Botulism in Metronidazole-Treated Conventional Adult Mice Challenged Orogastrically with Spores of Clostridium botulinum Type A or B

YINCHUN WANG† AND HIROSHI SUGIYAMA*

Food Research Institute and the Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53706

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Conventional adult mice were pretreated with metronidazole to make their intestinal tracts receptive to colonization by Clostridium botulinum. These mice, in groups of 10, were fed 0 (controls), 103, 104, or 105 C. botulinum type B spores and were placed for observation in filter-lid cages whose screen floors minimized the amounts of feces available for coprophagy. The opportunity to eat feces was made equal for all mouse groups by placing one mouse of every group in each of 10 cages. Mice given a spore inoculum began to develop botulism after incubation periods of slightly less than 2.75 days. Morbidity rates, which reached maxima within 5 days of challenge, were related to inocula levels. Mortality rates were also dose related. Mice given 105 spores and then type B antitoxin intraperitoneally, a treatment not affecting intraintestinal toxin production, remained healthy. Morbidity among control adult mice was seldom more than 10% and could be ascribed to toxin ingested with feces. A C. botulinum type A spore suspension gave similar results, although morbidity and mortality rates were generally lower than after challenge with a comparable number of type B spores. Mice challenged with 105 or 106 spores had similar toxin levels in their large intestines 48 h later. Morbidity rates correlated better with toxin levels in the small intestines.

Infant botulism is the toxic infection in which toxin produced by Clostridium botulinum colonizing the intestinal tract passes into the bloodstream and is carried by it to distant cholinergic neuromuscular junctions (1). The toxin poisons these junctions by preventing the release of normal amounts of acetylcholine by nerve stimuli and thereby causes a syndrome of flaccid paralysis (8).

The intraintestinal colonization phase of infant botulism has been duplicated by administering C. botulinum spores in toxin-free suspensions into the stomachs of 8- to 10-day-old mice, when the age-related susceptibility of the host species is highest (9). Additionally, normally resistant adult mice are colonized when challenged oroagastrically with the spores at the time their gut microfloras are suppressed by feedings of a mixture of kanamycin and erythromycin (2)—both broad-spectrum antibiotics—or metronidazole (3), whose antimicrobial activity is limited to obligate anaerobes. Although a recent publication reports that infant mice of certain age range develop botulism when given high challenge inocula of 107 or more C. botulinum type E spores (6), intraintestinal growth of C. botulinum type A in our animal models has been nonsymptomatic. Its occurrence is therefore monitored by testing for botulinum toxin production in the gut. When toxin in colonized guts is titrated by the intraperitoneal (i.p.) route in normal mice, some samples have ≥1,000 mean lethal doses (LD50). Since the intraintestinally formed toxin must be absorbed into the bloodstream before it can cause systemic effects, the failure of these toxin levels to cause overt botulism is attributed to most of the toxin being in the cecum and colon, from where it is poorly absorbed.

Although the colonization of conventional infant and antimicrobiially treated adult mice is nonsymptomatic, that of germfree adult mice almost always causes fatal botulism within 3 to 5 days (7), and gnotobiotic mice having floras of a few species also become ill (10). These illnesses could be responses to toxin absorbed directly after production, but the possible involvement of coprophagically acquired toxin cannot be eliminated.

The present communication describes findings differing from those reported previously (3): botulism was elicited when C. botulinum type A or B spores were administered to conventional adult mice that had been pretreated with metronidazole to make their guts susceptible to colonization by the inoculum. Mice were housed in cages with screen floors to reduce the amount of feces accessible to the animals and were distributed in a manner that made it possible to evaluate how much coprophagy contributed to morbidity rates.

MATERIALS AND METHODS

Spores. Spores of C. botulinum type A strain 62 and type B strain Okra were obtained by incubating the cultures in a freshly prepared medium of 2.5% Trypticase, 2.5% Thiotone, and 2.0% Phytone (all from BBL Microbiology Systems, Cockeysville, Md.) for 5 to 7 days at 37°C. Growth was harvested and washed 7 to 10 times with distilled water by centrifugation.

Viable spores were enumerated with the five-tube most-probable-number method, using cooked meat medium (Difco Laboratories, Detroit, Mich.) made with 0.5% glucose-0.2% soluble starch. Inocula were dilutions of a stock suspension portion that was heated in an 80°C water bath for 15 min. Growth was recorded after incubations of at least 7 days at 37°C, and those in the endpoint tubes were tested for the presence of the appropriate type of toxin. Viable counts were confirmed periodically.

The stock spore suspension was diluted with physiological saline so that 0.5 ml contained the highest spore dose of the experiment. This dilution was heated in an 80°C water bath.
for 15 min before being diluted for the other challenge doses. The heating assured the absence of toxin in the inocula; 0.5 ml of the highest challenge dose did not cause illness when injected i.p. into normal mice.

**Distribution of toxin.** Metronidazole. Tests were done with HA/ICR strain mice of 23 to 26 g (Sprague-Dawley, Madison, Wis.). An aqueous solution containing 13.2 mg of metronidazole (Sigma Chemical Co., St. Louis, Mo.) per ml was prepared by warming, and 0.5 ml was fed to mice with a 20-gauge feeding needle (Popper and Sons, New Hyde Park, N.Y.) at 10- to 14-h intervals for a total of 8 doses (3). Drinking water during this treatment period contained 1.0 mg of metronidazole per ml and was changed daily. Mice were transferred after each dose to filter-lid cages (American Scientific Products, Chicago, Ill.) which were autoclaved with bedding and Laboratory Chow 5010 (Ralston Purina, St. Louis, Mo.) inside.

**Challenge and caging.** Mice were divided randomly into experimental groups 18 h after the last dose of metronidazole. They were challenged orogastrically at this time with one of the spore doses, and one mouse of each group was placed in the same cage. Since 10 mice were used in a group, 10 cages were needed for an experiment.

Cages were filter-lid, polycarbonate boxes with approximate bottom measurements of 29 by 19 cm and a height of 13 cm (American Scientific Products), modified by using as floors a 4-mesh screen of 23-gauge wire raised ca. 2 cm above the cage bottom. Mice were transferred daily to sterile cages. Drinking water during the observation was sterile tap water changed daily.

Numbers of sick mice and the cumulative dead were recorded at least once a day.

**Toxin tests.** Intraintestinal toxin production was used to determine whether mice were colonized by the intragastrically administered spores. At 48 h postchallenge, up to three freshly excreted fecal pellets were collected from each mouse, or the large intestine with its digesta was removed after the mouse was sacrificed. The pools of feces or the gut samples were homogenized with 0.4% phosphate–0.2% gelatin (pH 6.2). The homogenate was held overnight at 4°C, and 0.5 ml of the supernatant (extract), obtained by centrifugation, was injected i.p. into a normal mouse. The extracts killing the toxin test mice within 3 days were considered to contain botulinum toxin, since the appropriate type of botulinum antitoxin neutralized the toxicity of representative extracts. It was necessary to dilute some extracts to reduce a nonbotulinum lethal agent(s) to levels that did not interfere with the test (3).

Specimens to be titrated were weighed. Feces were extracted with 9 times their amount (wt/vol) of buffer-gelatin to yield a 1:10 dilution; intestinal segments were extracted to yield 1:4 or 1:5 dilutions. Serial twofold dilutions of the extracts were injected i.p. in 0.5-ml amounts into their own pair of mice. The LD₅₀ dilution was the one killing one of the two mice within 3 days; when this effect was not obtained, the LD₅₀ dilution was calculated as midway between the highest dilution lethal for both mice and the next dilution killing neither of the pair. Toxin titrated in feces was measured as LD₅₀ per gram (wet weight); amounts in gut segments were totals in the samples.

**Determination of toxin.** Metronidazole-treated mice were challenged with 10² or 10³ C. botulinum spores and then distributed so that one mouse of each group was in each of 10 screen-floor cages. At 48 h postchallenge, five randomly chosen mice of each group were sacrificed. Their entire small and large (colon and cecum) intestines were removed separately, with the contents of the two segments being prevented from mixing. Feces were collected at this time from the remaining mice, and these animals were observed for illness for 4 more days. Samples were extracted, and those of feces and large intestines were tested qualitatively for toxin to determine the colonization rate of the mouse groups. Unused portions of gut extracts were stored at 4°C. When toxin was found in the large intestine, the saved extracts of that animal were titrated for toxin.

**Duration of colonization.** The previous study of C. botulinum colonization of metronidazole-treated mice included a determination of the total LD₅₀ of toxin in the large intestines of mice sacrificed on different days after challenge with 10² type A spores (3). A similar study was done except that test samples were feces from the same mice. Metronidazole-treated mice were challenged with 10² type B spores and were immediately given ip 20 IU of type B antitoxin, a treatment that does not affect intraintestinal toxin production but prevents overt botulism in C. botulinum-monoaerated mice (10). Freshly excreted feces were collected on successive days from each of three C. botulinum-colonized mice, and these samples were titrated for type B toxin.

**RESULTS**

**Responses to type B spores.** Metronidazole-treated mice were separated into five groups of 10 mice each. Mice of one group were controls fed saline; those of other groups were challenged with 10⁵, 10⁴, or 10³ C. botulinum type B spores. Intestinal colonization rates, determined with feces collected from five mice, were related to inoculum sizes: none of the tested control mice were colonized, but 60, 80, 100, and 100% of mice receiving the successively larger doses were colonized (Fig. 1A). Titrations of toxin-positive feces of three mice in each group indicated higher concentrations in the feces of mice given larger spore doses.

Mice were apparently healthy at the time feces were collected for the toxin test 48 h postchallenge, but some mice died during the following 18 h after developing a typical botulism syndrome (incubation of <2.75 days). These illnesses and those beginning during the next 2 days were usually fatal. Morbidity rates were related to challenge doses, but reached maxima by postchallenge day 5 regardless of challenge dose. Illnesses with incubations of more than 4 days did not usually progress beyond the mild stage. Observations were terminated after day 6 since new illness did not occur and sick mice began to recover after this time.

Although none of the five control mice tested were colonized 2 days after the start of the experiment, one of these tested mice was found dead on day 5. In other experiments, controls remained healthy, or (rarely) up to 2 of 10 developed botulism on day 4 or 5.

Although the syndrome of test mice was indistinguishable from that after i.p. injection of highly purified botulinum toxin, a different test was done to show that the illness was botulism. In the experiment (Table 1), metronidazole-treated mice were divided into two groups of 10 mice each. All mice were challenged with 10² type B spores, and 20 IU of type B antitoxin were administered i.p. immediately thereafter to mice of one group. Type B toxin in feces collected 2 days later indicated colonization rates of ≥90% for mice of both groups. During the 6 days of observation, 9 of 10 mice not receiving antitoxin became ill, but all antitoxin-treated mice remained healthy.

Toxin concentrations were highest in feces excreted on postchallenge day 2 or 3 and thereafter declined so that the animals no longer excreted toxin by day 5 or 6. The short
duration of type B colonization was similar to that indicated by a previous study of toxin in the large intestines of mice challenged with type A spores but not given prophylactic antitoxin (3).

**Response to type A spores.** The experiments with type B spores were repeated with the type A spore suspension (Fig. 1B). Results were similar except that type A tended to have fewer effects: comparable numbers of spores generally caused lower morbidity and mortality.

Type A challenges elicited the flaccid paralysis of botulism. In two separate experiments involving groups of 10 mice, challenges with 10^3 spores did not cause illness when mice were given 20 IU of type A antitoxin prophylactically, although among the two groups of 10 mice not given antitoxin, 5 and 7 developed botulism. The continued good health of the great majority of control mice not administered a spore inoculum indicated that ingested toxin was not the important cause of illness among test mice. In other experiments, no more than 2 of 10 controls became sick.

The 50% infective dose of type A spores is ca. 10^5 per mouse or significantly less than the 10^7 spores per mouse of the earlier study (3). Since the spore suspension of the previous study had been in the laboratory for several years and that of the present work was a newly prepared one made with the same culture strain, an experiment was done to determine whether spores lost infectivity with age. The old suspension was as infective as the new one. The 50% infective dose of the type B suspension was less than 10^2 spores per mouse. Excepting the possibility that mice started with a different gut flora, we are unable to explain the lower infectivity seen previously.

**Distribution of toxin in gut.** In the experiment concerned with the relationship between spore doses and toxin amounts formed, colonization rates of mice challenged with 10^5 and 10^6 type B spores were, respectively, 80 and 100% (Table 2). Excluding uncolonized mouse no. 3, toxin amounts in the large intestine 48 h after challenge with different numbers of spores were not significantly different (P > 0.05), although averages were 5,050 LD_50s after low challenge dose versus 9,900 LD_50 after the high dose. Average amounts in the small intestines were also not significantly different when all tested animals were considered. However, when mouse no. 7 was omitted because it differed from others of the group, the average in this part of the gut after the larger inoculum (2,280 LD_50) was greater (P < 0.05) than that after the smaller inoculum (210 LD_50).

Morbidity among the five observed mice of each group

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**TABLE 2.** Toxin in small and large intestines 48 h after orogastric challenges of metronidazole-treated mice with *C. botulinum* type B spores

<table>
<thead>
<tr>
<th>Challenge (logLD_50)</th>
<th>Mouse no.</th>
<th>No. of LD_50s in gut</th>
<th>% (small/large) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td>2^a</td>
<td>1</td>
<td>15</td>
<td>3,400</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>70</td>
<td>6,500</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>15</td>
<td>1,100</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>750</td>
<td>9,200</td>
</tr>
<tr>
<td>5^b</td>
<td>6</td>
<td>1,200</td>
<td>4,800</td>
</tr>
<tr>
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<td>7</td>
<td>30</td>
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<td>5</td>
<td>8</td>
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<td>5</td>
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</tr>
<tr>
<td>5</td>
<td>10</td>
<td>2,400</td>
<td>9,500</td>
</tr>
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</table>

^a 80% colonization rate; two of five mice kept for observation developed botulism.

^b 100% colonization rate; all five mice kept for observation developed botulism.
TABLE 3. Toxin in small and large intestines 48 h after orogastric challenges of metronidazole-treated mice with C. botulinum type A spores

<table>
<thead>
<tr>
<th>Challenge (log_{10})</th>
<th>Mouse no.</th>
<th>No. of LD_{50} in gut</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small</td>
<td>Large</td>
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<td>2</td>
<td>3</td>
<td>10</td>
<td>1,600</td>
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<td>2</td>
<td>4</td>
<td>530</td>
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</tr>
<tr>
<td>2</td>
<td>5</td>
<td>40</td>
<td>710</td>
</tr>
<tr>
<td>5^{b}</td>
<td>6</td>
<td>680</td>
<td>3,600</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>1,000</td>
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<td>400</td>
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<td>9</td>
<td>1,600</td>
<td>6,400</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>50</td>
<td>10,500</td>
</tr>
</tbody>
</table>

* 90% colonization rate for 10 mice: 1 of 5 mice kept for observation developed botulism.
* 100% colonization rate for 10 mice: 3 of 5 mice kept for observation developed botulism.

was within the ranges expected from earlier findings (Fig. 1A). All mice given the larger inoculum died of botulism within 3 days, and among mice given the low dose, two, both excreting toxin on postchallenge day 2, developed botulism on day 4 and died on day 5.

Type A spores gave similar results (Table 3). Toxin amounts in the large guts (average, 2,160 LD_{50} after low challenge versus 5,740 LD_{50} after high challenge) were not statistically different. Amounts in the small intestines (average, 160 versus 745 LD_{50}) also were not different statistically when findings of all mice were used, but were different when mouse no. 10 was excluded.

**DISCUSSION**

In the previous study of metronidazole-treated mice (3), illness was seen when mice were challenged with 10^4 type A spores. However, the infrequent and random occurrence of illness, together with the possibility of ingested toxin being the cause, led to the conclusion that the colonization is essentially nonsymptomatic. The findings of the present study permit the opposite conclusion: the animal model developed systemic botulism without ingesting toxin.

The new conclusion is made possible by the experimental design. Since most of the feces fell through the screen floors beyond reach of the occupants, the opportunity for coprophagy was minimized and the morbidity rate of control mice (treated with metronidazole but not receiving a spore inoculum) was usually ≤10%. The few illnesses among controls were acquired from test mice since they occurred (i) even if the animal was not colonized by the pathogen 48 h after the start of the experiment, and (ii) at least 1 day after one or more spore-challenged cagemates became ill. Moreover, frequencies of coprophagically acquired botulism were made equal for all mouse groups by having one mouse of every group in each cage. In this situation, the true morbidity rate, which includes only illness caused by toxin entering the circulation directly after production, is the difference between the observed morbidities of test and control groups. These true rates were related to the numbers of spores in the challenge dose.

LD_{50} per gram of feces were higher when mice were administered larger spore inocula, but toxin quantities in the large intestines of mice challenged with 10^2 versus 10^3 spores were not statistically different. The differences in results are partly due to the large intestine values being affected by the presence of variable amounts of digesta, in which toxin is present, whereas fecal values are expressed per gram of what can be considered to be digesta concentrates. Fecal toxin concentrations may be more indicative of the relative toxin amounts formed in different mice.

Intestinal segments assayed for toxin were obtained 48 h postchallenge since colonization rates and toxin amounts in the large intestines are usually maximal (3) and fecal toxin concentrations are highest at this time. Additionally, toxin causing the illnesses with the shorter incubation times would likely be absorbed into the bloodstream before or around this time. Since sampling time precedes illness, information is not available on the distribution of toxin in the gut of a mouse that developed botulism. However, if systemic botulism depends solely on the toxin levels in the large gut, it can be suggested that more mice should have developed botulism since toxin levels after 10^2 spores overlap those produced by the 10^3-spore inoculum.

Botulinum toxin injected into the lumen of the lower bowel is poorly absorbed into the bloodstream (4), but it is possible that the presence of a relatively high toxin level over an extended period of many hours may increase the chances of some toxin being absorbed. However, if this is the case, it would seem that ≥1,000 LD_{50} should be enough to cause botulism so that the 10^2-spore dose should have caused more illness than was found.

Few samples were tested, but both type A and B morbidity rates were better correlated with toxin levels in the small intestine than in the large gut. Although not determined in the present work, most of the toxin in the small intestine is probably in the ileum, as found in C. botulinum-monoassociated mice (10). Toxin in this part of the gut is more likely to cause systemic effects since it is more readily absorbed into the circulation than is toxin in the large gut (5).

Toxin amount in the small intestine was usually related to what was in the large bowel, but simple retrograde transfer from the main pool in the lower gut does not completely explain the relative amounts in the small versus the large intestine. The constipation that precedes systemic illness in clinical infant botulism (1) is not seen in mice, but it is possible that high levels of early formed toxin in the large bowel might act locally in a manner allowing more toxin into the ileum, or allowing the pathogen to multiply more in the ileum and concomitantly produce a larger amount of toxin. Whatever the correct reason, the finding of relatively large amounts of toxin in the large bowel of mice that remained healthy suggests that nonsymptomatic infections of human infants may be a more common occurrence than has been reported.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**