Demonstration of the Common Antigens of *Clostridium botulinum*, *C. sporogenes* and *C. novyi* by an Enzyme-linked Immunosorbent Assay and Electroblood Transfer

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(Received 19 October 1983)

EDTA extracts were prepared from whole cells of 16 strains of *Clostridium botulinum* (types A–E), 6 strains of *C. novyi* (types A–D) and 3 strains of *C. sporogenes*. They were reacted in an enzyme-linked immunosorbent assay (ELISA) with antisera raised against whole, UV-killed cells of *C. sporogenes* and *C. novyi* type A. Results showed significant cross-reactions between *C. sporogenes* antiserum and the *C. botulinum* type A (three out of four strains), proteolytic type B (all strains) and one type E strain, and between *C. novyi* type A antiserum and *C. botulinum* types C and D. All the *C. sporogenes* and *C. novyi* strains reacted with their homologous antiserum; these two species showed no cross-reactions. All the reactions were investigated further by running the EDTA extracts on SDS-polyacrylamide gels. The separated molecules were electrophoretically transferred to nitrocellulose membranes, reacted with antiserum and complexes visualized with horseradish peroxidase conjugate reagents. Only those extracts that reacted significantly in the ELISA gave a pattern of cross-reactive antigen bands and the number of bands and intensity of stain closely paralleled the strength of the ELISA reaction.

INTRODUCTION

The heterogeneous group of clostridia collectively known as *Clostridium botulinum* are alike in that they all produce toxins with a similar pharmacological action. In taxonomic terms, however, strains of *C. botulinum* can be divided into four reasonably distinct groups: Group I, proteolytic strains with serologically distinct A and B toxins; Group II, non-proteolytic strains producing toxins of types B, E and F; Group III, strains producing toxins of types C and D; and Group IV, proteolytic, non-saccharolytic, toxigenic type G strains (Smith, 1977). In culture and biochemistry, excluding toxin production, Group I strains are indistinguishable from *C. sporogenes*; accordingly *C. sporogenes* is considered by some workers to be a non-toxigenic variant of *C. botulinum* (Iida *et al.*, 1981). Various degrees of cross-reactivity between the two species have been revealed by immunofluorescence procedures (Walker & Batty, 1964; Hatheway & McCroskey, 1981) and by numerical taxonomy; for example, Kiritani *et al.* (1973) grouped 20 proteolytic strains of *C. botulinum* and 14 strains of *C. sporogenes* in the same phenon. Similarly Group III *C. botulinum* strains (i.e. types C and D) are indistinguishable from *C. novyi*. Type C *C. botulinum* can be cured of the prophage that codes for toxin production and converted by another phage to a *C. novyi* type A strain, and *C. botulinum* types C and D can be inter-converted by phage (Eklund & Poysky, 1981).

Our extensive study of cross-reactive antigens in the genus *Clostridium* (Poxton & Byrne, 1984) demonstrated shared antigens between *C. botulinum* types A and B (proteolytic) and *C. sporogenes*. Moreover, antigens from types C and D cross-reacted with antiserum to *C. novyi* type A. The present study extends the investigation of these cross reactions.

Abbreviations: npB, type B non-proteolytic strain; pB, type B proteolytic strain; TBS, Tris-buffered saline.

METHODS

Bacteria. All strains of *C. botulinum* were from the collection in the Botulinum Laboratory, Unilever Research, Sharnbrook, Bedford, UK: type A, strain nos 1, 7, 8, 9; type B (proteolytic, pB), strain nos 25, 26, 27; type B (non-proteolytic, npB), strain nos 188, 194, 229; type C, strain no. 205; type D, strain no. 154 and type E, strain nos 34, 118, 126, 250. The *C. novyi* strains NCTC 538 (type A), NCTC 9747 (type C) and NCTC 8350 (type D) and *C. sporogenes* NCTC 8594 were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, UK. Other strains from our laboratory culture collection were: *C. novyi* GR2A and GR3A (type A strains) and GR2B (type B), and *C. sporogenes* strains 24 and 33. The strains of *C. botulinum* were checked for toxin type by Mr C. Grimes of Unilever Research at the time of EDTA antigen preparation. The culture supernates were used in mouse toxin-antitoxin neutralization tests as outlined by Willis (1977). Neutralizing antitoxin was obtained from the Pasteur Institute, Paris, France. The identity of all strains was confirmed. The strains of *C. novyi* and *C. sporogenes* were fully characterized by the method of Holdeman *et al.* (1977).

Growth conditions. Stationary phase bacteria (0.2 ml) which had been cultured in Robertson's cooked meat broth and incubated anaerobically with 10% CO₂ for up to 48 h at 37 °C, were used to inoculate 100 ml proteose peptone/yeast extract broth (Holbrook *et al.*, 1977). Cultures were incubated anaerobically with 10% CO₂ for 16 h, or 40 h for *C. botulinum* types C and D, at 37 °C. Spore formation as judged by phase-contrast microscopy was negligible under these conditions.

Antigen extraction. Extraction with 10 mM-EDTA of washed bacteria from the 100 ml cultures was as previously described (Poxton & Byrne, 1981) except that the dialysis step was omitted after it was shown to be unnecessary.

Preparation of antisera. Antisera to *C. novyi* type A NCTC 538 and *C. sporogenes* NCTC 8594 were raised in New Zealand White rabbits by intravenous injection of whole, washed UV-killed bacteria over a 5 week period as detailed by Poxton (1979). A standard dose of 10⁹ organisms per injection was used. These sera are referred to as reference species antisera.

Indirect enzyme-linked immunosorbent assay (ELISA). The EDTA extracts were diluted to 30 µg protein ml⁻¹ in antigen buffer (50 mM-sodium carbonate buffer, pH 9.6) and 50 µl volumes were used to coat wells in flat-bottomed polystyrene microtitre plates. They were incubated for 4 h at 37 °C and overnight at 4 °C. After washing three times with 0.15 M-NaCl containing 0.05% (v/v) Tween 20 (Tween saline), doubling dilutions (1 in 400 to 1 in 102400) of rabbit antiserum in Tween PBS (50 mM-sodium phosphate buffer pH 7.4 containing 0.15 M-NaCl, 0.05% Tween 20 and 0.02% sodium azide) were added to each well (50 µl) and incubated for 4 h at room temperature. The plates were washed three times in Tween saline, and anti-rabbit IgG-alkaline phosphatase conjugate (Miles, Slough, UK) diluted 1 in 500 in Tween PBS was added to each well (50 µl) and incubated overnight at room temperature. After washing three times in Tween saline, 50 µl *p*-nitrophenyl phosphate (Sigma) at a concentration of 1 mg ml⁻¹ in 50 mM-sodium carbonate buffer pH 9.8, containing 1 mM-MgCl₂, was added to each well. After incubation for 1 h at room temperature, the plates were read in a Titertek Multiskan (Organon Teknika, St Neots, UK): the highest dilution of serum that gave an *A*₄₀₅ > 1.0 was recorded as the titre.

Polyacrylamide gel electrophoresis and electroblot transfer. SDS-polyacrylamide gel electrophoresis was done on 10% slab gels with the buffer system of Laemmli (1970) as described by Poxton & Brown (1979). Approximately 50 µg protein (based on the Lowry method) was run in each track.

Electroblot transfer was essentially by the method of Towbin *et al.* (1979). Material was transferred to nitrocellulose membrane (Transblot Transfer Medium, Bio-Rad) in Tris/glycine/methanol buffer, pH 8.3 over a 16 h period at 12 V, 40 mA. The transferred antigens were detected with the ImmunBlot immunoassay (Bio-Rad). Briefly, the membrane, after washing for 10 min in Tris-buffered saline (20 mM-Tris, 500 mM-NaCl, pH 7.5; TBS) was placed in 3% (w/v) gelatin TBS for 45 min to block any unbound sites. It was then transferred into the *C. sporogenes* or *C. novyi* antiserum diluted 1 in 200 in 1% gelatin TBS and incubated for 3 h at room temperature. After two 10 min washes in 0.025% Tween 20 TBS the membrane was placed into goat anti-rabbit IgG-horse-radish peroxidase conjugate (Bio-Rad) diluted 1 in 3000 in 1% gelatin TBS and incubated for 1 h at room temperature. After a further two 10 min washes in Tween TBS it was placed into HRP colour development solution (Bio-Rad) which contains 4-chloro-1-naphthol and hydrogen peroxide. Colour developed within 5–15 min. The reaction was stopped by placing into distilled water and washing with several changes of water over 1 h. All of the above steps were done in clean glassware and with gentle agitation throughout.

RESULTS

ELISA studies

EDTA extracts of all of the test strains were reacted in an ELISA system with our reference antisera to *C. sporogenes* and *C. novyi* (Table 1). All the proteolytic type B, three out of four of the type A and one type E strains reacted strongly with the *C. sporogenes* reference species

Table 1. Reactions obtained with EDTA extracts of *C. botulinum*, *C. sporogenes* and *C. novyi* in ELISA with antisera to *C. sporogenes* and *C. novyi*

The figures presented are typical of results obtained in repeated experiments. —, Titre of less than 400.

EDTA antigen prepared from:	Titre of reaction of the stated antigen with antiserum raised to whole cells of:	
	<i>C. sporogenes</i> NCTC 8594	<i>C. novyi</i> NCTC 538
<i>C. botulinum</i>		
A1	1 600	—
A7	3 200	—
A8	400	—
A9	—	—
pB25	6 400	—
pB26	400	—
pB27	800	—
npB188	—	—
npB194	—	—
npB229	—	—
C205	—	1 600
D254	—	3 200
E34	—	—
E118	6 400	—
E126	—	—
E250	—	—
<i>C. sporogenes</i>		
NCTC 8494	6 400	—
24	1 600	—
33	1 600	—
<i>C. novyi</i>		
A NCTC 538	—	12 800
A GR2A	—	800
A GR3A	—	1 600
B GR2B	—	1 600
C NCTC 9747	—	1 600
D NCTC 8350	—	1 600

antiserum. The type A strain that did not react (A9) was not even a weak reaction; no colour was detectable by the ELISA plate reader at the lowest dilution (1 in 400) used. The two heterologous *C. sporogenes* strains reacted significantly with the reference *C. sporogenes* antiserum. No reaction was demonstrated between the non-proteolytic B, the C, the D, three out of four of the E strains and all of the *C. novyi* strains with the *C. sporogenes* antiserum.

With the *C. novyi* antiserum only *C. novyi* strains and *C. botulinum* strains of types C and D showed a reaction.

Polyacrylamide gel electrophoresis and electroblot transfer

Initially, EDTA extracts from one strain of each type of *C. botulinum* and from one strain each of *C. sporogenes* and *C. novyi* were run on 10% SDS-PAGE slab gels and stained with Coomassie blue (Fig. 1a). All patterns were complex but there were marked similarities between those of *C. botulinum* types A and B (proteolytic) and those of types C and D (tracks 2 and 3, and 5 and 6 respectively).

Two duplicates of this gel (unstained) were electrophoretically transferred to nitrocellulose membranes and were probed with the *C. sporogenes* and *C. novyi* antisera. The results, after development with the horseradish peroxidase conjugate reagents, are shown in Fig. 1(b, c). The antiserum to *C. sporogenes* (Fig. 1b) reacted significantly with *C. botulinum* type A1 (track 2) and proteolytic type B25 (track 3) and the homologous *C. sporogenes* (track 8) EDTA extracts. In the cross-reaction with the *C. botulinum* proteolytic type B25 extract many strongly staining bands were visible, the overall intensity being stronger than with the homologous reaction. The cross-

Fig. 1. SDS-polyacrylamide gel electrophoresis and corresponding electroblot transfers of EDTA extracts of strains of *C. botulinum*, *C. novyi* and *C. sporogenes*. Approximately 50 μg protein in 50 μl sample buffer were run on each track of a 10% acrylamide gel. Track 1, *C. novyi* type A NCTC 538; track 2, *C. botulinum* A1; track 3, *C. botulinum* pB25; track 4, *C. botulinum* npB188; track 5, *C. botulinum* C205; track 6, *C. botulinum* D154; track 7, *C. botulinum* E34; track 8, *C. sporogenes* NCTC 8594. (a) Coomassie blue-stained gel. (b) Electroblot transfer of (a) reacted with antiserum to *C. sporogenes* NCTC 8594. (c) Electroblot transfer of (a) reacted with antiserum to *C. novyi* NCTC 538.

reaction with the type A extract was less strong but several bands, which were common to the type B, were stained. Single faint cross-reacting bands were also apparent in the *C. novyi* and the *C. botulinum* non-proteolytic type B extracts. The antiserum to *C. novyi* (Fig. 1c) only cross-reacted with extracts from *C. botulinum* types C and D, one major antigen being present in each.

Fig. 2. Electroblot transfers of EDTA extracts of strains of *C. botulinum* and *C. sporogenes* separated as in Fig. 1 (a) and reacted with antiserum to *C. sporogenes* NCTC 8594. Track 1, *C. botulinum* A1; track 2, *C. botulinum* A7; track 3, *C. botulinum* A8; track 4, *C. botulinum* A9; track 5, *C. botulinum* pB25; track 6, *C. botulinum* pB26; track 7, *C. botulinum* pB27; track 8, *C. botulinum* E118; track 9, blank; track 10, *C. sporogenes* NCTC 8594; track 11, *C. sporogenes* 24; track 12, *C. sporogenes* 33.

In order to demonstrate fully the cross-reactions revealed by ELISA between *C. botulinum* strains and the two antisera and also to compare these inter-species cross-reactions with the reactions within *C. sporogenes* and *C. novyi* strains, two further electroblot transfers were done. EDTA extracts of all the strains of type A and proteolytic B, strain E118 (see Table 1) of *C. botulinum* and the three *C. sporogenes* strains were run together on an SDS polyacrylamide gel. They were transferred to nitrocellulose and reacted with the *C. sporogenes* antiserum (Fig. 2). Extracts from A1 and A7 (tracks 1 and 2) showed almost identical patterns with a series of diffusely staining bands at the bottom of the gel and three common bands at molecular weights of approximately 90000, 77000 and 47000. The A8 extract (track 3) was similar to A1 and A7 except that the bands were weaker in staining intensity and some high molecular weight bands were faintly visible at the top of the gel. The proteolytic type B strains (tracks 5–7) produced a much more variable reaction than the type A strains. The extract from B25 (track 5) produced a very complex pattern of strongly staining bands as already described. Reactions with extracts from B26 and B27 were much weaker but the patterns produced were similar to each other. A significant reaction was also produced by the extract from strain E118 (track 8). A series of strongly staining low molecular weight bands at the front of the gel was the main feature of this pattern with a few faint higher molecular weight bands. When compared with the homologous reaction (track 10) the two other strains of *C. sporogenes* produced much less complex patterns (tracks 11 and 12). Again the series of low molecular weight bands was common, while there were other common bands with molecular weight of approximately 47000 and 31000.

Similarly the type C and D strains of *C. botulinum* and all the *C. novyi* strains were run together and reacted with the *C. novyi* type A antiserum (Fig. 3). The *C. botulinum* types C and D (tracks 1 and 2) both produced similar patterns. A band with a molecular weight of approximately 47000 was the major cross-reacting antigen, but several high molecular weight bands were apparent at the top of the gel and there was a series of regularly spaced cross-reacting low molecular weight bands at the bottom of the gel. The reactions between the three type A *C. novyi* strains were very

Fig. 3. Electroblot transfers of EDTA extracts of strains of *C. botulinum* and *C. novyi* separated as in Fig. 1(a) and reacted with antiserum to *C. novyi* type A NCTC 538. Track 1, *C. botulinum* C205; track 2, *C. botulinum* D154; track 3, blank; track 4, *C. novyi* A NCTC 538; track 5, *C. novyi* A GR2A; track 6, *C. novyi* A GR3A; track 7, *C. novyi* B GR2B; track 8, *C. novyi* C NCTC 9747; track 9, *C. novyi* D NCTC 8350.

similar to each other (tracks 4, 5 and 6). The major cross-reacting antigens had molecular weights of 48 000 and 31 000 with a series of regularly spaced bands at the bottom of the gel. The *C. novyi* types B, C and D produced similar patterns (tracks 7, 8 and 9): there was a major band with a molecular weight of 47 000 and a series at the bottom of the gel which was similar to all the other tracks on the gel.

DISCUSSION

The results obtained with the ELISA and the electroblot procedures agree closely and show conclusively that most strains of *C. botulinum* types A and proteolytic B (Group I) share antigens with *C. sporogenes*, whereas types C and D (Group III) share antigens with *C. novyi*. This provides further evidence of the close taxonomic relationships between these species. In view of the limited number of strains investigated, it is not yet certain how widely these cross-reactions occur and how many exceptions there may be to the general rule demonstrated here. For example, one of the strains of *C. botulinum* type A did not react, whilst one of the type E strains did react with *C. sporogenes* antiserum. Moreover, varying degrees of cross-reactivity could be demonstrated with the three type A and three proteolytic type B strains that cross-reacted with the *C. sporogenes* antiserum. This was also true for the three strains of *C. sporogenes* investigated, and indicates antigenic heterogeneity. Reactions within the *C. novyi* strains were much more uniform. No major differences were seen between the three *C. novyi* type A strains. The *C. novyi* strains of types B, C and D showed close antigenic similarity with each other and with the *C. botulinum* strains of types C and D.

The patterns of cross-reactivity revealed by electroblot transfer all showed some similarities. Most of the major cross-reactive bands corresponded to predominant protein bands on the Coomassie blue-stained gels. All strains that reacted with antiserum, however, also showed

patterns of regularly spaced bands at the bottom of the gels. These bands did not appear to correspond to any of the protein bands on the Coomassie blue-stained gels. They were reminiscent of the patterns produced by lipopolysaccharides of Gram-negative bacteria when stained with silver (Tsai & Frasch, 1982). It is possible that these cross-reacting antigens were lipocarbohydrate molecules from the cytoplasmic membrane (lipoteichoic acid analogues). We know that, at least for *C. difficile* and *C. sordelli*, the membrane lipocarbohydrates are cross-reacting antigens and that they are solubilized by EDTA treatment (Poxton & Cartmill, 1982).

There was no attempt made in this study to use antiserum raised to any *C. botulinum* strain. The aim was to use antisera raised against cells that were as close as possible to living organisms: washing and killing with a minimum dose of UV light were the only treatments used. Obtaining undenatured cells of toxigenic *C. botulinum* devoid of toxin is not possible. No attempt was made to work with *C. botulinum* types F and G as the author was not protected against these types.

This study was funded by a grant from the Biomedical Research Committee of the Scottish Home and Health Department (grant no. K/MRS/50/C247). I am extremely grateful to Dr J. S. Crowther and Mr C. Grimes of Unilever Research, Sharnbrook, Bedfordshire for the advice, assistance and facilities made available during my visit to their laboratory. Professor J. G. Collee's interest and encouragement is gratefully acknowledged.

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