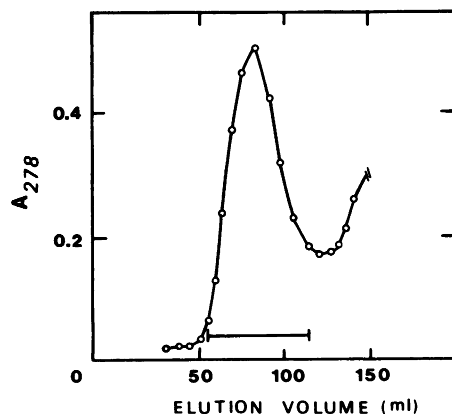

FIG. 1. DEAE-Sephadex chromatography of butyricum toxin in concentrated cell extract. Conditions: column, 2.7 by 55 cm; 0.05 M sodium citrate buffer, pH 5.5; flow rate, 30 ml/h; 6-ml fractions; 20°C. The sample was 40 ml containing 189 mg of protein (Bio-Rad assay) and 2.2×10^9 LD_{50}s. The pool of fractions of the first protein peak (bar) had an A_{260}/A_{278} ratio of 0.53 and is called DEAE-Sephadex toxin.

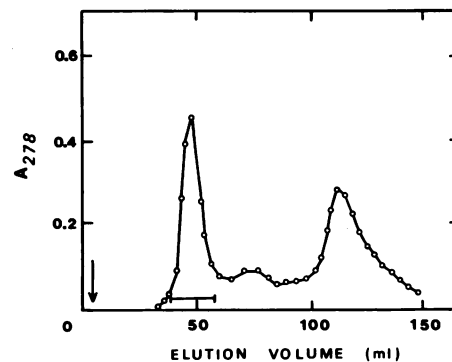

FIG. 3. DEAE-Sephacel chromatography of CM-Sepharose toxin. Conditions: column, 1.6 by 25 cm; 0.01 M sodium phosphate buffer, pH 7.4; flow rate, 30 ml/h; 4-ml fractions; 4°C. The NaCl gradient (started at the arrow) was 80 ml of phosphate buffer plus 80 ml of buffer containing 0.3 M NaCl. The sample was 80 ml containing 7.8 mg of protein and 1.6×10^9 LD_{50}s. The pool of fractions of the first peak (bar) is purified toxin.

TABLE 1. Purification of *C. butyricum* toxin in 12 liters of culture using three chromatographic steps

Treatment	Total		Specific toxicity (10^7 LD_{50}s/mg of protein)
	Protein (mg)	10^6 LD_{50}s[a]	
Concentrated cell extract	189	2,200	1.16
DEAE-Sephadex	13[b]	2,000	15.3
CM-Sepharose	7.8	1,600	20.5
DEAE-Sephacel	2.75	605	22.0
		192[c]	7.0

[a] i.v. titrations.
[b] Obtained by the Bio-Rad assay; the others were obtained spectrophotometrically.
[c] Intraperitoneally determined toxicity.

Toxicity. Trypsinization of all toxin preparations increased toxicity about 200-fold, but butyricum and type E toxins reached maximal toxicities at different incubation times. With a 2:1 (wt/wt) toxin-enzyme ratio and a temperature of 35°C, full activation of butyricum toxin occurred in less than 5 min, while type E toxin required at least 20 min.

Although the phenomenon did not occur with less purified preparations, the toxicity of purified butyricum toxin depended on the assay method such that the i.v. assayed value was about three times higher than the intraperitoneally determined toxicity (Table 1). The difference could not be eliminated by using other toxin-enzyme ratios and incubation times.

Even the higher toxicity of purified toxin obtained with the i.v. assay was less than that theoretically possible; specific toxicity was only 1.1 times that of CM-Sepharose toxin instead of the twofold difference expected when the nontoxic protein of the bimolecular complex is removed. Type E toxin was different in this respect; the last purification step increased its toxicity to the expected degree and, when it was purified, it showed comparable toxicities in the two assay methods (6). The trypsinization times needed for full activation also indicated some difference between the two purified toxins.

Nevertheless, when i.v. assayed toxicities were used, the specific toxicity calculated for butyricum toxin was about three times that of type E toxin (22×10^7 versus 6×10^7/mg of protein). As previously reported for type E toxin, the specific toxicity of butyricum toxin on the basis of the i.v. assayed LD_{50} was about 15% higher when the calculation used protein amounts determined by the Bio-Rad method instead of by spectrophotometry.

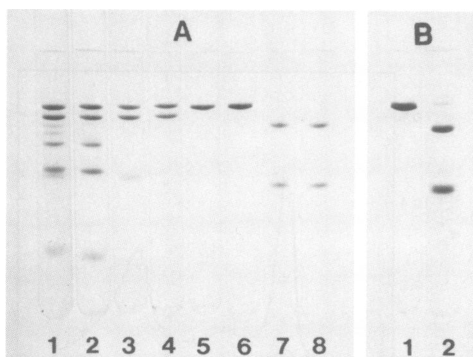

FIG. 4. (A) Polyacrylamide gel electrophoresis of 12 μg of butyricum (odd-numbered lanes) and type E botulinum (even-numbered lanes) toxins obtained at different steps. Lanes: 1 and 2, DEAE-Sephadex toxins; 3 and 4, CM-Sepharose toxins; 5 and 6, DEAE-Sephacel (purified) toxins; 7 and 8, DEAE-Sephacel toxins trypsinized and reduced with 2-mercaptoethanol. (B) Lanes: 1, coelectrophoresis of purified butyricum and type E toxins (12 μg each); 2, coelectrophoresis of the two purified toxins that were trypsinized and reduced with 2-mercaptoethanol.

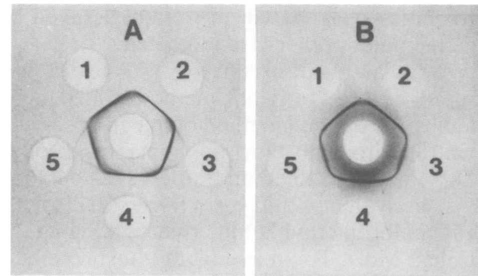

FIG. 5. (A) Immunodiffusion tests of purified butyricum and type E botulinum toxins. The center well was charged with 5 IU of type E antitoxin. Wells 1, 2, and 4 contained 5 μg of type E toxin, and wells 3 and 5 contained 5 μg of butyricum toxin. (B) Immunodiffusion tests of nontoxic proteins of a *C. butyricum* toxigenic strain and *C. botulinum* type E. The center well was charged with type E antitoxin. Wells 1, 2, and 4 contained 4 μg of type E nontoxic protein, and wells 3 and 5 contained 4 μg of butyricum protein.

Antigenicity. When reacted with antitoxin raised against type E toxin, each of the two toxins formed a single immune precipitate line which joined in a line of identity except for a spur formed by type E toxin (Fig. 5A). The spur indicated that the known type E toxin has an antigen that is absent in butyricum toxin. Antitoxin raised against butyricum toxin was not available, so that it was not possible to determine whether butyricum toxin has an antigen not shared by type E toxin.

The M_r 127,000 nontoxic proteins (Fig. 5B) in the bimolecular complexes of the two toxins, recovered in the second protein eluted from DEAE-Sephacel (Fig. 3), were antigenically indistinguishable (Fig. 5B). This nontoxic protein is apparently not produced by nontoxigenic *C. butyricum*, since the concentrated cell extract of strain ATCC 19398 did not form a precipitate line in the immunodiffusion test (data not shown). The absence of a precipitate line also indicated that nontoxigenic *C. butyricum* does not produce a protein whose antigens include one that is serologically related to an antigen in type E botulinum toxin.

The present results indicate that the toxigenic *C. butyricum* strains produce a toxin very similar, although not identical, to the toxin of a known *C. botulinum* type E strain. Since *C. butyricum* strains are normally nontoxigenic, the similarities of the toxins make it possible to suggest that the toxigenic *C. butyricum* strains resulted when nontoxigenic strains acquired the toxin gene of *C. botulinum* type E. This interspecies transfer of genetic material probably includes the gene for the nontoxic protein with which type E botulinum toxin normally forms bimolecular complexes.

ACKNOWLEDGMENTS

This study was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by contributions of food companies.

J. A. Giménez is a fellow of the Universidad de Buenos Aires and Consejo Nacional de Investigaciones Cientificas y Técnicas de la República Argentina.

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