Use of Ganglioside Affinity Filters to Identify Toxigenic Strains of Clostridium botulinum Types C and D

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Clostridium botulinum neurotoxin is synthesized by toxic clones grown anaerobically on ganglioside affinity filters. The toxin binds to the filters and is detected by reaction with 125I-immunoglobulin G from type-specific antitoxin. Toxin spots from culture filtrates were similarly identified. The C. botulinum type C and D strains were selected for developing this affinity filter assay because synthesis of the C1 and D toxins is bacteriophage dependent. Toxigenic clones were distinguished from prophage-cured atoxigenic derivatives. These studies represent a first step toward the development of a general nonbiological screening procedure for identifying botulinan toxin and toxigenic cells. The affinity filter methodology should facilitate genetic analysis of the basis of C. botulinum toxicity.

Seven types of Clostridium botulinum strains (A through G) are distinguished by differences in the antigenic specificity of their pharmacologically similar neurotoxins (3). Human foodborne botulism is associated with types A, B, E, and F. Types C and D are implicated in animal botulism. Toxin from the type C strain is termed C1. Many C and D cultures also produce an unrelated C2 toxin. Infant botulism, characterized by toxin-induced paralysis of neuromuscular transmission and respiratory arrest, is a recently described, little understood disease which was identified through isolation of toxigenic C. botulinum types A and B from feces of symptomatic infants. It is one cause of the sudden infant (crib) death syndrome (1, 2, 27, 34, 40).

Newer laboratory methods are needed to allow rapid identification of botulinal toxin and C. botulinum in clinical specimens and to establish the proportion of sudden infant death syndrome due to C. botulinum infection, estimated to be about 5% (2). Demonstration of botulinal toxin and C. botulinum in contaminated materials is now done by mouse toxin neutralization tests and by isolation of the organism using anaerobic culture techniques. The necessity for bioassay, however, considerably limits the potential for genetic analysis of clonal toxicity.

Bacteriophages are associated with C. botulinum types A through F (6, 7, 9, 10, 15, 17, 29, 30, 41). Several authors have suggested a relationship between culture toxigenicity and phage (4, 7, 17, 19). There is a direct association between bacteriophage from the C and D strains and the synthesis of type-specific botulinal neurotoxin (6, 9). Toxigenic (Tox+) C and D strains cured of their prophage, respectively, cease producing C1 and D toxin. Cured cultures are converted to toxin production by reinfection with either the type C or D phage. These phages form turbid plaques on lawns of the cured nontoxic (NT) cells, but not on cells which have been infected and converted by the same type of phage (6, 8-10, 15, 32). This result suggests that cell conversion arises by a lysogenic mechanism that could depend on the production of an immunity substance or phage-coded repressor. The C. botulinum type C and D phages are suggested to exist in the host as pseudolysogens, as opposed to true lysogens, because most strains can be cured of prophage by growth in the presence of phage-specific antisera.

Because of their potential for prophage curing and phage conversion, the C. botulinum C and D strains were selected for use in developing a rapid plating assay for distinguishing between Tox+ and NT clones. The assay is based on the affinity filter procedure which was recently described for screening toxigenic cholera clones (24). This assay depends upon binding of cholera toxin to the brain ganglioside GM1. Botulinal toxins and tetanospasmin elaborated by Clostridium tetani also bind to gangliosides at low toxin concentrations (13, 14, 22, 25, 36). Tetanus toxin binds to gangliosides GD1a and GT1b (16). The specific gangliosides bound by C. botulinum toxin have not been determined. However, because botulinal toxin has an action almost identical to that of tetanus toxin at certain neuromuscular junctions (22, 26), it probably binds to the same gangliosides. Accordingly, I tested whether a ganglioside affinity assay can be used for identifying type-specific toxigenic C. botulinum clones.
The general features of the affinity filter assay are as follows. Ganglioside-albumin conjugates are covalently bound on the surface of cellulose filter disks. Colonies are replicated to filter disks placed over agar plates. The replicated clones grow on the filter disks, and toxin from Tox+ colonies binds tightly to the associated gangliosides. After removal of the cells from the filter, the bound toxin is detected by incubating the filter with 125I-labeled antibody against the toxin and autoradiography of the washed and dried filter. Dark exposed spots appear on the X-ray film corresponding to the positions where the Tox+ clones were printed on the filter. The prints of the NT clones do not expose the film. The affinity filter methodology could have general applicability as a clinical and research tool for eventually detecting all the C. botulinum toxin types.

MATERIALS AND METHODS

Bacterial strains. The toxigenic and phase-cured nontoxicogenic type C and D C. botulinum strains employed are listed in Table 1. In this report, toxogenic strains are defined as those which synthesize the dominant bacteriophage-dependent type C1 or D neurotoxins. These toxins are usually not enhanced in activity by trypsin (5, 21, 28) and can be present at 10^4 to 10^5 mouse lethal doses (LD_{50}) per ml of culture filtrate. Subsequent to trypsin activation, C2 toxin (which has no known relationship to the tox C gene) can also be detected in cultures of many tox and tox strains at 20 to 200 LD_{50} per ml (31). The C2 toxin is not inactivated by antisera to either the C1 or D toxin (5).

Antitoxin. The antisera to C1 toxin, prepared against C1 toxin from a culture of strain 468 tox C, was obtained from M. W. Eklund. The procedure used for preparing the antisera was described by Eklund and Poyssy (5). The type D antisera was obtained from M. Cardella via M. W. Eklund and was prepared against toxin from strain D6F (part of inaccessible Fort Detrick collection). It is suggested (M. W. Eklund, personal communication) that whenever C1 toxin is the major one should add "and possibly D toxin," and whenever D toxin is mentioned one should add "and possibly C1 toxin" (5). This is because antisera to D toxin slightly reduce measured C1 toxicity and vice versa. The observation is explained two ways. (i) The C1 and D toxins share an antigenic determinant and weakly cross-react. (ii) Alternatively, low levels of D toxin may be made in C1-producing cultures, and vice versa.

Preparation of ganglioside affinity filters. Crude preparations (50 mg) containing mixtures of gangliosides (Sigma type II [39], type III [12], and type VI) were separately dissolved in 6 ml of water and adjusted to pH 4.6. EDAC (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide HCl, 100 mg; Bio-Rad Laboratories) was added and the solution was maintained at pH 4.7 for 15 min. Albumin (500 mg/10 ml, pH 4.6) was added to the solution, which was stirred and continuously maintained at pH 4.6 for 2 h. The reaction was terminated by addition of (NH_4)_2SO_4 to 100% saturation. The precipitate was resuspended in 50 ml of acetate buffer (0.1 M sodium acetate, pH 5.0), dialyzed against the same buffer (plus 0.02% sodium azide), and diluted to 12 mg/ml based on an absorbancy at 280 nm of 0.68 for 1 mg of the conjugate per ml. Unsubstituted albumin (26% of a 30 mg/ml solution in acetate buffer) was mixed with the albumin-ganglioside conjugate (4.0 ml; then 6 ml of 2.5% glutaraldehyde was added dropwise over 3 min. Whatman no. 1 cellulose filters were dipped into the protein ganglioside conjugate solution, drained, placed on Mylar sheets, and dried. The unreacted glutaraldehyde groups on the filters were blocked by soaking in 0.25 M ethanolamine in acetate buffer for 20 min. The filters were washed on each side with PBS (10 mM NaHPO_4, 0.85% NaCl, pH 7.4) and dried. They contain a network of covalently cross-linked albumin and ganglioside-albumin molecules which cannot be eluted from the filters.

Isolation of immunoglobulin from antisera. The immunoglobulin G (IgG) fractions were purified from high potency rabbit anti-C sera and equine anti-D sera described in above. The IgG fraction from the equine sera was purified by three successive precipitations at 36% (NH_4)_2SO_4. The rabbit IgG fraction was

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Designation</th>
<th>Reference</th>
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<tbody>
<tr>
<td>153 toxC</td>
<td>153(4 toxC)</td>
<td>6</td>
</tr>
<tr>
<td>153 toxC</td>
<td>HS15</td>
<td>6</td>
</tr>
<tr>
<td>Stockholm toxC</td>
<td>SKM(c-stoxC)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>(2C&quot;NT&quot;)</td>
<td></td>
</tr>
<tr>
<td>SKM toxC</td>
<td>A050</td>
<td>6</td>
</tr>
<tr>
<td>SKM toxC</td>
<td>1620 toxC</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1620(2 toxC)</td>
<td></td>
</tr>
<tr>
<td>1873 toxD</td>
<td>1873(d-1873 toxD)</td>
<td>29, 32</td>
</tr>
<tr>
<td>1873 toxD</td>
<td>A0A113</td>
<td>6</td>
</tr>
<tr>
<td>468 toxC</td>
<td>468(1 toxC)</td>
<td>10</td>
</tr>
</tbody>
</table>

* Strains cured of tox and tox phase are designated as "nontoxogens." Strains shown as "lysogens" harbor either tox or tox phase and are described (8, 10) as pseudolysogens.

* C1 and C2 toxins synthesized.

* C2 toxin synthesized.

* C1 toxin synthesized.

* The tox of lysogen A050(c-7 tox C) was constructed by M. W. Eklund and F. T. Poyssy by infecting A050 with tox phage c-7 tox C which they had isolated as a turbid single plaque from SKM tox C. This phase is probably identical to phase c-stox C isolated by Oguna et al. (32) from SKM tox C.

* No toxin synthesized.

* D and C2 toxins synthesized.

Table 1. C. botulinum strains used
prepared by three successive precipitations at 40% (NH₄)₂SO₄. The globin fraction was dialyzed against phosphate buffer (15 mM Na₂HPO₄, pH 8.0) and passed over an activated (washed with 0.1 M NaOH) diethylaminoethyl column equilibrated and eluted with phosphate buffer. The pooled first major peak was adjusted to 0.15 M NaCl, concentrated by (NH₄)₂SO₄ precipitation, dialyzed against PBS, and frozen.

Iodination of immunoglobulin. The IgG fractions were iodinated as follows, using a solid-state reaction (11; J. J. Mekalanos, personal communication). Each preparation was diluted to 2.0 mg/ml, dialyzed against pH 7.4 phosphate buffer (0.1 M Na₂HPO₄), and centrifuged at 12,000 rpm for 30 min to remove insoluble precipitates. IodoGen (Pierce) (1 mg/ml in CHCl₃) was added (0.01 ml) to glass tubes (12 by 100 mm) and spread around the bottom 1 cm of each tube. After this solution dried, 0.01 ml of 125I and 0.20 ml of IgG were added. (The carrier-free Na125I, pH 10.4, was diluted about fivefold with 0.1 M phosphate buffer, pH 7.4, to yield 1.0 mcg/0.01 ml of 125I solution.) The reaction mixtures were shaken at 22°C for 10 min, pooled, and passed over an 11-ml Bio-Gel P-30 column which was eluted with pH 7.4 phosphate buffer. The pooled void volume peak was adjusted to 1 mg of albumin/ml and 0.02% sodium azide and was held at 4°C.

Affinity filter assay. The dry ganglioside filters were sterilized by irradiating both sides for 1 h at 20 cm with a 254-nm, 30-W ultraviolet light. The filters were placed on 1.2% TYG agar plates (6). A period of 30 min was required for moisture from the plates to wet the filters. The filter plates were deoxygenated by incubation for 1 h in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) with palladium catalysis, flushed with H₂. (Control filter plates made with non-irradiated filters were contaminated after 24 to 48 h of incubation at 37°C by growth of a single, highly motile spore-forming anaerobe. Similarly incubated ultraviolet-irradiated filters were only occasionally contaminated.)

Pure cultures of Tox⁺ and NT C. botulinum type C and D strains were inoculated to pre-reduced 2% TYG agar plates which were placed in GasPak jars with palladium catalysts. The jars were flushed with hydrogen gas until the residual oxygen was converted to water vapor and then were incubated for 16 to 24 h at either 30 or 37°C. (Longer incubation times, especially at 37°C, result in unmanageable spreading of the colonies.) The TYG plates were either inoculated by spotting about 0.001 ml of a cooked meat medium (18) culture containing 10⁵ to 10⁶ viable spores per ml or by stabbing the agar with a needle touched to a vegetatively growing 30°C TYG broth (6) culture which had been inoculated with the same cooked meat medium stock culture. The colonies from the 2% agar TYG plates were transferred to sterile deoxygenated velvet, and the imprint was replicated to a moist ganglioside filter on a TYG plate. The printed filter plates were incubated anaerobically for 24 h at 37°C.

The incubated printed filter was subsequently removed from the TYG plate with tweezers, and visible cell debris was rinsed off with a stream of PBS. The filter was placed on a wire-mesh screen in a Büchner funnel and rinsed on both sides, under vacuum, with 75 ml of PBS. The washed wet filters were incubated about 1.5 h at 37°C in petri dishes in a mixture of 5.0 ml of PBS, pH 7.4, 0.05 ml of albumin (100 mg/ml in PBS, 0.02% Na₂S₀₄, adjusted to pH 7.4), and 0.01 to 0.05 ml of 125I-IgG. They were removed, rinsed with a stream of PBS, rinsed on both sides with 75 ml of PBS, and then pinned to foil over cardboard and dried under infrared lamps. The filters were taped to glass plates and autoradiographed using either Kodak Royal X-Omat or NS-5T X-ray film.

RESULTS

Efficiency of iodination. The overall efficiency of the solid-state iodination reaction was determined by multiplying the percentage recovery of the pooled IgG peak from the second Bio-Gel A-1.5M exclusion column (Fig. 1) times recovery of iodinated protein from the P-30 column. In the initial separation of iodinated anti-C and anti-D IgG’s from unbound 125I⁻, 86% of the added label eluted in the void volume of the P-30 column. Overall, 57% of added 125I was transferred to anti-C IgG, 41% was transferred to anti-D IgG, and 16% was transferred to the large unknown protein in the anti-D preparation (Fig. 1) eluting ahead of the thyroglobulin marker. The initial anti-C and anti-D IgG fractions from the P-30 column contained about 35% unbound 125I.

Replicate plating technique. The ability of the ganglioside plating assay to discriminate between Tox⁺ and NT C. botulinum C and D strains was examined (Fig. 2). Results obtained with the type II and III ganglioside filters are shown. Assays were also performed using what proved to be unsatisfactory type VI ganglioside filters prepared from a Sigma bovine brain extract containing phosphatidyl ethanolamine, cerebrosides, cerebroside sulfate, sphingomyelin, and gangliosides. The anti-C 125I-IgG bound to areas on the type II and III filters (Fig. 2, left side) corresponding to the printing of toxigenic type C strains 153toxC (dark spot, upper left), Stockholm strain SKMtoxC (dark spot, left), and AO50(c-7toxC) (dark spot, middle), but not to the phage-cured, nontoxic HS15toxC⁻ and AO560toxC⁻ derivatives of these strains (Table 1), or to the type D Tox⁺ strain 1873 and NT strain OA113. There was no reaction with either strain 162toxC or its cured host HS37toxC⁻. In contrast, anti-D 125I-IgG (Fig. 2, right side) bound to filter areas 153toxC, SKMtoxC, AO50(c-7toxC), and 1873toxD, but not to the regions where the toxC⁻ and toxD⁻ strains were printed. Thus, anti-C 125I-IgG binds to filter regions where only type C1 toxin is synthesized, whereas anti-D 125I-IgG binds to regions producing either the D or the C1 toxin.
Filter spotting technique and ganglioside affinity. Toxic culture filtrates adjusted at half pH intervals between pH 5.0 and 8.0 were spotted to type II and III filters (Fig. 3) to test whether the ganglioside assay can detect botulinum toxin in liquid culture. A strong positive reaction is evident between the applied 1,000 LD50 spots of type C1 toxin from strain 468toxC and anti-C125I-IgG. There was essentially no binding of anti-C125I-IgG with culture filtrate spots of toxin (100 LD50 applied) from strain 162toxC (Fig. 3), in agreement with the results for printed clones (Fig. 2). The filter assay was able to detect an equivalent level of type D toxin. A strong reaction (data not shown) was observed between anti-D125I-IgG and 100 LD50 of the strain 1873 toxin. There was reduced antibody binding to undiluted spots of the type D, but not type C, culture filtrates. The severe haloing effect observed with undiluted type D TPGY (6) culture filtrates was only slightly noticeable (Fig. 3) with undiluted type C culture filtrate spots. (The C strains were grown in TYG broth (6), which is similar to TPGY but lacks 2% peptone.) The sensitivity of the filter spot assay for 100 LD50 of toxin is illustrated by noting that purified D neurotoxin from strain 1873 has a specific toxicity of $5 \times 10^8$ LD50/mg of N (28) (for the M toxin complex, see below). The C1 neurotoxin is equally toxic.

DISCUSSION

Both the interaction between the neurotoxins and filter-bound gangliosides and the recognition of this complex by type-specific antitoxins require further investigation. The lack of binding of anti-C125I-IgG with printed clones or culture spots of strain 162toxC implies either that differences in ganglioside binding affinities occur between C1 toxin from different strains or that the cellular release or synthesis of C1 toxin from strain 162toxC was below the sensitivity of the assay.

The observed cross-reactivity between anti-D125I-IgG and the replicate prints of Tox+ C clones can be understood from an analysis of the biochemically characterized forms of the C1 and D toxins. Type D toxin is isolated from hemagglutinin (HA)-positive Tox D strains as a 19S dimer of 16S (9.0 \times 10^5 daltons) L-toxin molecules. The L-toxin form consists of the $5 \times 10^5$-dalton HA molecule and a 12S M-toxin. The M-toxin com-
plex includes one or two nontoxic proteins plus the (1.7 × 10^6-dalton) type D neurotoxin (3, 28, 33). This is the largest toxin form which is isolated from HA-negative type D strains such as 1873 (28, 33). In contrast, the C1 toxin is isolated as a 12S complex between HA and the (1.5 × 10^5-dalton) neurotoxin (33, 38). Some C1 and D toxins cross-react with each other (5, 20, 23, 35). This occurs because the HAs associated with each toxin are antigenically similar (37). Strains 468toxC and SKMtoxC used in this study are both HA positive. Antiserum to the formalinized type C1 toxin includes antibodies against the toxin and HA. The prepared anti-C {\textsuperscript{125}I}-IgG does not bind to imprints of 1873toxD because they are HA negative. It appears that the anti-D sera was prepared from formalized D toxin isolated from an HA-positive strain. Thus, one would expect, as observed, that the anti-D {\textsuperscript{125}I}-IgG will bind to replicated prints of HA-positive type C clones.

The production of HA as well as toxin synthesis by C. botulinum type C and D strains is phage dependent (31). The release of phage by
toxic strains should not obscure the sensitivity of the ganglioside assay since neither protein appears to be part of the phage coat (15, 31, 32).

Needed future studies include the use of ganglioside affinity filters for identifying toxigenic C. botulinum type A, B, E, F, and G strains, and partial modification of the described procedure for routine toxin titration. The spot assay could be developed into a routine clinical diagnostic test for identifying botulinum toxin in the serum or intestinal contents of infected infants, or in contaminated foods: by eliminating filter autoradiography and directly measuring the activity of ganglioside-bound filter strips which have been exposed to toxin and then allowed to react with type-specific 125I-IgG, the time required for the assay might be reduced to several hours.

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