Emergence of Clostridium botulinum type B-like nontoxigenic organisms in a patient with type B infant botulism

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Emergence of *Clostridium botulinum* Type B-Like Nontoxigenic Organisms in a Patient with Type B Infant Botulism

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We encountered a patient with infant botulism caused by a single clone of *Clostridium botulinum* type B. In the early convalescent phase, a *C. botulinum* type B-like nontoxigenic organism emerged in the feces instead. Growth inhibition of toxigenic strains by nontoxigenic strains was examined.

The clinical spectrum of infant botulism ranges widely, from a symptomless state to severe paralysis (5). Treatment of symptons is still a principle in the treatment of the disease (6), and the mechanism of recovery remains unclear. We encountered a 6-month-old patient with infant botulism caused by *Clostridium botulinum* type B, which was the first case of type B infant botulism in Japan to be reported (3). In this patient, the causative type B organisms disappeared from the feces in early recovery phase and nontoxigenic organisms indistinguishable from the causative organisms emerged.

In brief, the patient's symptoms started with constipation, and neuromuscular illness characteristic of infant botulism was observed on the 10th day from onset. On the 26th day from onset, a gradual improvement in baseline function was observed while mild constipation still continued. These symptoms almost completely disappeared by the 43rd day. The route of ingestion of the type B organism was unknown.

Fecal samples were obtained on the 10th, 26th, and 43rd days from onset. The supernatant of the fecal suspension was tested for lethal toxicity in mice. To isolate *C. botulinum*, the fecal samples were spread on egg yolk-nutrient agar, which was incubated anaerobically at 37°C for 2 days (9). The biological and biochemical properties of the isolates were determined as described previously (9).

Chromosomal DNAs were prepared from fecal isolates. Cultures were grown in brain heart infusion (BHI; BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) containing 0.05% cysteine at 37°C for 5 h and were treated with penicillin G before harvesting. The cells were heated at 70°C for 10 min and were treated with lysozyme, sodium dodecyl sulfate, and proteinase K. Chromosomal DNAs were prepared from the lysate by phenol-chloroform extraction and RNase treatment. The 5-μg DNA samples were digested with *Hin*dIII or *Eco*RI and separated through a 1% agarose gel. The gel was stained with ethidium bromide, and DNA fragments were visualized under UV light. To detect the type B neurotoxin gene, the DNA fragments were transferred onto a nylon membrane (7). The membrane was hybridized with a ^{32}P-labelled DNA probe for the type B toxin gene and autoradiographed. DNA fragments for the probe were prepared by PCR with the primer set B3 and B4 (8).

Botulinum type B toxicity values were 100 minimum lethal doses (MLD)/g of feces obtained on the 10th day from onset and 50 MLD/g of feces obtained on the 26th day. No toxicity in feces obtained on the 43rd day was detected. Colonies characteristic of proteolytic *C. botulinum* had developed on the culture of feces obtained on the 10th day. Three strains, which were randomly selected from the colonies, produced 4,000-MLD/ml type B toxin in chopped-meat medium containing 1% glucose (2, 9).

On the culture of feces obtained on the 26th day from onset, colonies similar to those from feces obtained on the 10th day had developed. However, none of 20 colonies randomly selected was toxigenic. Six strains from these colonies were further examined and showed the same biological and biochemical properties as those of the three toxigenic strains (Table 1). The restriction enzyme patterns of the chromosomal DNAs from these nine strains were identical on the basis of visual comparison (Fig. 1). Southern blot analysis of the *Eco*RI digest showed that the toxigenic strains possessed a 15-kb fragment containing the type B toxin gene and that the nontoxigenic strains lost the fragment (Fig. 1). Colonies similar to proteolytic *C. botulinum* were not found in feces obtained on the 43rd day from onset.

To analyze the phenomenon of the toxigenic organisms disappearing and the nontoxigenic organisms emerging in the convalescent phase, three experiments concerning growth inhibition among all the toxigenic and nontoxigenic isolates were performed. First, the toxigenic strains were cross-streaked against the nontoxigenic strains on BHI agar and cultured. Second, the actively growing toxigenic strains in BHI broth were mixed with culture supernatants of the nontoxigenic strains. No nontoxigenic strains inhibited growth of the toxigenic strains. Then, 1.8×10^8 cells of a toxigenic strain and 1.2×10^8 cells of a nontoxigenic strain (ratio, 1:0.67) were cocultured in BHI broth overnight and five consecutive passages were performed. Then, the ratio of toxigenic cells to nontoxigenic cells in the coculture was measured by colony counting after plating. To distinguish toxigenic colonies from nontoxigenic ones, the toxin gene was detected by PCR. The final ratio was 1:0.81. A pure culture of the toxigenic strains did not yield any nontoxigenic derivative after five consecutive passages.

Analyses of the biochemical properties and restriction enzyme patterns of chromosomal DNAs suggest that the case we report was caused by a single clone of a type B organism and that the nontoxigenic strains might be derived from the toxigenic clone. It is also suggested that the toxigenic strains firmly retain their toxigenicity in vitro. Therefore, it may be more

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TABLE 1. Properties of the toxigenic and nontoxigenic isolates obtained from feces

Property	Result for:	
	Toxigenic isolates (three strains)	Nontoxigenic isolates (six strains)
Liquefaction of 10% gelatin	+	+
Digestion of casein, milk, and meat	+	+
Volatile fatty acid from peptone-yeast extract-glucose medium	A, ib, b, iv, and ic[a]	A or a, ib, B or b, iv, and ic[a]
Indole production	−	−
Nitrate reduction	−	−
Acid production from arbutin, dextrin, glucose, glycerol, maltose, salicin, sorbitol, starch, sucrose, and trehalose	+	+
Acid production from 21 other sugars[b]	−	−
Hydrolysis of:		
Esculin	+	+
Starch	−	−
NaCl tolerance (%)	4	4
Motility	+	+

[a] a, acetic acid; ib, isobutyric acid; b, *n*-butyric acid; iv, isovaleric acid; ic, isocaproic acid. Capital letters indicate at least 10 mM, and lowercase letters indicate less than 10 mM. Isocaproic acid was detected in two toxigenic strains and in three nontoxigenic strains.

[b] Amygdalin, adonitol, arabinose, cellobiose, dulcitol, erythritol, fructose, galactose, glycogen, inositol, inulin, lactose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, sorbose, and xylose.

likely that a small population of nontoxigenic organisms derived from the toxigenic organism was ingested by the patient with the toxigenic one. Since growth-inhibitory substances such as bacteriocin (1, 4) were not found in the present study, it may be supposed that another kind of interaction, such as competition in attachment to the intestinal surface, plays an important role in the overgrowth of the nontoxigenic organisms during the convalescent phase. Accumulated information gathered from cases similar to the present case will lead to a better understanding of the role of nontoxigenic organisms in infant botulism.

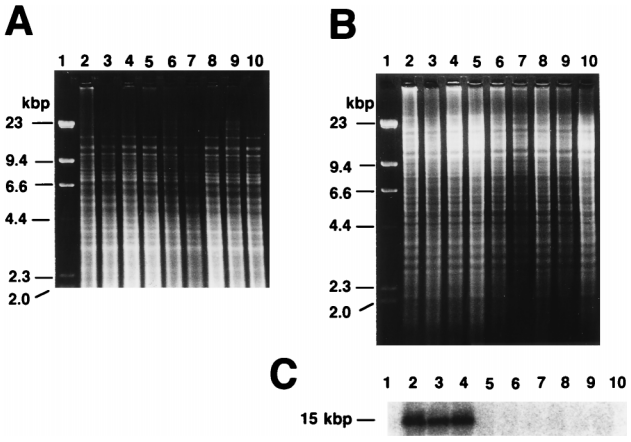

FIG. 1. Restriction enzyme patterns of chromosomal DNAs of the isolates obtained from feces. (A) *Hin*dIII digestion; (B) *Eco*RI digestion; (C) Southern blot analysis of the *Eco*RI digest with a probe specific to the type B neurotoxin gene. Lanes: 1, marker; 2 to 4, the toxigenic strains; 5 to 10, the nontoxigenic strains.

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