Heterogeneities of Two Components of C₂ Toxin Produced by *Clostridium botulinum* Types C and D

By IWAO OHISHI* AND YUKITAKA OKADA†

College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan

(Received 2 April 1985; revised 26 July 1985)

Botulinum C₂ toxin (C2T) is composed of two dissimilar protein components, designated components I and II, which are linked with neither covalent nor noncovalent bonds. The heterogeneity of these two components of C2T produced by *Clostridium botulinum* type C and D strains was examined. Of 21 strains examined, 19 strains produced the two components, while the others produced neither component I nor component II. The 19 producers of C2T could be divided into three groups based on the differences in antigenicity, molecular weight and biological activity of components I and II. The results provide evidence of heterogeneity in the molecular structure of the two components of C2T, which is possibly a cause of the differences in the biological activity of the toxin observed in different strains.

INTRODUCTION

Most isolates of *Clostridium botulinum* types C and D produce C₂ toxin (C2T), in addition to their respective C₁ and D neurotoxins (Eklund & Poysky, 1972; Jansen & Knoetze, 1977; Ohishi & Sakaguchi, 1982). C2T is also produced by strains producing no C₁ or D toxin; these strains could be obtained *in vitro* by curing types C and D of their prophages (Eklund & Poysky, 1972) and were also isolated from soil in Japan (Nakamura *et al.*, 1978).

C2T is composed of two separate protein components, designated components I and II (Ohishi *et al.*, 1980). The toxin, as well as being lethal, has novel biological activities, including enterotoxic, cytotoxic and vascular permeability activities, all of which are elicited by the co-operation of the two components; the components alone have very little activity, but, together, act as a toxin of high activity (Ohishi *et al.*, 1980, 1981, 1984; Ohishi, 1983). These findings indicate that C2T differs entirely in molecular structure and in biological activity from any neurotoxins of *C. botulinum* types A through F. Indeed, these biological and molecular characteristics of C2T were obtained from studies on toxin purified from strain 92-13, which produces neither C₁ nor D neurotoxin (Nakamura *et al.*, 1978). In preliminary studies we observed that the two components of C2T produced by certain strains of *C. botulinum* types C and D differed immunologically from those of strain 92-13. In the present study, therefore, we attempted to compare the properties of the two components of C2T elaborated by *C. botulinum* type C and D strains.

METHODS

Bacterial strains. Strains of *C. botulinum* types C and D and their sources were as follows: type C strain Stockholm and type D strain 1873 from M. Magot, Institut Pasteur, Paris; type C strains 6812, 6813, 6814 and 6816 from K. Oguma, Sapporo Medical College, Sapporo, Japan; type D strain N24 from D. F. Giménez, Universidad Nacional de San Luis, San Luis, Argentina; type C strains 4946 and C_β and type D strain OP from C. M. Cameron, Veterinary Research Institute, Onderstepoort, South Africa; type C strains CB1, CB11, CB13,

† Present address: Takeda Chemical Industries Ltd, Juso-Honmachi, Yodogawa-ku, Osaka 532, Japan.

Abbreviations: C2T, C₂ toxin; CCS, concentrated culture supernate.

CB15 and CB19 and type D strain CB16 from T. Itoh, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo; strains 92-13, 98-2 and 93-1, which produce no C₁ but C2T, from S. Nakamura, School of Medicine, Kanazawa University, Kanazawa, Japan. *C. botulinum* type C strain Stockholm and type D strain 1873 were treated with acridine orange (AO) according to the method described by Eklund *et al.* (1971); these strains, StockholmAO and 1873AO, stopped producing C₁ and D neurotoxins, respectively, when screened for neurotoxin by neutralization with anti-C₁ and anti-D toxin sera. All strains were maintained in cooked meat medium (Difco Laboratories) supplemented with 1% (w/v) glucose, 1% (w/v) ammonium sulphate and 0.2% cysteine hydrochloride, pH 7.6, and stored at -20 °C.

Cultural conditions. To produce C2T, the stock culture (0.1 ml) was inoculated into the medium (10 ml) prepared as described above. After incubation for 2 d at 37 °C, the culture supernate obtained by centrifugation at 12000 g for 20 min was concentrated to approximately one-tenth of its original volume with Ficoll (Pharmacia) at 4 °C, dialysed against 50 mM-potassium phosphate/sodium phosphate buffer, pH 7.3, and stored at -20 °C until used.

Agar gel double immunodiffusion. Double immunodiffusion tests were done in 1% (w/v) Noble agar (Difco) containing 100 mM-NaCl and 50 mM-potassium phosphate/sodium phosphate buffer, pH 7.3. Wells, 7.5 mm from centre to centre, contained 25 µl of either antigen or antiserum. Gels were incubated at 4 °C for 1 to 2 d in a humid atmosphere in a Petri dish. For enhancement of precipitin lines, the immunodiffusion plates were stained in 0.1% Thiazine red in 7% (v/v) acetic acid, followed by destaining with 7% (v/v) acetic acid, soaking in 1% (v/v) glycerol/7% (v/v) acetic acid for 1 h and drying at 37 °C overnight.

Anti-component I and anti-component II sera. Antisera against components I and II of strain 92-13 were prepared in rabbits as described previously (Ohishi, 1983).

Electrophoretic immunoblotting. The electrophoretic immunoblotting of two components of C2T was done according to the method of Towbin *et al.* (1979). The first electrophoresis of concentrated culture supernate (CCS) was done in 7.5% (w/v) polyacrylamide gel containing 0.1% SDS by the method of Weber & Osborn (1969); the sample, in 8 M-urea and 0.1% SDS, was applied to the gel plate and was run at 60 mA for 6 h at room temperature. The separated proteins were then transferred electrophoretically to a nitrocellulose sheet (0.45 µm pore size; Toyo, Osaka, Japan) at 40 V for 18 h at 4 °C. Transferred components I and II were detected by an immunological staining method; the sheet was soaked in 0.1% Tween 20 in 0.15 M-NaCl-10 mM-Tris/HCl, pH 7.4, (buffer A) containing 3% (w/v) gelatin for 30 min at room temperature to block the binding sites for protein and rinsed in buffer A. The sheet was incubated at room temperature for 30 min with antiserum to either component I or II appropriately diluted with buffer A. After washing with buffer A, the sheet was incubated at room temperature with horseradish peroxidase coupled antibody against rabbit IgG (Cappel Laboratories, West Chester, Pa., USA). For colour reaction, the blot was washed with buffer A and soaked in buffer A containing 0.02% H₂O₂ and 0.05% 4-chloro-1-naphthol, which was prepared fresh. The reaction was terminated after 20 to 30 min by washing with water. The molecular weights of components I and II were determined by SDS-PAGE by comparing their relative mobilities to the following protein standards: phosphorylase a (mol. wt 94000); bovine serum albumin (67000); ovalbumin (45000) and chymotrypsinogen A (25000). These protein standards were detected by staining the blot with 0.1% amido black containing 45% (v/v) methanol and 10% (v/v) acetic acid and by destaining with 7% (v/v) acetic acid.

Assay for vascular permeability activity. Increase in vascular permeability produced by C2T in the culture supernate was determined by the method described previously (Ohishi *et al.*, 1981). The culture supernate was treated with trypsin, since this enhances the activity of the toxin. The reaction mixture contained 0.2 ml sample and 200 µg trypsin (type III; Sigma) in a final volume of 1.0 ml 50 mM-potassium phosphate/sodium phosphate buffer, pH 8.0. After incubation at 35 °C for 30 min, the enzyme reaction was terminated by adding 400 µg soybean trypsin inhibitor (Worthington).

Preparation of two components of C2T. Components I and II of C2T were purified from the culture fluid of strain 92-13 as described by Ohishi *et al.* (1980).

Enzyme-linked immunoassay. Alkaline phosphatase-IgG conjugate was prepared by the method of Engvall & Perlmann (1971). Polypropylene balls, 6.0 mm in diameter, were coated for 18 to 24 h at 4 °C with affinity purified IgG, at a protein concentration of 50 µg ml⁻¹, in 0.15 M-potassium phosphate/sodium phosphate buffer, pH 7.5, containing 0.1% NaN₃. After transferring into glass test tubes, the balls were washed four times with 500 µl 10 mM-potassium phosphate/sodium phosphate buffer, pH 7.0, containing 0.1% NaN₃, 1% (w/v) BSA, 1 mM-MgCl₂ and 0.1 M-NaCl (buffer B). Culture supernate (50 to 200 µl) and buffer B (400 µl) were then added to the test tubes and incubated at room temperature for 1 h. Each ball in the test tube was again washed four times with 500 µl buffer B, and then 10 µl alkaline phosphatase-IgG conjugate and 400 µl buffer B were added. After incubation for 1 h at room temperature, excess conjugate was removed by washing the balls with buffer B before they were transferred into another test tube. Then, 100 µl 3.8 mM-sodium *p*-nitrophenyl phosphate in 0.1 M-Na₂CO₃/NaOH, pH 10.0, was added. The test tubes were incubated at 35 °C for 1 h, and then 1.0 ml 1 M-NaOH was added. The absorbance of the fluid was measured at 400 nm (1 cm cuvette; Beckman DB-T spectrophotometer). Components I and II were diluted serially with buffer B and used as standards in every assay.

A buffer blank was assayed to determine the nonspecific colour reaction, which was subtracted from the absorbance of the sample. Absorbance readings of all replicates were averaged and the component concentration was calculated from the standard curve, which was obtained by plotting the absorbance against the log concentration of the standards. No cross-reaction in the assay system between components I and II was observed up to 500 ng of component per test tube.

RESULTS

All agar gel double immunodiffusion tests and immunoblotting analyses were repeated five to six times; representative results are shown.

Immunodiffusion analyses

Agar gel double immunodiffusion patterns of CCSs of 21 strains against anti-component I serum are shown in Fig. 1(a-d). Reactions of identity were formed between CCS of strain 92-13 and the CCSs of strains 98-2, 93-1, 6812, 6813, 6814, 6816 and N21 (subsequently termed group 1), and between the CCS of strain 92-13 and the CCSs of strains 4946, OP, C_β, Stockholm, StockholmAO, 1873 and 1873AO (subsequently termed group 2). Reactions of partial cross-reactivity were formed between CCS of strain 92-13 and CCSs of strains CB1, CB11, CB13 and CB15 (subsequently termed group 3). All of these strains formed a single precipitin line against anti-component I serum. No precipitin line was formed with CCSs of strains CB19 and CB16 against anti-component I serum. Immunodiffusion patterns of representative strains of each group against anti-component I serum are shown in Fig. 1(e); reactions of identity were formed between CCSs of group 1 strains 92-13 and 6812 and group 2 strains 4946 and 1873, and between CCSs of group 2 strains and group 3 strains CB13 and CB15, whereas reactions of partial cross-reactivity were formed between CCSs of group 1 strains and those in group 3.

Double immunodiffusion patterns of the CCSs against anti-component II serum are shown in Fig. 2(a-d). Reactions of identity were formed between the CCS of strain 92-13 and the CCSs of group 1 strains. Reactions of partial cross-reactivity were formed between CCS of strain 92-13 and the CCSs of group 2 strains, and between the CCS of strain 92-13 and the CCSs of group 3 strains. All of the strains in groups 1 to 3 produced a single precipitin line against anti-component II serum, and no precipitin line was formed with CCSs of strains CB19 and CB16. Immunodiffusion patterns of representative strains of each group against anti-component II serum are shown in Fig. 2(e); reactions of partial cross-reactivity were formed between CCSs of group 1 strains 92-13 and 6812 and those of group 2 strains 4946 and 1873, and between CCSs of group 1 strains and those of group 3 strains CB13 and CB15 whereas reactions of identity were formed between CCSs of strains in groups 2 and 3.

Immunoblotting analyses

Figure 3(a, b) shows immunoblotting patterns of the CCSs of 21 strains stained with anti-component I and II sera, respectively. Component I produced by all strains, except for strains CB19 and CB16, gave one band, with an electrophoretic mobility the same as that of strain 92-13; strains CB19 and CB16 gave no band. The molecular weight of component I, estimated from the relative mobility, was 55000. Component II of the strains in groups 1 and 3 gave a single band with the same electrophoretic mobility as that of strain 92-13, whereas component II of the group 2 strains gave one band with a different electrophoretic mobility from that of component II of the strains in groups 1 and 3. The molecular weights of component II of strains in groups 1 and 3 and component II of group 2 strains were estimated as 105000 and 95000, respectively.

Vascular permeability activity

To determine the biological activity of C2T, the vascular permeability activity, which is highly specific for the detection of C2T in the culture supernate of type C and D strains (Ohishi & Sakaguchi, 1982), was assayed. In addition, the production of components I and II by type C and D strains was also determined by enzyme-linked immunoassay. The results are shown in Table 1. Although group 1 strains were high toxin producers, the specific activity of components

Fig. 1. Double immunodiffusion of anti-component (I) serum (centre well) with CCSs of *C. botulinum* type C and D strains. (a) 1, 92-13; 2, N21; 3, 6812; 4, 1873; 5, Stockholm; 6, 4946. (b) 1, 92-13; 2, 6813; 3, 6814; 4, 6816; 5, 1873AO; 6, StockholmAO. (c) 1, 92-13; 2, 98-2; 3, 93-1; 4, OP; 5, C_β; 6, CB19. (d) 1, 92-13; 2, CBI; 3, CBI1; 4, CBI3; 5, CBI5; 6, CBI6. (e) 1, 92-13; 2, CBI3; 3, 1873; 4, 6812; 5, CBI5 and 6, 4946.

Fig. 2. Double immunodiffusion of anti-component (II) serum (centre well) with CCSs of *C. botulinum* type C and D strains. Wells 1-6 in (a)-(e) are as in Fig. 1.

Fig. 3. Immunoblotting analyses of components I and II in CCSs of *C. botulinum* type C and D strains. Proteins were transferred electrophoretically from an SDS-polyacrylamide gel to a nitrocellulose sheet and components I and II were stained with (a) rabbit anti-component I serum or (b) anti-component II serum; they were then reacted with horseradish peroxidase coupled antibody against rabbit IgG, and with peroxidase substrate (see Methods). The lanes in (a) and (b) are as follows. 1, 6812; 2, CB1; 3, 4946; 4, 1873; 5, 1873AO; 6, Stockholm; 7, StockholmAO; 8, N21; 9, C_β; 10, OP; 11, 6813; 12, 6814; 13, 6816; 14, 92-13; 15, 98-2; 16, 93-1; 17, CB19; 18, CB16; 19, CB11; 20, CB13; 21, CB15.

I and II of these strains was lower than that of group 2 strains (Table 1). The amount of C2T produced by group 3 strains, as determined by enzyme-linked immunoassay, was almost the same as that produced by group 2 strains, but the vascular permeability activity of the toxin produced by group 3 strains was very low or not detectable (Table 1). However, the higher vascular permeability activity of these strains was demonstrated when purified component I, but not component II, of strain 92-13 was added to the CCSs of group 3 strains (Table 1).

The ratio of components I and II produced by the strains examined in the present study varied from 1 to 6 and did not correlate with the neurotoxin type of the strains.

DISCUSSION

The present study provides evidence that components I and II of C2T produced by *C. botulinum* types C and D are heterologous and that type C and D strains that produce C2T can be divided into three groups on the basis of the immunological heterogeneities of components I and II. The classification of the strains that produce C2T into three groups correlates well with the heterogeneity in biological activity of C2T produced by the strains in each group. The vascular permeability activity, based on the amount of components I and II in the culture supernate, varied depending on the group; the activity of C2T of group 2 strains was higher than that of strains in groups 1 and 3. The C2T produced by group 3 strains had little or very low vascular permeability activity, although the amount of both components I and II produced by these strains as determined by enzyme-linked immunoassay was almost the same as that produced by group 2 strains. This is possibly due to the fact that component I produced by group 3 strains is immunologically active but not biologically active, because the addition of purified component I of strain 92-13 to the culture supernate of these strains restored the vascular permeability activity. The molecular weight of component II as determined by SDS-PAGE also differed among the groups; component II produced by group 2 strains had a molecular weight of 95 000, whereas that produced by the strains in groups 1 and 3 had a molecular weight of 105 000. All of these results indicate that the molecular structure of each of the two components of C2T produced by *C. botulinum* types C and D is heterogeneous and varies depending on the strain.

Table 1. *Vascular permeability (VP) activity and production of components I and II of C2T by C. botulinum type C and D strains*

Group (see Results)	Strain	VP activity (BU ml ⁻¹) of culture supernate*		Component I† + Component II†		Component concn § [µg (ml culture supernate) ⁻¹]		Component ratio (II:I)	Specific VP activity (BU µg ⁻¹) of components I and II
		No additions	+ Component I†	+ Component II†	I	II			
1	92-13	860	ND	ND	8.32 ± 0.87	13.5 ± 1.56	1.6	39.4	
	98-2	181	ND	ND	1.74 ± 0.22	6.08 ± 1.33	3.5	23.2	
	93-1	81	ND	ND	0.87 ± 0.10	0.96 ± 0.16	1.1	44.3	
	6812	780	ND	ND	3.88 ± 0.25	17.0 ± 3.84	4.4	37.3	
	6813	1050	ND	ND	13.8 ± 0.20	20.3 ± 4.08	1.5	30.7	
	6814	880	ND	ND	4.32 ± 0.77	5.32 ± 0.38	1.2	91.3	
	6816	410	ND	ND	3.78 ± 0.65	5.33 ± 0.06	1.4	45.0	
2	N21	485	ND	ND	2.89 ± 0.36	6.55 ± 0.72	2.3	51.4	
	4946	365	ND	ND	0.31 ± 0.03	1.79 ± 0.22	5.8	173.8	
	Stockholm	416	ND	ND	0.19 ± 0.02	1.04 ± 0.21	5.5	338.2	
	StockholmAO	530	ND	ND	0.45 ± 0.18	1.15 ± 0.06	2.6	331.3	
	1873	420	ND	ND	0.73 ± 0.04	1.13 ± 0.06	1.6	225.8	
	1873AO	210	ND	ND	0.54 ± 0.05	1.26 ± 0.11	2.3	116.7	
	C _β	170	ND	ND	0.97 ± 0.14	1.00 ± 0.08	1.0	86.3	
3	OP	310	ND	ND	1.17 ± 0.16	1.78 ± 0.16	1.5	105.1	
	CB1	<20	120	<20	1.17 ± 0.31	1.79 ± 0.22	1.5	<6.8	
	CB11	42	1020	72	1.86 ± 0.24	3.03 ± 0.36	1.6	8.6	
	CB13	<20	131	<20	0.29 ± 0.03	0.32 ± 0.06	1.1	<32.8	
	CB15	71	700	145	0.43 ± 0.04	1.22 ± 0.14	2.8	43.0	
	CB16	<20	<20	<20	<0.05	<0.05	—	—	
	CB19	<20	<20	<20	<0.05	<0.05	—	—	

ND, Not determined.

* One blueing unit (BU) was defined as the quantity of the toxin producing a blueing lesion 10 mm in diameter (Ohishi *et al.*, 1980).

† The activity was determined by adding 5 µg component I purified from strain 92-13 to 1 ml of the culture supernate.

‡ The activity was determined by adding 10 µg component II purified from strain 92-13 to 1 ml of the culture supernate.

§ Mean ± SE (n = 4).

Unlike *C. botulinum* neurotoxin, C2T elicits various biological activities. However, what role(s) C2T plays in the pathogenesis of infection with strains that produce C2T has not yet been clarified. As discussed elsewhere (Ohishi & Odagiri, 1984), if C2T causes a necrotic enteritis in animals, the severity of the disease seems to depend on the colonizing strain in the intestine, because the biological activity of C2T differs with the strain, as demonstrated in this study.

Of 21 strains examined, 19 strains, including two strains treated with acridine orange, produced C2T as detected by immunodiffusion, immunoblotting and enzyme-linked immunoassay; the other two did not produce C2T. None of the strains produced either only component I or II. This is consistent with previous results, which have shown that most of *C. botulinum* type C and D strains produce C2T in addition to C₁ or D neurotoxin (Ohishi & Sakaguchi, 1982). Components I and II, produced by acridine orange-treated strains which ceased to produce the C₁ or D neurotoxin, were immunologically identical to those produced by the untreated strains. This indicates that the treatment of the strains with acridine orange at the concentration required to cure them of their prophage does not change the structural gene encoding the two components of C2T.

Immunological analyses show that each of the two components of C2T produced by the strains in groups 1, 2 and 3 has specific epitope(s), in addition to common antigenic site(s). Immunodiffusion patterns with the CCSs of representative strains of each group clearly showed immunological heterogeneity of components I and II among the three groups (Figs 1e and 2e). However, some features of immunological cross-reactivity still require clarification. Component I produced by group 3 strains may be differentiated immunologically from component I of strains in groups 1 and 3, because group 3 strains produced component I of little or no activity, possibly due to defects in the molecular structure of the component. Similarly, the two types of component II in group 2 and group 3 strains may be differentiated from each other by immunodiffusion, because the molecular weights of the two types of component II were different. These results seem to be due to the fact that the anti-component I and anti-component II sera used in the present study were produced by immunizing rabbit with components I and II purified from group 1 strain 92-13. More detailed analyses of the antigenic differences between the two components of C2T must await the purification of the two components from strains in groups 2 and 3.

REFERENCES

- EKLUND, M. W. & POYSKY, F. T. (1972). Activation of a toxic component of *Clostridium botulinum* types C and D by trypsin. *Applied Microbiology* **24**, 108-113.
- EKLUND, M. W., POYSKY, S. M., REED, S. M. & SMITH, C. A. (1971). Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. *Science* **172**, 408-482.
- ENGVAL, E. & PERLMANN, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 874-879.
- JANSEN, B. C. & KNOETZE, P. C. (1977). The taxonomic position of *Clostridium botulinum* type C. *Onderstepoort Journal of Veterinary Research* **44**, 53-54.
- NAKAMURA, S., SERIKAWA, T., YAMAKAWA, K., NISHIDA, S., KOZAKAI, S. & SAKAGUCHI, G. (1978). Sporulation and C₂ toxin production by *Clostridium botulinum* type C strains producing no C₁ toxin. *Microbiology and Immunology* **22**, 591-596.
- OHISHI, I. (1983). Response of mouse intestinal loop to botulinum C₂ toxin: enterotoxic activity induced by cooperation of nonlinked protein components. *Infection and Immunity* **40**, 691-695.
- OHISHI, I. & ODAGIRI, Y. (1984). Histopathological effect of botulinum C₂ toxin on mouse intestine. *Infection and Immunity* **43**, 54-58.
- OHISHI, I. & SAKAGUCHI, G. (1982). Production of C₂ toxin by *Clostridium botulinum* types C and D as determined by its vascular permeability activity. *Infection and Immunity* **35**, 1-4.
- OHISHI, I., IWASAKI, M. & SAKAGUCHI, G. (1980). Purification and characterization of two components of botulinum C₂ toxin. *Infection and Immunity* **30**, 668-673.
- OHISHI, I., IWASAKI, M. & SAKAGUCHI, G. (1981). Vascular permeability activity of botulinum C₂ toxin elicited by cooperation of two dissimilar protein components. *Infection and Immunity* **31**, 890-895.
- OHISHI, I., MIYAKE, M., OGURA, K. & NAKAMURA, S. (1984). Cytopathic effect of botulinum C₂ toxin on tissue-culture cells. *FEMS Microbiology Letters* **23**, 281-284.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350-4354.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406-4412.