A Novel Strain of *Clostridium botulinum* That Produces Type B and Type H Botulinum Toxins

Jason R. Barash and Stephen S. Arnon
Infant Botulism Treatment and Prevention Program, California Department of Public Health, Richmond, California

(See the major article by Dover et al on pages 192–202, and the editorial commentaries by Popoff on pages 168–9 and Hooper and Hirsch on page 167 and Relman on pages 170–2.)

**Background.** *Clostridium botulinum* strain IBCA10-7060, isolated from a patient with infant botulism, produced botulinum neurotoxin type B (BoNT/B) and another BoNT that, by use of the standard mouse bioassay, could not be neutralized by any of the Centers for Disease Control and Prevention–provided monovalent polyclonal botulinum antitoxins raised against BoNT types A–G.

**Methods and Results.** The combining of antitoxins to neutralize the toxicity of known bivalent *C. botulinum* strains Ab, Ba, Af, and Bf also failed to neutralize the second BoNT. Analysis of culture filtrate by double immunodiffusion yielded a single line of immunoprecipitate with anti-A, anti-B, and anti-F botulinum antitoxins but not with anti-E antitoxin. A heptavalent F(ab′)2 botulinum antitoxin A–G obtained from the US Army also did not neutralize the second BoNT. An antitoxin raised against IBCA10-7060 toxoid protected mice against BoNT/B (Okra) and against the second BoNT but did not protect mice against BoNT/A (Hall) or BoNT/F (Langeland).

**Conclusion.** The second BoNT thus fulfilled classic criteria for being designated BoNT/H. IBCA10-7060 is the first *C. botulinum* type Bh strain to be identified. BoNT/H is the first new botulinum toxin type to be recognized in >40 years, and its recognition could not have been accomplished without the availability of the mouse bioassay.

**Keywords.** botulinum toxin; botulism; *Clostridium botulinum*; botulinum antitoxin; mouse bioassay.

Modern knowledge of *Clostridium botulinum* originates with the discovery of the bacterium in 1897 by van Ermengem, who isolated it from an incompletely salted ham eaten in the now-famous foodborne botulism outbreak in the small Belgian town of Ellezelles that sickened 23 members of a musical club, of whom 13 became quite paralyzed and 3 died. Van Ermengem’s study (112 pages in the original German) established that the illness of botulism resulted from an extremely potent, heat-labile toxin that was produced by a spore-forming, obligately anaerobic bacterium [1].

In 1910, Leuchs in Berlin showed that 2 European strains of *C. botulinum* produced toxins that had different antigenicities because an antitoxin raised against one toxin did not cross-neutralize the toxin of the other strain [2]. In 1919, working with US strains of *C. botulinum*, Georgina Burke designated the 2 antigenically distinguishable botulinum toxins (BoNTs) as type A (BoNT/A) and type B (BoNT/B) [3]. She thereby established the present-day alphabetical designations of the several toxin types whose defining characteristic remains the absence of cross-neutralization in the mouse bioassay by type-specific monovalent botulinum antitoxin [4].

In the decades that followed Burke’s work, 5 more toxin types were discovered; these consist of types C (discovered in 1922), D (1928), E (1937), F (1960), and most recently, G (1970) [5–10]. Although no new BoNT types have been recognized in the past 40 years, strains that produce 2 toxins (usually in different amounts) have been identified, with the lesser amount of toxin indicated by a lower case letter. These strains, termed “bivalent,” include those with the dual toxicities of Ab, Ba, Af, and Bf, as well as AB strains that produce equivalent amounts of both toxins [11–14]. A(B) strains also exist; they are termed “A silent B” because
their additional gene for BoNT/B contains a stop-codon mutation that yields an inactive BoNT/B [15, 16]. Also, subtypes of BoNT/A, B, E, and F have been described based on differences in the toxin gene nucleotide and toxin amino acid sequences, surface epitopes, and physiological characteristics [17–19].

We now report the isolation and characterization of a novel strain of proteolytic C. botulinum, recovered from a patient with infant botulism, that produces a type H botulinum toxin by the established criterion of absence of neutralization in the mouse bioassay with any of the 7 monovalent anti-A through anti-G botulinum antitoxins, either when used individually or when combined in pairs to neutralize bivalent strains. This novel toxin could only be neutralized by an antitoxin raised against it in rabbits, even though double-immunodiffusion analysis of the BoNT-containing culture filtrate produced by this novel strain identified /A-like, /B-like, and /F-like epitopes in it. The characteristics of the type H toxin were further elucidated by molecular studies reported in the companion article by Dover et al [20].

**MATERIALS AND METHODS**

**Primary Isolation**

A 5-g fecal sample submitted for infant botulism diagnostic testing from a patient treated with Human Botulism Immune Globulin (BIG-1V; BabyBIG) [21] was evaluated using standard methods (Supplementary Materials). Colonies characteristic of C. botulinum were isolated in pure culture on egg yolk agar and subcultured to cooked meat glucose starch broth for toxin testing and cryopreservation for further studies. The strain was designated IBCA10-7060.

**Characterization Studies**

Most botulinum antitoxin neutralization studies were performed with cooked meat glucose starch cultures of IBCA10-7060 incubated at 35°C for 72 hours, diluted 1:20 with gelatin phosphate diluent, and filter sterilized, which is the standard method used in our laboratory for typing toxins in pure cultures of C. botulinum. Mouse bioassays were performed in repeated replicates, using monovalent polyclonal equine antitoxins A, B, C, D, E, F, and G (Centers for Disease Control and Prevention [CDC], Atlanta, GA). The 1:20 cooked meat glucose starch culture dilutions were also tested with bivalent and trivalent mixtures of monovalent botulinum antitoxins AB, AF, BE, BF, ABE, and ABF. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of the California Department of Public Health.

The standard mouse bioassay requires that 2 mice each receive a 0.6-mL intraperitoneal dose composed of 0.1 mL of monovalent equine botulinum antitoxin and 0.5 mL of fecal extract or diluted culture filtrate. The antitoxin and fecal extract or culture filtrate are mixed and incubated for 30 minutes at room temperature before being injected into mice. The 0.1-mL dose of monovalent equine antitoxin is standardized to contain ≥1 international unit (IU) of type-specific antitoxin. For BoNT/A, B, C, D, E, F, and G, 1 IU of botulinum antitoxin is defined as the amount of antibody that neutralizes 10 000 intraperitoneal mouse lethal doses (50% end point; MLD_{50}) of BoNT. For BoNT/E, 1 IU of type E antitoxin is defined as the amount neutralizing just 1000 intraperitoneal MLD_{50} [4]. Each mouse injected with a 1:20 dilution of filter-sterilized culture combined either with a single monovalent antitoxin or with a mixture of 2 antitoxins received a total intraperitoneal dose volume of 0.6 mL or 0.7 mL, respectively. CDC monovalent antitoxins are suspended in 50% glycerol, so care was taken to avoid glycerol toxicity when creating antitoxin combinations (Supplementary Materials).

Culture filtrates were also tested against a heptavalent botulinum antitoxin containing polyclonal F(αb′)₂ antibodies against all 7 BoNT serotypes A–G (USAMMMDA, Ft. Detrick, MD). Concentrations of the 7 BoNT-neutralizing antibodies in the Ft. Detrick product were approximately 10–80 times those of the CDC monovalent immunoglobulin G (IgG) antitoxins. Concentrations of antitoxins in the heptavalent antitoxin product were as follows (rounded IU units): anti-type A, 238 IU/mL; anti-type B, 295 IU/mL; anti-type C, 95 IU/mL; anti-type D, 836 IU/mL; anti-type E, 836 IU/mL; anti-type F, 291 IU/mL; and anti-type G, 102 IU/mL. As needed to adjust for differences in toxin-neutralizing potency between the monovalent and heptavalent products, the heptavalent antitoxin was diluted ≤1:25 with gelatin phosphate diluent to enable equivalent efficacy comparisons with the CDC diagnostic monovalent antitoxins that each contained ≥10 IU/mL.

The concentration of BoNT expressed by IBCA10-7060 in a 72-hour cooked meat glucose starch culture was evaluated with a MLD_{50} study, using 2 mice per 10-fold serial dilution. The residual of each dilution was retained, and serial 1:2 dilutions of the 10-fold dilution immediately above the 50% end point were then injected into groups of 6 mice. The MLD_{50} per milliliter was calculated using the method of Reed and Muench [22]. The digoxigenin enzyme-linked immunosorbent assay (DIG-ELISA) method was also used qualitatively to evaluate BoNT production [23].

The API 20a (Biomerieux, Hazelwood, MO) and Rapid ANA (Remel, Lenexa, KS) identification systems were used to establish biochemical profiles of IBCA10-7060 isolates. DNA extracted from a trypticase peptone glucose yeast extract culture of IBCA10-7060 was used to identify and characterize boNT gene clusters and proteolytic group I 16s ribosomal RNA sequences and to perform whole genome sequencing studies reported by Dover et al in the companion article [20].

**Immunodiffusion**

BoNT produced by IBCA10-7060 was studied with an immunodiffusion assay adapted from the classic methods of
Ouchterlony and Nilsson [24] that have proved useful in identifying BoNT (Supplementary Materials) [25, 26]. We evaluated our immunodiffusion method by first using equine monovalent polyclonal botulinum antitoxins (CDC, Atlanta, GA) against unpurified BoNTs (culture filtrates) from the following infant botulism treatment and prevention program reference strains: C. botulinum type A (Hall), C. botulinum type B (ATCC 7949), C. botulinum type F (Langeland), and C. botulinum type E (Detroit) incubated in trypticase peptone glucose yeast extract broth for 72 hours at 35°C.

Production of Antitoxin to Strain IBCA10-7060 Culture Filtrate

We produced polyclonal rabbit anti-IBCA10-7060 antitoxin for additional strain characterization studies by using the culture and ammonium sulfate precipitation methods of Hatheway et al to prepare a toxoid for injection into rabbits (Supplementary Materials) [27].

RESULTS

Diagnostic Testing of Patient Feces for BoNT

All mice injected with filter-sterilized stool extract alone or combined with ≥1 IU monovalent botulinum antitoxin A or with ≥1 IU of monovalent botulinum antitoxin B developed typical symptoms of botulism and died. Mice injected with boiled stool extract survived (BoNT is heat labile). Mice injected with stool extract plus antitoxin A died within 24 hours, while mice injected with stool extract plus antitoxin B died within 48 hours. Further studies to characterize the presumptive botulinum toxin(s) produced by IBCA10-7060 were performed with pure cooked meat glucose starch culture filtrates.

Identification of C. botulinum IBCA10-7060

Stool cultured on egg yolk agar grew mixed enteric flora that included rare lipase-positive colonies. Egg yolk agar subcultures of the unheated cooked meat glucose starch culture also grew lipase-positive colonies with an appearance consistent with that of C. botulinum. Four lipase-positive colonies were directly isolated from the original stool culture grown on egg yolk agar. API 20a and Rapid ANA II biochemical profiles identified these 4 proteolytic, lipase-producing isolates as either C. botulinum or Clostridium sporogenes, 2 species that are differentiated by identifying the expression of BoNT by C. botulinum [4]. A cooked meat glucose starch culture filtrate was tested in triplicate by DIG-ELISA, using test wells individually coated with monovalent capture antibodies to BoNT/A, /B, /E, and /F. BoNT/A, /B, and /F but not /E epitopes were detected in the culture filtrate.

Initial Mouse Bioassay Testing

Culture filtrates of the 4 IBCA10-7060 isolates contained heat-labile toxin that produced classic signs of botulism in mice. However, standard BoNT neutralization procedures failed to protect mice (Table 1). No monovalent antitoxin (anti-A through anti-G) or mixtures of monovalent antitoxins (AB, AF, BE, and BF) protected mice from IBCA10-7060 culture filtrates.

Table 1. Inability of Polyclonal Monovalent Botulinum Antitoxins A–G, Used Singly and in Combination, to Protect Mice From Botulinum Neurotoxin (BoNT) Produced by Strain IBCA10-7060

<table>
<thead>
<tr>
<th>Time After Injection, Mean</th>
<th>No Antitoxin</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>AB</th>
<th>AF</th>
<th>BF</th>
<th>BE</th>
<th>ABE</th>
<th>ABF</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>1/2</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>20 h</td>
<td>0/2</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>44 h</td>
<td></td>
<td>0/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/2</td>
<td></td>
<td>0/2</td>
<td>0/2</td>
<td>4/6</td>
<td>6/6</td>
</tr>
<tr>
<td>66 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/6</td>
<td>3/6</td>
</tr>
<tr>
<td>90 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Data are no. of mice that survived/no. injected. Abbreviation: MLD50, mouse lethal dose (50% end point).

a All antitoxins used in these triplicate experiments were intact immunoglobulin G (IgG; equine) polyclonal monovalent botulinum antitoxins supplied by the Centers for Disease Control and Prevention for diagnostic botulism testing. By definition, 1 IU neutralizes 10,000 MLD50 of types A, B, C, D, F, or G botulinum toxin and 1000 MLD50 of type E botulinum toxin.

b Concentrations of all antitoxin mixtures were adjusted to deliver 1 IU of the type-specific antitoxin per mouse. The toxicity of the strain IBCA10-7060 culture filtrate (CF) was approximately 40,000 MLD50/mL, with a BoNT/B to /H ratio of 24:1 (see Results). Thus, the 0.5 mL of the 1:20 dilution injected into each mouse contained approximately 960 MLD50 BoNT/B and 40 MLD50 BoNT/H. In the ABF antitoxin mixture, the antitoxin:toxin neutralization ratio, expressed in MLD50, for BoNT/B was 1000:96, and for BoNT/A and /F it was 1000:4 (see Methods), yet the mice were not protected.

c No Clostridium botulinum toxin types BE, ABE, or ABF are known to exist. However, the combinations of monovalent antitoxins B and E; A, B, and E; and A, B, and F were created to account for the a priori possibility of such novel toxin production by strain IBCA10-7060.

d Note the prolonged time to death for all mice that received either monovalent antitoxin B alone or in combination with other monovalent antitoxins (bolded cells). The trivalent mixtures protected mice the longest. The absence of monovalent antitoxin B resulted in the death of all mice by 20 hours, which was the same as that for mice that received no antitoxin.

C. botulinum BoNT Type Bh • JID 2014:209 (15 January) • 185
even at 1:80 dilutions. Based on the ≥1 IU/0.1 mL (the intra-peritoneal dose per mouse) potency of the CDC botulinum antitoxins and their inability to protect mice from a 1:80 dilution of IBCA10-7060 culture filtrate, the filtrate was calculated to contain BoNT in excess of 1.6 × 10⁶ MLD₅₀/mL. In an analysis involving 6 mice per serial doubling dilution, the 72-hour cooked meat glucose starch culture filtrate actually had a total toxicity of only 4 × 10⁴ MLD₅₀/mL.

Further Mouse Bioassay Characterization
The identification of BoNT/A, /B, and /F epitopes by ELISA prompted additional mouse bioassays (all performed in triplicate) with the 7 monovalent botulinum antitoxins, used either singly or in various bivalent and trivalent combinations. When used singly, only antitoxin B protected mice against death for the first 24 hours. When monovalent antitoxin B was used in bivalent combination either with monovalent antitoxin A or with monovalent antitoxin F, the mice were protected against death for up to 48 hours (Table 1). In all experiments that combined antitoxin B with antitoxin A or antitoxin F, mice were protected for about twice as long as they were with monovalent antitoxin B alone. However, characteristic botulism illness and death were always the final outcome when either the BA or BF antitoxin combination was used. Pairs of mice that received IBCA10-7060 culture filtrate combined with trivalent antitoxins mixtures of either A, B, and E monovalent antitoxins or A, B, and F monovalent antitoxins survived longer than mice that received either monovalent antitoxin B alone or one of the bivalent antitoxin mixtures (ie, AB or BF). When mixed with IBCA10-7060 culture filtrate, the trivalent antitoxin combinations prolonged the time to death of mice but were unable to protect the mice against eventual botulism-associated death (Table 1).

Addition mouse bioassays were performed using 6 mice per group to pursue the observed prolongation of time to death. When monovalent antitoxins A, B, and E or A, B, and F were combined to form a trivalent antitoxin, death of mice was prevented for up to 48 hours, compared with untreated control mice. However, by 48 hours mice given the trivalent antitoxin mixtures showed classic signs of botulism. Their illness then slowly progressed over the following 24–48 hours, and by day 4 (96 hours) almost all mice had died (Table 1). Thus, despite identification by DIG-ELISA and double immunodiffusion of BoNT/A, /B and /F epitopes in IBCA10-7060 culture filtrates, the toxicity of the culture could not be neutralized with a trivalent mixture of monovalent A, B, and F diagnostic antitoxins. The experiment of mixing IBCA10-7060 culture filtrate with combined diagnostic monovalent antitoxins A, B, and F was repeated >10 times and always yielded the same result of all mice dying by days 4–7.

Heptaivalent equine botulinum antitoxin, an F(ab')₂ product that contains antitoxins to BoNT types A–G in substantially higher concentrations than in the monovalent antitoxins, was diluted to make the neutralizing potency of individual anti-A, anti-B, and anti-F components of the heptavalent antitoxin closer to that of the CDC monovalent antitoxins. The undiluted and diluted heptavalent antitoxin was proven to be 100% effective in protecting all mice in control studies against toxic culture filtrates produced by the BoNT/A-, BoNT/B-, and BoNT/F-producing reference strains of C. botulinum listed previously (Table 2). When injected with 0.5 mL of a 1:20 dilution of IBCA10-7060 culture filtrate combined with 0.1 mL of heptavalent A–G botulinum antitoxin that was diluted either 1:10, 1:20, or 1:25, all mice in all groups (6 mice per group) became symptomatic by 48 hours. The 0.5 mL of the 1:20 culture filtrate actually had a total of 23 800 MLD₅₀ of anti-A, 29 500 MLD₅₀ of anti-B, and 10 12 IU/mL of anti-F. Each mouse received 0.1 mL intraperitoneally, which provided the 72-hour cooked meat glucose starch broth at 35°C for 72 hours, then diluting 1:20 in gelatin phosphate diluent and filter sterilizing.

### Table 2. Ability of Polyclonal F(ab’)_2 Heptavalent Equine Botulinum Antitoxin to Protect Mice Against Botulinum Neurontoxin (BoNT) Types A, B, and F Contained in Culture Filtrates of Known Clostridium botulinum Strains

<table>
<thead>
<tr>
<th>Mouse Survival 7 d After Challenge With BoNT, With or Without Heptavalent Antitoxin</th>
<th>BoNT*</th>
<th>No Antitoxin</th>
<th>Undiluted Antitoxin</th>
<th>Diluted Antitoxin, by Dilution Ratio</th>
<th>Data are no. of mice that survived/no. injected.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hall A</td>
<td>0/4</td>
<td>4/4</td>
<td>1.5</td>
<td>1:10</td>
<td>1:20</td>
</tr>
<tr>
<td>BoNT A + Ff</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>BoNT A + B + Fg</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

Abbreviation: MLD₅₀, mouse lethal dose (50% end point).

**a** Crude BoNT produced by culturing listed strains in cooked meat glucose starch broth at 35°C for 72 hours, then diluting 1:20 in gelatin phosphate diluent and filter sterilizing.

**b** All control mice that did not receive heptavalent antitoxin died within 20 hours of intraperitoneal injection with culture filtrate.

**c** Undiluted USAMM IDA heptavalent Fab'₂ equine botulinum antitoxin contains the following neutralizing capacities: type A, 238 IU/mL; type B, 295 IU/mL; type C, 95 IU/mL; type D, 836 IU/mL; type E, 836 IU/mL; type F, 291 IU/mL; and type G, 102 IU/mL. Each mouse received 0.1 mL intraperitoneally, which provided the following toxin-neutralizing amounts: anti-A, 23.8 IU; anti-B, 29.5 IU; anti-C, 9.5 IU; anti-D, 83.6 IU; anti-E, 83.6 IU; anti-F, 29.1 IU; and anti-G, 10.2 IU. By definition, 1 IU neutralizes 10 000 MLD₅₀ of types A, B, C, D, F, or G botulinum toxin and 1000 MLD₅₀ of type E botulinum toxin.

**d** The 0.1-mL intraperitoneal dose of Fab'₂ heptavalent antitoxin provided neutralizing capacities of 23 800 MLD₅₀ of anti-A, 29 500 MLD₅₀ of anti-B, and 10 12 IU/mL of anti-F.

**e** The 0.1-mL intraperitoneal dose of the 1:25 dilution of the heptavalent antitoxin had the following neutralizing capacity: type A, 9500 MLD₅₀; type B, 12 000 MLD₅₀; type C, 3800 MLD₅₀; type D, 3300 MLD₅₀; type E, 3300 MLD₅₀; type F, 12 000 MLD₅₀; and type G, 4100 MLD₅₀. This heptavalent dose containing diluted anti-A, anti-B, and anti-F antibodies is roughly equivalent in potency to the neutralizing capacity contained in the Centers for Disease Control and Prevention monovalent antitoxins A, B, and F.

**f** Mixture contained a combination of Hall A and Langeland F culture filtrates with a final dilution of 1:20.

**g** Mixture contained a combination of Hall A, Okra B, and Langeland F culture filtrates with a final dilution of 1:20.
filtrate dilution contained approximately 1000 MLD<sub>50</sub> of BoNT activity, and the 0.1 mL of the 1:10 heptavalent antitoxin dilution contained anti-A, anti-B, and anti-F neutralizing potencies of approximately 23 800 MLD<sub>50</sub>, 29 500 MLD<sub>50</sub>, and 29 100 MLD<sub>50</sub>, respectively. Thus, mice injected with the diluted heptavalent product received an enormous excess of anti-A, anti-B, and anti-F neutralizing antibodies that nonetheless was unable to prevent their death from botulimum intoxication.

Each successive dilution of the heptavalent antitoxin provided less protection for the mice; that is, as the dilution of heptavalent antitoxin increased, the mice more rapidly developed symptoms of botulism and died. Death of symptomatic mice was first observed by day 3 (Table 3). All 6 mice injected with culture filtrate combined with heptavalent antitoxin diluted 1:25 died by day 4; when the antitoxin was diluted to 1:10, all 6 mice died by day 6; and when the antitoxin was diluted to 1:10, all 6 mice died by day 7 (Table 3).

Paradoxically, however, when used undiluted and at the 1:5 dilution, the heptavalent A–G F(ab′)<sub>2</sub> antitoxin protected the mice from death, probably via formation of nonspecific immune complexes between nonneutralizing antibodies in the F(ab′)<sub>2</sub> polyclonal antitoxin and the multiple epitopes on the surface of the toxin (see Discussion; Table 3, footnote d). These surviving mice were observed for 4 months for any late-developing signs of botulism that might follow gradual renal clearance of unbound F(ab′)<sub>2</sub> antibodies, as others have found when using F(ab′)<sub>2</sub> antitoxins [28–31]. All mice remained healthy for the full 4 months, as assessed on the basis of weight gain and the absence of illness.

Relative Amounts of Toxins B and H in Culture Filtrates
After it was realized that strain IBCA10-7060 produced BoNT/B and an unneutralizable second BoNT with A- and F-like epitopes, culture filtrate was serially diluted and injected intraperitoneally into mice to determine the relative amounts of the 2 toxins. Cooked meat glucose starch culture filtrate of IBCA10-7060 containing approximately 40 000 MLD<sub>50</sub>/mL of total BoNT activity was serially diluted and inoculated intraperitoneally into 2 parallel sets of mice. Four mice per dilution received filtrate with or without excess anti-B monovalent antitoxin. The 50% lethal end points among the mice that did not receive anti-B antitoxin and the mice that received anti-B antitoxin were compared, and the ratio of BoNT/B to BoNT/H was approximately 24:1. Although type B toxicity always exceeded type H toxicity, this ratio varied in subsequent experiments.

Efficacy of Antitoxin Raised Against IBCA10-7060 Culture Filtrate
Neutralization of strain IBCA10-7060 toxins was evaluated in mice, using a rabbit polyclonal IgG antitoxin (Rα7060) raised against a toxoid of culture filtrate. The apparent potency of Rα7060 antitoxin was relatively low, compared with that of the CDC monovalent antitoxins; its exact potency was not determined. A 0.1 mL amount of Rα7060 antitoxin neutralized a 1:500 dilution of IBCA10-7060 culture filtrate but not more-concentrated culture filtrates (Table 4). Based on an IBCA10-7060

<table>
<thead>
<tr>
<th>Time After Injection, Mean</th>
<th>Mouse Survival After Challenge With Culture Filtrate Mixed With Heptavalent Antitoxin&lt;sup&gt;A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>Undiluted&lt;sup&gt;B&lt;/sup&gt; 1:5 1:10 1:20 1:25</td>
</tr>
<tr>
<td>20 h</td>
<td>6/6 6/6 6/6 6/6</td>
</tr>
<tr>
<td>44 h</td>
<td>6/6 6/6 6/6 6/6</td>
</tr>
<tr>
<td>66 h</td>
<td>6/6 6/6 4/6 4/6</td>
</tr>
<tr>
<td>90 h</td>
<td>6/6 6/6 4/6 4/6</td>
</tr>
<tr>
<td>114 h</td>
<td>6/6 6/6 4/6 1/6</td>
</tr>
<tr>
<td>140 h</td>
<td>6/6 6/6 3/6 0/6</td>
</tr>
<tr>
<td>163 h</td>
<td>6/6 6/6 0/6&lt;sup&gt;C&lt;/sup&gt; 0/6</td>
</tr>
<tr>
<td>14 mo</td>
<td>6/6 6/6 0/6&lt;sup&gt;C&lt;/sup&gt; 0/6</td>
</tr>
</tbody>
</table>

Data are no. of mice that survived/no. injected. Abbreviations: BoNT, botulinum neurotoxin; MLD<sub>50</sub>, mouse lethal dose (50% end point).

<sup>A</sup> Each mouse received 0.5 mL of a 1:20 dilution of culture filtrate that contained approximately 40 000 MLD<sub>50</sub>/mL of BoNT, equivalent to a challenge dose of approximately 1000 MLD<sub>50</sub> per mouse (ie, just 0.1 IU equivalent of BoNT activity; see Methods). Heptavalent equine botulinum antitoxin was provided by USAMMMA.

<sup>B</sup> Undiluted USAMMMA heptavalent equine botulinum antitoxin contains the following neutralizing capacities: type A, 238 IU/mL; type B, 295 IU/mL; type C, 95 IU/mL; type D, 836 IU/mL; type E, 836 IU/mL; type F, 291 IU/mL; and type G, 102 IU/mL. Each mouse received 0.1 mL intraperitoneally, which provided these amounts of toxin-neutralizing activity: anti-A, 23.8 IU; anti-B, 29.5 IU; anti-C, 9.5 IU; anti-D, 83.6 IU; anti-E, 83.6 IU; anti-F, 29.1 IU; and anti-G, 10.2 IU. By definition, 1 IU neutralizes 10 000 MLD<sub>50</sub> of types A, B, C, D, F, or G botulinum toxin and 1000 MLD<sub>50</sub> of type E botulinum toxin.

<sup>C</sup> At the 1:25 dilution, the heptavalent antitoxin had the following neutralizing capacity per 0.1 mL mouse intraperitoneal dose: type A, 0.95 IU; type B, 1.2 IU; type C, 0.38 IU; type D, 3.3 IU; type E, 3.3 IU; type F, 1.2 IU; and type G, 0.41 IU. This diluted heptavalent antitoxin dose containing diluted anti-A, anti-B, and anti-F antibodies is approximately equivalent to the neutralizing capacity contained in the undiluted Centers for Disease Control and Prevention monovalent antitoxins A, B, and F, which could not protect mice against culture filtrate when used either singly or combined.

<sup>D</sup> The 1:10 dilution of antitoxin did not protect against mouse death from intraperitoneal injection of 1000 MLD<sub>50</sub> BoNT (960 MLD<sub>50</sub> of BoNT/B and 40 MLD<sub>50</sub> of BoNT/H) despite providing the following amounts of BoNT-neutralizing activity: anti-A, 23 800 MLD<sub>50</sub>; anti-B, 29 900 MLD<sub>50</sub>; and anti-F, 29 100 MLD<sub>50</sub>. Because the 1:10 dilution contained a >500-fold excess of anti-A and anti-F toxin neutralizing activity and a >30-fold excess of anti-B toxin neutralizing activity with no mouse survival, the survival of mice that received either the 1:5 dilution or the undiluted heptavalent antitoxin is believed to have resulted from nonspecific immune complex (ie, antigen-antibody lattice) formation during the preinjection incubation of the mixture, with clearance of the immune complexes after injection by the mononuclear phagocyte system (see Methods and Discussion). At the 1:5 dilution of heptavalent antitoxin at which all mice survived, the antitoxin A:toxin H ratio was 1190:1, the antitoxin B:toxin H ratio was 61:1. At the 1:10 dilution of heptavalent antitoxin at which all mice died, the antitoxin A:toxin H ratio was 595:1, the antitoxin F:toxin H ratio was 728:1, and the antitoxin B:toxin H ratio was 30:1.
culture filtrate titer of $4 \times 10^4 \text{MLD}_{50}/\text{mL}$, 0.2 mL of Ra7060 antitoxin product neutralized approximately 800 MLD$_{50}$/mL of BoNT/B and /H. Ra7060 antitoxin also neutralized toxic culture filtrates of $C.\ botulinum$ type B (Okra) but did not neutralize toxic culture filtrates of $C.\ botulinum$ type A (Hall) or of $C.\ botulinum$ type F (Langeland), even when they were diluted to 1:8000 (Table 4).

**Immunodiffusion Studies**

The monovalent equine botulinum antitoxins, the rabbit polyclonal antitoxin raised against IBCA10-7060 toxoid, and the equine heptavalent F(ab')$_2$ botulinum antitoxin were used as immunoprecipitating antibodies. When pure BoNT/A, /B, /E, and /F and their corresponding monovalent antitoxins were evaluated, immunoprecipitate lines formed only between each pure BoNT and its homologous monovalent antitoxin; no cross-reactivity between pure neurotoxins and antitoxins of differing serotypes was observed [26]. No immunoprecipitate formed between the normal horse serum control and the pure BoNTs. No immunoprecipitate was seen between antitoxin E and crude BoNT/F or between antitoxin F and crude BoNT/E, indicating the absence of cross-reactivity (Supplementary Figure 1).

Toxin in strain IBCA10-7060 trypticase peptone glucose yeast extract culture filtrates concentrated 10-fold was easily observed by agar immunodiffusion. A confluent line of immunoprecipitate formed between monovalent antitoxins A, B, and F and the toxins produced in culture by the 4 individual isolates of IBCA10-7060. In contrast, no immunoprecipitate line formed between strain IBCA10-7060 culture filtrate and monovalent antitoxin E (Figure 1). A single line of immunoprecipitate formed between Ra7060 antitoxin and the toxic culture filtrates produced by each of the 4 isolates of IBCA10-7060 (Figure 2).

Ra7060 antitoxin also formed a single immunoprecipitate line against pure BoNT/B and against culture filtrates of bivalent $C.\ botulinum$ strains cultured in cooked meat glucose starch and incubated for 72 hours at 35°C. Table 4.

<table>
<thead>
<tr>
<th>Culture Filtrate Containing BoNT$^a$</th>
<th>C. botulinum Strain</th>
<th>Alive/Injected With Ra7060, by Dilution of Concomitantly Administered Toxic Culture Filtrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:80</td>
</tr>
<tr>
<td>A Hall</td>
<td>0/2</td>
<td>ND</td>
</tr>
<tr>
<td>B Okra</td>
<td>2/2$^a$</td>
<td>ND</td>
</tr>
<tr>
<td>F Langeland</td>
<td>0/2</td>
<td>ND</td>
</tr>
<tr>
<td>Bh IBCA10-7060</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

Data are no. of mice that survived/no. injected.

Abbreviations: BoNT, botulinum neurotoxin; MLD$_{50}$, mouse lethal dose (50% end point); ND, not done.

$^a$ All Clostridium botulinum strains were cultured in cooked meat glucose starch and incubated for 72 hours at 35°C.

$^b$ Ra7060 antitoxin neutralized BoNT/B culture filtrate at a 1:80 dilution but was unable to neutralize BoNT/A or BoNT/F culture filtrates at 1:80, 1:800, or 1:8000 dilutions.

$^c$ Ra7060 antitoxin was able to neutralize BoNT/Bh culture filtrate at the 1:500 dilution, which, at the initial culture filtrate toxin concentration of 40 000 MLD$_{50}$/mL, was calculated as 38.4 MLD$_{50}$ BoNT/B and 1.6 MLD$_{50}$ BoNT/H injected intraperitoneal per mouse.

Figure 1. Immunodiffusion of strain IBCA10-7060 botulinum neurotoxin with monovalent botulinum antitoxins A, B, E, and F. Pictures 1, 2, and 4 show confluent lines of immunoprecipitate that formed between monovalent botulinum antitoxins A, B, E, and F, respectively, and 10-fold concentrated cooked meat glucose starch toxic culture filtrates from the 4 isolates of strain IBCA10-7060 that are loaded in peripheral wells 1–4 of each plate, clockwise from top. Wells 5 and 6 are blank. Picture 3 demonstrates that no immunoprecipitate forms between monovalent botulinum antitoxin E and IBCA10-7060 culture filtrates. Note the absence of any lines of partial identity between the 4 isolates of IBCA10-7060; that is, all isolates are producing identical toxin molecules. One-percent molecular-grade agarose was stained with 0.1% thiazine red.
DISCUSSION

The 7 botulinum toxin types A–G were discovered between 1897 and 1970 [1–3, 5–10] and were distinguished from each other by the defining experimental mouse bioassay principle that a polyclonal antitoxin raised against one toxin type is unable to neutralize any of the other 6 toxin types. With subsequent recognition of bivalent Ab, Ba, AB, Af, and Fb toxin-producing strains of *C. botulinum*, this experimental criterion for defining toxin types was enlarged to include neutralization of culture filtrate by bivalent mixtures of type-specific antitoxins (eg, AB, AF, or BF) [11–14].

By these criteria, *C. botulinum* strain IBCA10-7060 produced a novel botulinum toxin type—BoNT/H—because its culture filtrates could not be neutralized by antitoxin excess of any of the 7 A–G monovalent botulinum antitoxins, whether used individually, in double (AB, AF, and BF) or triple (ABE and AFB) combinations or by a comprehensive heptavalent (A–G) F(ab′)2 antitoxin (Tables 1, 3, and 5). Although the double-immunodiffusion assay and ELISA identified BoNT/A-like and /F-like epitopes in the type H toxin, and despite having excess toxin-neutralizing potency, the AF double-monovalent antitoxin mixture and the heptavalent A–G antitoxin were both unable to neutralize the lethality of IBCA10-7060 culture filtrates in mice once the BoNT/B activity had been neutralized by excess monovalent anti-B antitoxin. This experimental finding indicated that BoNT/H does not possess the BoNT/A and BoNT/F epitopes that enable their neutralization by type-specific antitoxins. Detailed molecular characterization of the bont/H gene and toxin gene cluster may be found in the companion article by Dover et al [20].

In the double-immunodiffusion assay, visible immunoprecipitate lines formed in the “zone of equivalence,” where the concentrations of an antigen and an antibody to that antigen are approximately equal (Figures 1 and 2 and Supplementary Materials) [24, 33–36]. The immunoprecipitate consists of a 3-dimensional lattice of immune (antibody:antigen) complexes [34–36]; when such antibody:antigen lattices form in the circulation, they are rapidly cleared by the liver [33] and spleen [37]. We postulate that the survival of mice that received IBCA10-7060 culture filtrate mixed either with undiluted or 1:5 diluted F(ab′)2 heptavalent botulinum antitoxin occurred because the very high concentrations of antibody in the undiluted and 1:5 antitoxin dilutions resulted in formation of antibody:antigen (immune complex) lattice formation. We suspect that immune complexes formed between the F(ab′)2/anti-A and anti-F polyclonal antibodies and the many BoNT/A-like and /F-like epitopes on the surface of BoNT/H during the 30-minute incubation that preceded intraperitoneal injection (Supplementary Materials) and that the subsequent immediate clearance of these immune complexes by liver and spleen enabled the mice to survive.

The in vitro ELISA and double-immunodiffusion assay used to analyze IBCA10-7060 culture filtrate identified epitopes in it that are also present on BoNTs/A, /B, and /F, but neither in vitro assay could determine whether antitoxins to BoNTs/A, /B, and /F would neutralize IBCA10-7060 culture filtrates. Furthermore, the in vitro assays were incapable of recognizing that a novel botulinum toxin type, BoNT/H, that contained nonneutralizing type A and type F epitopes was present in the culture filtrate. Only the mouse bioassay for BoNT detection and typing was capable of making this determination.

Strain IBCA10-7060 is the first *C. botulinum* type Bh strain to be identified and is the first *C. botulinum* strain known to...
produce BoNT/H. Botulinum toxin type H is the first new BoNT type to be identified in >40 years. As with other BoNTs, the possibility of misuse of BoNT/H exists and requires appropriate anticipatory measures [38]. The recognition of novel BoNT/H after an interval of almost half a century serves as a reminder that additional novel botulinum toxin types likely await discovery and that for this purpose, the mouse bioassay remains indispensable.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. The contributions to knowledge of Clostridium botulinum and botulinum toxin by Louis DS. Smith provided inspiration for this work. We thank Karen Hill of Los Alamos National Laboratory for helpful discussions; the Environmental Microbial Diseases Laboratory, CDPH, for performing the ELISA assay; and the US Army Medical Materiel Development Activity, Ft. Detrick, MD, for providing the heptavalent F(ab')2 botulinum antitoxin.

Financial support. This work was supported by the Infant Botulism Treatment and Prevention Fund of the California Department of Public Health.

Potential conflicts of interest. All authors: No reported conflicts.

Table 5. Summary of Experimental Evidence for the Existence of Botulinum Neurotoxin (BoNT) Type H

<table>
<thead>
<tr>
<th>Experimental Finding</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Strain IBCA10-7060 (Str 7060) is a Gram-positive, obligately anaerobic, spore-forming, lipase-positive, lecithinase-negative, proteolytic bacterium.</td>
<td>The biochemical and morphological characteristics of the bacterium Str 7060 match those of proteolytic C. botulinum.</td>
</tr>
<tr>
<td>2. 16 S ribosomal RNA characterization identified the nucleic acid sequence of proteolytic C. botulinum in Str 7060 [20].</td>
<td>Bacterial species identification by 16 S ribosomal RNA characterization confirmed Str 7060 to be a proteolytic C. botulinum [20].</td>
</tr>
<tr>
<td>3. Mice injected with Str 7060 culture filtrate developed classical symptoms of botulism.</td>
<td>Str 7060 produces at least 1 BoNT.</td>
</tr>
<tr>
<td>4. Mice injected with heated Str 7060 culture filtrate remained healthy.</td>
<td>The BoNTs produced by Str 7060 are heat-labile, as are all known BoNTs.</td>
</tr>
<tr>
<td>5. The lethal effects of Str 7060 culture filtrate for mice were not neutralized by any of the 7 monovalent BoNT types A–G when used individually.</td>
<td>Either Str 7060 produces &gt;1 BoNT or the toxin it produces is not one of the BoNT types A–G.</td>
</tr>
<tr>
<td>6. Double immunodiffusion testing of Str 7060 culture filtrate with monovalent botulinum anti-A, anti-B, and anti-F antitoxins produced a single confluent immunoprecipitate line. DIG-ELISA of Str 7060 culture filtrate gave a positive reaction in the wells coated with botulinum anti-A, anti-B, and anti-F capture antibodies but not with anti-E antibodies.</td>
<td>Str 7060 produces BoNTs that have nonneutralizing epitopes in common with typical BoNT/A, /B, and /F but that lack epitopes in common with BoNT/E.</td>
</tr>
<tr>
<td>7. The lethality of Str 7060 culture filtrate for mice was not neutralized by any 2-fold combination of monovalent antitoxins against the known bivalent BoNT-producing strains (ie, antitoxin combinations AB, BF, and AF).</td>
<td>Either Str 7060 produces a novel BoNT or a novel combination of ≥ 2 botulinum toxins, or the toxins it produces are not typical A, B, or F botulinum toxins.</td>
</tr>
<tr>
<td>8. Mixtures of monovalent antitoxins that contained antitoxin B (eg, BA and BF) prolonged the time to death among mice injected with Str 7060 culture filtrate, compared with those injected with mixtures that did not contain antitoxin B (eg, AF).</td>
<td>Str 7060 produces a botulinum toxin type B and at least 1 other lethal toxin.</td>
</tr>
<tr>
<td>9. The Rx7060 antitoxin raised against a toxoid of Str 7060 culture filtrate protected mice against the otherwise lethal effects of the Str 7060 culture filtrate and of BoNT/B culture filtrate. However, Rx7060 antitoxin did not protect mice against either BoNT/A or BoNT/F culture filtrates.</td>
<td>The Str 7060 culture filtrate contains at least 2 BoNTs. One toxin is BoNT/B, and the other(s) is a novel toxin(s) whose antitoxin(s) does not cross-neutralize either BoNT/A or BoNT/F.</td>
</tr>
<tr>
<td>10. The lethal effects of Str 7060 culture filtrate for mice were not neutralized by a trivalent mixture of monovalent BoNT/A, /B, and /F.</td>
<td>The BoNTs produced by Str 7060 do not consist of the 3 individual type A, type B, and type F BoNTs.</td>
</tr>
<tr>
<td>11. At dilutions of the US Army heptavalent A–G polyvalent antitoxin that resulted in a &gt;500-fold excess of neutralizing potency for each toxin type, the A–G heptavalent antitoxin did not protect mice against the lethal effect of Str 7060 culture filtrate.</td>
<td>Despite identification in Str 7060 of epitopes in common with BoNT/A, /B, and /F, the BoNTs Str 7060 produces include a unique nonneutralizable antigenicity (ie, a novel toxin type) that differs from that of the 7 known BoNTs, A–G. In alphabetical sequence, this unique nonneutralizable lethal antigenicity becomes BoNT/H.</td>
</tr>
</tbody>
</table>

The definition and operational differentiation of botulinum toxin types was as follows: the inability of a polyclonal immunoglobulin G antitoxin raised against each of the 7 known (A–G) botulinum toxins to neutralize the lethal effects of any of the other 6 toxin types. A modern caveat encompasses the “bivalent” (ie, dual toxin–producing strains Ab, Ba, AB, At, and Bf) by demonstrating neutralization of their culture filtrate by a combination of 2 monovalent immunoglobulin G antitoxins [4].

Abbreviations: C. botulinum, Clostridium botulinum; DIG-ELISA, digoxigenin enzyme-linked immunosorbent assay.
All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References


