Volume 252, number 1,2, 83–87 FEB 07411 July 1989

Immuno-crossreactivity between botulinum neurotoxin type C1 or D and exoenzyme C3

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Received 1 June 1989

Botulinum neurotoxin type D and exoenzyme C3 have been separately purified from *Clostridium botulinum* strain D-1873 to apparent homogeneity. Both ADP-ribosylated a rat liver cytosolic protein of 24 kDa. The N-terminal amino acid sequence of C3 was determined and showed a low degree of homology with those of the light and heavy chains of neurotoxins of various types which have been reported previously. However, a polyclonal antibody raised against C3 cross-reacted with the light chains, but not with the heavy chains, of type C1 and D neurotoxins. Furthermore, a monoclonal antibody recognizing the light chains of type C1 and D neurotoxins interacted with C3. These results suggest that the light chain of type C1 or D neurotoxin and exoenzyme C3 share at least one epitope in common with each other.

Botulinum neurotoxin; Exoenzyme C3; ADP-ribosylation; Monoclonal antibody

1. INTRODUCTION

Botulinum neurotoxin is a potent exotoxin produced by *Clostridium botulinum* and has been classified into seven groups (types A–G) on the basis of antigenicity [1]. The molecular masses of all of the types are approx. 150 kDa. They consist of a single polypeptide chain, which is nicked by the action of proteases released from the organisms or artificially added. The nicked neurotoxin is composed of two polypeptide chains, viz. the heavy (approx. 100 kDa) and light (approx. 50 kDa) chains, which can be separated from each other by disulfide reduction. It has been proposed that the neurotoxin shows toxic activity through inhibition of acetylcholine release at peripheral synapses [1]. Detailed mechanisms of inhibition of acetylcholine release, however, have not been well elucidated.

Recently, botulinum type C1 and D neurotoxins have been shown to ADP-ribosylate GTP-binding proteins of 21–26 kDa [2–4]. On the other hand, it has been reported that ADP-ribosyltransferase activity may originate from another enzyme, exoenzyme C3, produced by the same bacteria [5] and, therefore, it has been proposed that ADP-ribosylation is not relevant to the neurotoxicity [6].

Here, we performed an investigation of immunoreactivity between botulinum neurotoxin type C1 or D and exoenzyme C3, together with a comparison of their N-terminal amino acid sequences in order to ascertain whether they are structurally related to each other. The present paper describes several lines of evidence for the presence of at least one common epitope in both type C1 and D neurotoxins and exoenzyme C3.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

[^{32}P]NAD (800 Ci/mmol) was purchased from New England Nuclear; QAE-Sephadex A-50, Sephadex G-75 and G-200, Mono Q and S from Pharmacia; DEAE-cellulose, affigel-protein A and 3,3'-diaminobenzidine from Brown, Bio-Rad and Wako (Osaka), respectively; and peroxidase-conjugated anti-mouse and anti-rabbit IgGs from Dakopatt.

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2.2. *Assay for ADP-ribosylation*

ADP-ribosylation was performed according to Matsuoka et al. [7] using rat liver cytosolic proteins as substrates.

2.3. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the presence of 0.1% SDS on 8 or 15% polyacrylamide gels as in [8] using bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (26 kDa) and myoglobin (17 kDa) as molecular mass standards. Gels were stained with Coomassie brilliant blue R-250 [9] and subjected to autoradiography.

2.4. *Purification of botulinum type C1 and D neurotoxins and exoenzyme C3*

Botulinum type C1 and D neurotoxins were purified from culture supernatants of *C. botulinum* strain C-Stockholm and strain D-1873, respectively, as described [10].

Exoenzyme C3 was purified from the culture supernatant of strain D-1873. Ammonium sulfate was added to the supernatant to 55% saturation. The resulting pellet was collected by centrifugation, suspended in borax-phosphate buffer (42 mM $Na_2B_4O_7$-22 mM NaH_2PO_4, pH 8.0) and applied to a column (6.5 × 42 cm) of Sephadex G-75 previously equilibrated with the same buffer, which was also employed in developing of the column. Fractions containing C3 were pooled and applied to a column (2.2 × 55 cm) of QAE-Sephadex A-50 previously equilibrated with the above buffer. C3 was detected in the breakthrough fractions. Fractions containing C3 were pooled and applied to a column (0.5 × 5 cm) of Mono S previously equilibrated with 20 mM phosphate buffer (pH 7.5). After washing the column with the same buffer, adsorbed materials were eluted with a linear gradient of 0–0.5 M NaCl. Fractions containing C3 were pooled and applied to a column (2.2 × 84 cm) of Sephadex G-75 previously equilibrated with 20 mM Tris-HCl (pH 7.5) and the column developed with the same buffer. A single peak of C3 was eluted, which was superimposable with a protein peak. Fractions containing C3 were pooled and used as a preparation of purified C3. The purified C3 gave a single band on SDS-PAGE, suggesting apparent homogeneity.

2.5. *Determination of N-terminal amino acid sequence of exoenzyme C3*

The N-terminal amino acid sequence of C3 was determined on an Applied Biosystems 470A automatic protein sequencer and a 120A phenylthiohydantoin derivative analyzer.

2.6. *Enzyme-linked immunosorbent assay (ELISA)*

ELISA for detection of antibody was carried out as described by Oguma et al. [11] except that peroxidase-conjugated anti-mouse or anti-rabbit IgG was used as second antibody. Peroxidase reaction was performed at room temperature for 30 min in 10 mM citrate-25 mM Na_2HPO_4 using both 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (0.2 mg/ml) and H_2O_2 (0.02%) as substrates. Subsequently, the absorbance at 405 nm was measured.

2.7. *Preparation of monoclonal antibody*

Methods of immunization, details of formation and cloning of hybridoma cell lines and preparation of ascites fluid have been described [12]. In this study, we employed five monoclonal antibodies, two (N-CDA-5 and N-CDA-135) of which are reactive with the light chains of type C1 and D neurotoxins, the other three (N-CDA-14, N-CDA-69, N-CDA-175) being reactive with the heavy chains [12]. Each monoclonal antibody was used after isolation from ascites fluids by two stepwise chromatographies on Mono Q and Mono S columns: ascites fluids were diluted 10-fold with 20 mM triethanolamine-HCl (pH 7.7) and applied to a column (0.5 × 5 cm) of Mono Q previously equilibrated with the same buffer. After extensive washing of the column with the same buffer, adsorbed antibody was eluted with a linear gradient of 0–0.5 M NaCl. Fractions containing antibody were dialyzed against 20 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (Bes)-NaOH (pH 6.0) and applied to a column (0.5 × 5 cm) of Mono S previously equilibrated with 20 mM Bes-NaOH (pH 6.0). Adsorbed antibody was eluted with a linear gradient of 0–0.25 M NaCl. Fractions containing antibody were pooled and used as a purified monoclonal antibody preparation. Detection of antibody was performed by ELISA as described above.

2.8. *Preparation of polyclonal antibodies against botulinum type D neurotoxin and exoenzyme C3*

Rabbit antiserum against botulinum neurotoxin type D was prepared as in [11]. Anti-exoenzyme C3 serum was prepared as follows: C3 (100 μg) was emulsified with an equal volume of Freund's incomplete adjuvant and injected subcutaneously. An initial injection was followed by intravenous boosters of 20 μg each without adjuvant at the third and each subsequent weeks. Anti-neurotoxin and anti-C3 IgGs were isolated from their respective rabbit antisera by chromatography on Affigel-protein A (antibody purification kit MAPS II, Bio-Rad) before use.

2.9. *Immunoblot analysis*

Immunoblotting was performed according to Towbin et al. [13] with peroxidase-conjugated anti-mouse or anti-rabbit IgG as second antibody and both H_2O_2 and 3,3′-diaminobenzidine as peroxidase substrates.

2.10. *Protein determination*

Protein was determined by the method of Lowry et al. [14] using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Recently, two conflicting proposals concerning ADP-ribosylation have been presented; one viewpoint considers botulinum neurotoxin to possess ADP-ribosyltransferase activity [2–4], whereas the other suggests that this activity originates from exoenzyme C3 rather than from neurotoxin [5,6]. In order to clarify this discrepancy, we isolated both botulinum type D neurotoxin and exoenzyme C3 from culture supernatants of the same strain, D-1873, and measured their ADP-ribosyltransferase activities. Highly purified preparations of neurotoxin and C3 gave two bands of 50 and 100 kDa, and a single band of 25 kDa, respective-

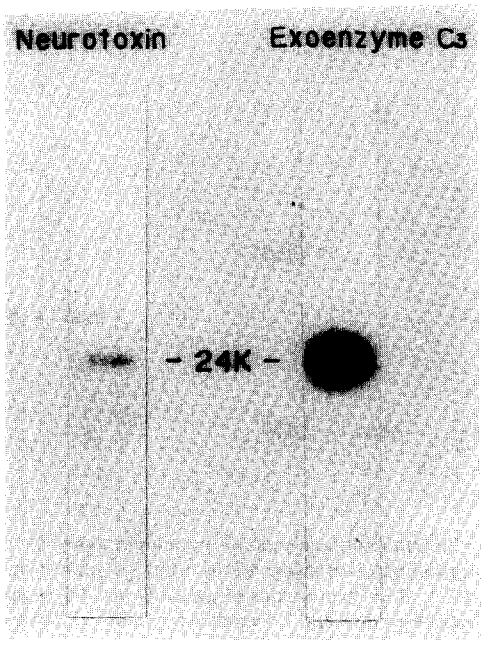

Fig.1. ADP-ribosylation of 24 kDa protein of rat liver cytosol by botulinum neurotoxin type D (10 μg/ml) and exoenzyme C3 (1 μg/ml). Electrophoresis was carried out on 15% polyacrylamide gel in the presence of 0.1% SDS.

ly, on SDS-PAGE (not shown). Both the purified neurotoxin and C3 were found to ADP-ribosylate a protein of 24 kDa among rat liver cytosolic proteins (fig.1) although the activity of the former preparation was less than 1% of that of the latter. Although one cannot ascertain whether the neurotoxin would actually exhibit ADP-ribosyltransferase activity, we examined the possibility of structural similarity existing between the neurotoxin and C3.

Firstly, the N-terminal amino acid sequence of C3 was determined and compared with those reported for both chains of neurotoxins of several types [15,16]. As shown in table 1, little homology was detectable between C3 and any of the chains of neurotoxins of various types, at least with respect to comparisons of N-terminal amino acid sequences.

Secondly, immuno-crossreactivity between the neurotoxin and C3 was examined by immunoblotting using five monoclonal antibodies raised against the neurotoxin (fig.2) and polyclonal antibody against C3 (fig.3). Among the monoclonal antibodies used, one (N-CDA-5) which commonly reacts with the light chain components of type C1 and D neurotoxins [12], recognized C3 (fig.2). In contrast, all monoclonal antibodies specific for the heavy chains showed little crossreactivities with C3 (fig.2). The fact that polyclonal antibody raised against type D neurotoxin was able to recognize C3 (not shown) supports the above observation on the reactivity of anti-light chain monoclonal antibody. Furthermore, it was found that anti-C3 polyclonal antibody recognized not only C3 but also the light

Table 1

N-terminal amino acid sequences of exoenzyme C3 and botulinum neurotoxins

Protein	Amino acid sequence		
	1	10	20
C3	A-Y-S-N-T-Y-Q-E-F-N-T-N-I-D-Q-A-K-A- -G-N-A-Q-		
LC			
Type A[a]	P-F-V-N-K-Q-F-N-Y-K-D-P-V-N-G-V-D-		
Type B[a]	P-V-T-I-N-N-F-N-Y-N-D-P-I-D-N-N-		
Type C[b]	-I-T-I-N-N-F-N-Y-S-D-P-V-D-N-K-N-I-L-Y-L-D-T-H-L-		
Type E[a]	P- -K-I-N-S-F-N-Y-N-D-P-V-N-D-R-T-I-L-Y-I-		
HC			
Type A[a]	A-L-N-D-L-C-I-K-V-N-N-I-D-L-K-F-		
Type B[a]	A- -P-G-I-C-I-D-V-D-N-E-D-L-F-F-I-A-D-		
Type E[a]	K-S- - -I-C-I-E-I-N-N-G-E-L-F-		

[a] Data from [15]
[b] Data from [16]

LC and HC: light and heavy chain, respectively, of neurotoxin

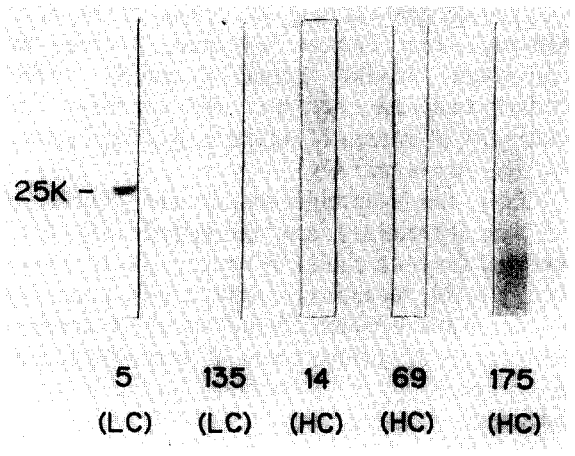

Fig.2. Immunoblot analyses of exoenzyme C3 by using monoclonal antibodies reactive with botulinum type C1 and D neurotoxins (N-CDA-5, N-CDA-135, N-CDA-14, N-CDA-69, N-CDA-175). C3 (5 μg) was electrophoresed on 15% polyacrylamide gel under reducing conditions in the presence of 0.1% SDS. Immunoblotting was performed as in [13]. LC and HC: monoclonal antibodies specific for light and heavy chain, respectively, of neurotoxin.

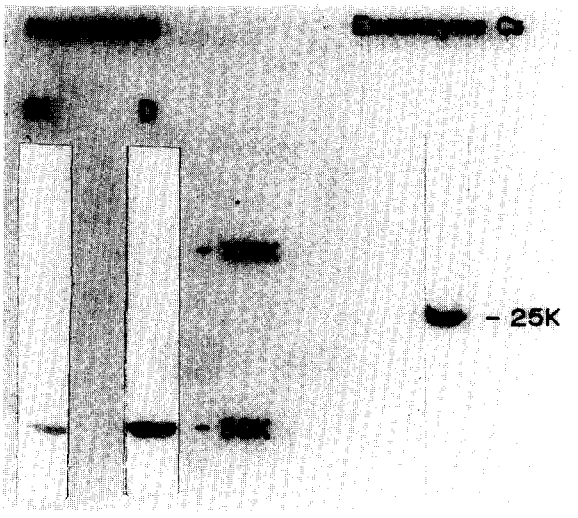

Fig.3. Immunoblot analyses of botulinum type C1 and D neurotoxins and exoenzyme C3 by using polyclonal antibody against C3. Both neurotoxins (5 μg each) and C3 (5 μg) were electrophoresed on 8 and 15% polyacrylamide gel, respectively, under reducing conditions in the presence of 0.1% SDS. Immunoblotting was according to [13].

chains of type C1 and D neurotoxins, whereas it was unable to do so in the case of the heavy chains (fig.3). The results on the reactivity of anti-C3 polyclonal antibody appear to be inconsistent with those reported by Rosener et al. [5] and Rubin et al. [17] that polyclonal antibody against C3 was unable to recognize type C1 neurotoxin. The fact that anti-C3 antiserum, reported by Rosener et al. [5], almost completely inhibited the ADP-ribosyltransferase activity of C3 at a dilution of 1:1000, whereas our anti-C3 antibody showed only about 20% inhibition at almost the same dilution (not shown), would suggest the possibility that these two anti-C3 polyclonal antibodies may recognize different epitopes in the C3 molecule, although the reason for the discrepancy has not been elucidated.

In conclusion, the light chains of botulinum type C1 and D neurotoxins and exoenzyme C3 have at least one common antigenic determinant, perhaps present in the interiors of their amino acid sequences. Our preliminary result that N-CDA-5 monoclonal antibody recognizing C3 is unable to inhibit ADP-ribosyltranferase activity of C3 would imply that common epitope(s) may be present outside the active site in C3. Whether or not botulinum neurotoxin has intrinsic ADP-ribosyltransferase activity remains unclear. Analyses of the active sites for ADP-ribosyltransferase activity of the neurotoxin and C3, together with determination of the amino acid sequence(s) of common epitope(s), would help to clarify this point.

Acknowledgement: We are grateful to Dr I. Matsuoka, Hokkaido University, for helpful discussions.

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