INFECTION AND IMMUNITY, July 1978, p. 59-63
0019-9567/78/0021-0059$02.00/0

Vol. 21, No. 1
Printed in U.S.A.

Intraintestinal Toxin in Infant Mice Challenged Intragastrically with *Clostridium botulinum* Spores

H. SUGIYAMA[1,2]* AND DANIEL C. MILLS[1]

Food Research Institute[1] and Department of Bacteriology,[2] University of Wisconsin, Madison, Wisconsin 53706

Received for publication 27 March 1978

Conventionally raised suckling mice were injected intragastrically with 10^5 spores of a *Clostridium botulinum* type A culture. Botulism was not observed, but 80% or more of mice challenged when 8 to 11 days old had botulinum toxin in the large intestine 3 days later. Mice younger than 7 days or older than 15 days were resistant to the challenge. When in vivo toxin production was started by spores given to 9-day-old mice, toxin was present in the intestine at 1 through 7 days postchallenge but with greatest consistency between 1 and 4 days. Total toxin in an intestine ranged up to 1,920 50% lethal doses as titrated intraperitoneally in adult mice. The dose infecting 50% of a group of 9-day-old mice was 700 (95% confidence limits of 170 to 3,000) spores per animal. Toxin was formed in the lumen of the large intestine; it was not associated with the ileum. Injection of 10^5 spores intraperitoneally into 9-day-old mice resulted in toxin production in the large intestines of 30% of the test animals.

Botulism of human infants undoubtedly has occurred in the past, but its recognition as a clinical entity did not take place until 1976 (1, 9). Its importance is shown by a recent summary which records the distribution of 33 type A and 25 type B cases, including two deaths, in 15 states in the United States (3). The illness will likely be found increasingly in this country and in others as awareness of the syndrome becomes more general.

Apart from having occurred exclusively in children 3 to 26 weeks old (3), infant botulism apparently differs importantly from the classically known botulism food poisoning in how the causative toxin is acquired. Intensive studies of the cases and their epidemiological circumstances strongly support the conclusion that infant botulism has its genesis with the ingestion of *Clostridium botulinum* only (1, 9). The spore form is likely important because vegetative cells are unlikely to survive transit through the stomach. These spores can be acquired with many potential vehicles because they are present in soils (15). In at least some infants, outgrowth of the swallowed spores occurs, and the ensuing intraintestinal vegetative multiplication results in toxin production.

This chain of events contrasts with the previously known pathogenesis in which the toxemia is caused solely or primarily by toxin that is consumed with a food in which *C. botulinum* has grown (15). Additional toxin may become available if the botulinum organisms that are swallowed with the toxin multiply intraintestinally (10, 13, 15). Practical experience has shown that a food containing some botulinum spores but no toxin will not become dangerous to anyone other than an infant if the organism cannot grow in it. Additionally, adult laboratory animals are highly tolerant of botulinum spores given per os; with suspensions not containing free toxin, 10^8 to 10^9 or more spores per animal must be fed to elicit botulism (4, 6, 11, 16).

The increasing rate at which infant botulism cases are being identified makes highly desirable an understanding of why the organism alone can start an intestinal infection in infants but not in adults. Experimental studies with animals might provide clues but have not been possible because a suitable subject is not known. This communication presents data that intraintestinal toxin formation can occur in suckling mice when they are intragastrically (i.g.) administered botulinum spore doses much smaller than that required to initiate intraintestinal infection of adult animals.

MATERIALS AND METHODS

Mice. Infant mice (HA/ICR strain) were raised and maintained conventionally in a room reserved for this study. Growth rates of pups in different litters were made as uniform as possible. This was done by breeding females which had raised at least one previous litter and by keeping the number of pups in litters the same by sacrificing the excess on day of birth. The present standard litter has 10 infants. Occasionally a

pup can be lost before an experiment is started. Each litter was kept in its own cage, with food (Mouse Chow formulation 5015, Ralston Purina, St. Louis, Mo.) and water always available.

When several litters were needed for a test, not all were ready on a given day; the challenges were administered as the animals became available. Generally, all pups of a litter received the same dose, but occasionally mice were marked so that up to three different challenges could be tested on members of the same litter. So far, cross-infection has not been recognized.

Challenges. A spore suspension of *C. botulinum* type A strain 62A prepared several years ago was used throughout. Doses were calculated from the average of similar numbers obtained in four separate five-tube most-probable number tests (18) done on the stock since the present work was started. Grown in cooked meat medium (Bacto, Difco Laboratories, Detroit, Mich.), the culture produced 10^5 or more 50% lethal doses (LD_{50}) per ml for adult mice.

Inoculations were made i.g. by the percutaneous method. A hypodermic needle (27 gauge, 0.5 inch [ca. 1.27 cm]) was used to penetrate the abdominal wall and enter the stomach, which in suckling mice is easily located as a milk-filled pouch. Working spore suspensions in water were heated for 15 min at 80°C and injected in 0.05-ml volumes. These suspensions had no free toxin as determined by lack of effect of 10^6 spores injected intraperitoneally (i.p.) into adult mice.

Tests for toxin. Inoculation and sacrifice of infant mice were done in the afternoon. Animals were killed in groups of about five by holding in a plastic bag and flushing out the air with carbon dioxide gas. The abdominal organs were exposed, and the large intestine (cecum plus colon) was rapidly transferred into 1.0 ml of 0.2% phosphate buffer–0.1% gelatin, pH 6.4, contained in a tissue grinder being held in an ice-water bath. Time between death and sampling did not exceed 10 min.

The tissue was ground while being cooled, and the homogenate was centrifuged in a small test tube for 10 min at 10,000 × *g* at 4°C. The resulting extract was tested for toxicity by injecting 0.25 ml i.p. into a mouse of 20- to 26-g weight. In the early stages of work, all lethal extracts were retested with type A, and sometimes additionally with type B, antitoxin to confirm that the mouse-killing agent was type A botulinum toxin. When nonbotulinum lethal factors were not encountered in the extracts, the confirmatory tests were limited to representative specimens.

Total toxin in a large intestine was assayed with serial, twofold dilutions of the extract made with buffer-gelatin. Each dilution was tested separately by injecting 0.5 ml i.p. into each of four adult mice. The LD_{50} in the extract was calculated from deaths occurring within 4 days (18).

RESULTS

In the first trials, mice ranging from 3 to 14 days old were challenged i.g. with 10^6 spores and observed for 2 weeks. When none developed indications of botulism, consideration was given to the possibility of toxin being formed intraintestinally without the animals becoming overtly ill.

The possibility was examined with a modified experiment in which toxin was sought in the gut (ileum plus large intestine) of infant mice injected i.g. with spores 3 to 8 days previously. Several positive extracts indicated that *C. botulinum* had grown in the intestinal tract without the hosts becoming ill. Other tests showed the toxin to be limited to the large intestine and present in the lumen contents.

Determination was made of the age range during which suckling mice are susceptible to intestinal infection from i.g.-administered botulinum spores (Table 1). The slightly different results of the two experiments are typical of the variability encountered throughout this work. However, common trends were evident. Very young mice (through about 6 days of age) were refractory to intraintestinal colonization by the inoculum. Mice 7 through 13 days of age at the time of challenge were susceptible, but maximum susceptibility (>80% positives) was during the 8- through 11-day age period. Older animals were not as receptive and were completely resistant by the time they were 14 to 15 days old.

Optimum time for demonstrating toxin after spore inoculation was ascertained by the kind of experiments summarized in Table 2. In this experiment, examination for toxin in mice more than 17 days old at the time of sacrifice was modified because of nonbotulinum agent(s) that killed the adult test mice. These extracts were routinely frozen overnight at −20°C in small-diameter test tubes. They were thawed the next morning without disturbance, and the top portion of the fluid column was used for the toxin test. This method has successfully identified botulinum toxin of low LD_{50} levels in specimens having interfering amounts of "nonspecific"

TABLE 1. *Age of mice during which i.g.-administered C. botulinum spores produce intestinal infection*[a]

Age (days)	Mice with toxin in intestine/no. tested	
	Expt 1	Expt 2
4	0/12	0/12
5	0/11	0/12
6	0/11	0/12
7	5/10	9/12
8	NT[b]	11/12
9	10/10	10/12
10	NT	10/11
11	9/10	9/11
12	NT	1/12
13	4/10	1/12
14	NT	0/12
15	0/15	0/12

[a] A total of 10^5 spores per mouse were administered. Large intestines were tested for toxin 3 days later.
[b] NT, Not tested.

TABLE 2. *Botulinum toxin in intestines of mice[a] at different times after spore challenge*

Day after challenge	Mice with toxin present/no. tested
0.5	0/20
0.75	1/20
1	15/20
2	20/20
3	18/19
4	16/20
5	11/20
6	6/20
7	7/20
8	0/20

[a] Nine-day-old mice were injected i.g. with 10^5 spores.

mouse-killing agents (2, 14). Confirmatory tests used types A and B botulinum antitoxins and normal rabbit serum.

Toxin was present in the majority of mice at 1 through 4 days after administration of spores to 9-day-old mice. The difference in positive percentages at 2 and 3 days postchallenge was not significant. In other trials comparing 2 through 4 days, the percents positive at the two intervals were the same or just as often slightly higher after 3 days.

Toxin production by 18 h was probably more frequent than indicated by the one positive in the tabulated data. Extracts of 18 other intestines of this group of mice elicited signs of botulism in the adult mice without killing, indicating that 0.25 ml of the extracts had a sublethal amount of toxin.

Total toxin in intestines was quantitated at different days after 9-day-old mice were injected i.g. with 10^5 spores. When assayed in adult mice by i.p. injection of intestinal extracts, toxin quantities in four mice sacrificed 2 days after challenge were 60, 120, 120, and 160 LD_{50}. At 3 days postchallenge, toxin values for different mice were 30, 40, 160, 254, and 810; at 5 days, titers were <20, <20, 80, and 1,920 LD_{50}. The wide variation in values for each testing time and the limited numbers of samples titrated did not permit a valid determination as to titers at the tested days being significantly different.

Aside from possible rare exceptions, overt botulism has not been recognized among infected suckling mice. The absence of toxic manifestations could result if the titers determined with adult mice are much lower when quantitation is done with infant mice. An experiment showed that even if it exists, the sensitivity difference between infants and adults is not great enough to explain the continuing well-being of infected hosts. Extracts of large intestines from seven infected suckling mice were injected i.p. into 9-day-old mice. Test volume per pup was 0.1 ml, or 1/10 of the homogenate volume of the intestines. All extracts killed the pups within 24 h after causing respiratory and locomotor difficulties characteristic of botulism.

The number of spores needed to produce intestinal infection in half of a representative group of infant mice (ID_{50}) was determined by using two litters, each of 10 pups, for each dose of a decimally decreasing series. Mice were 9 days old when administered spores, and tests for toxin were performed 3 days after the challenges.

A flat dose response plot was obtained. Of the 20 mice receiving 10^5 spores each, 14 showed growth of *C. botulinum*. The successively lower spore numbers initiated intestinal infection in 13, 11, 9, 1, and 0 mice. Thus, the maximum positive percentage was slightly less than normally obtained, but even 10 spores caused in vivo toxin production in 1 of 20 mice. When the data were treated by the method of Litchfield and Wilcoxon (7), an ID_{50} of 700 spores per mouse with 95% confidence range of 170 to 3,000 spores was calculated.

In giving the inoculum, spores were injected only when the needle tip was seen inside the stomach. However, as suggested by a droplet sometimes seen on the skin upon withdrawing the needle, a few spores could have been getting into the peritoneal cavity itself. It seemed unlikely that toxicity in the intestinal extracts was due to growth from these spores producing toxin that contaminated the serosal side of the large intestine. Toxin in the cavity should be readily absorbed and affect the animal; moreover, it should result in toxic small intestines as often as large intestines.

Nevertheless, it was important to obtain experimental evidence. Mice of age when most can be infected by i.g. challenge with 10^5 spores were given varying spore numbers by the i.p. route. They were sacrificed 3 days later, and the peritoneal cavity was rinsed with 1.0 ml of buffer-gelatin. The ileum and large intestine were then removed separately. The lumen contents of the large intestine were collected by flushing with 1.0 ml of buffer, and separate extracts were made of the contents and the remaining tissue (Table 3).

As shown for the two higher challenge doses, toxin was not present in any of the peritoneal lavage specimens or in the extracts made of the ileums. When toxin was associated with the large intestine, it was almost always completely removed with the lumenal contents. The one toxic tissue specimen probably resulted from incomplete washing out of the lumen.

In the studies on pathogenicity to adult laboratory animals of i.g.-administered *C. botulinum*

TABLE 3. *Toxin in mice injected i.p. with botulinum spores*[a]

Spores/ mouse	Specimen[b]	Mice with toxin/20 mice tested
10^5	Peritoneal lavage and ileum	0
	Large intestine contents	6
	Large intestine tissue	1[c]
10^4	Peritoneal lavage and ileum	0
	Large intestine tissue	2
	Large intestine tissue	0
10^3	Large intestine	1
10^2	Large intestine	0

[a] Mice were 9 days old at challenge and were examined for toxin 3 days later.

[b] With two higher doses, toxin tests were made on four specimens prepared from each mouse (see text); with lesser challenges, tests were made on extracts made of entire large intestine.

[c] Tissue of intestine whose contents had toxin.

spores (4, 6, 11, 16), botulism was not developed with spore numbers which in infant mice can cause intraintestinal toxin production. Apparently, no examination was made for toxin in the intestinal tract of animals that remained healthy. In the present work, separate groups of at least 20 adult mice each were challenged by i.p. or i.g. route with 10^5 spores. The animals developed no ill effects, and at 3 days postchallenge did not have botulinum toxin in the gut.

DISCUSSION

Most mice given 10^5 botulinum spores i.g. when 7 to 11 days old had botulinum toxin in their large intestines 3 days later. This is toxin produced in vivo during multiplication of the inoculum because the titers far exceeded the intrasporal toxin (6, 19) that could be present in the numbers of spores being injected.

Toxin quantitated in the intestines are amounts present at the time mice were sacrificed and represent only a part of the total toxin produced during the course of an infection. The data obtained with separate pups indicate that, in a given infected mouse, toxin would be present nearly always from day 1 through 4, and in some through day 7, after the challenge. During at least part of this time, turnover of toxin is occurring because infected mice did not have notable constipation; some toxin is being excreted while new molecules are being synthesized until *C. botulinum* multiplication stops. This growth probably ceases when mice reach the age when they can no longer be infected by spore challenges.

Suckling mice may be slightly less sensitive than adults to botulinum toxin given by the i.p. route (20), but at 3 days postchallenge the infected infants had in their large intestines at least 10 times more toxin than the minimum needed to kill them if the toxin had been in the peritoneal cavity. The localization of the toxin would explain the failure of these toxin amounts to affect the infected mice. Toxin was found only in the large intestines, never associated with the small intestines. Under these circumstances, absorption into the systemic circulation would be negligible; in the animal species studied, ingested toxin is absorbed primarily from the small intestine (8, 17) and minimally from the colon (5). The interpretation is consistent with the generally uneventful recoveries of human infant cases who, without receiving therapeutic antitoxin, are released from hospitalization when they are still excreting toxin in their feces (1, 9).

The intestinal infections produced by i.g.-inoculated spores cannot be explained satisfactorily by spores that may accidentally be deposited in the peritoneal cavity during the injections. With 1% leakage of a 10^5-spore dose, 10^3 spores would be left in the cavity. By themselves, the 10^3 would infect about 5% of the mice instead of the 50% that would be infected when this number is injected into the stomach. A similar discrepancy can be seen even when an unlikely 10% leakage is considered. The i.g. route is more effective than the i.p. in starting intraintestinal toxin production.

Relative to growth of *C. botulinum* in their gut, infant mice pass sequentially through three stages. They are refractory during the first few days after birth but become susceptible when they reach 1 week of age. The susceptible period lasts a few days and is followed by a resistance that probably continues through adulthood. The reasons for these stages cannot be specified at present but are likely related to the different microorganisms that successively colonize the intestinal tract during the first weeks of life (12). The start of the final resistant state seems to correlate with the time when infants start to sample solid food.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; Public Health Service grant T32-ES07015 from the National Institute of Environmental Health Sciences to the Center for Environmental Toxicology, University of Wisconsin, Madison; and contributions of food companies. D.C.M. is a postdoctoral appointee for a grant to the Center for Environmental Toxicology.

LITERATURE CITED

1. **Arnon, S. S., T. F. Midura, S. A. Clay, R. M. Wood, and J. Chin.** 1977. Infant botulism: epidemiological, clinical and laboratory aspects. J. Am. Med. Assoc. **237**:1946–1951.

2. **Bott, T. L., J. Johnson, E. M. Foster, and H. Sugiyama.** 1968. Possible origin of the high incidence of *Clostridium botulinum* type E in an inland bay (Green Bay of Lake Michigan). J. Bacteriol. **95**:1542–1547.
3. **Center for Disease Control.** 1978. Follow-up on infant botulism—United States. Morbid. Mortal. Weekly Rep. **27**:17–23.
4. **Coleman, G. E., and K. F. Meyer.** 1922. Some observations on the pathogenicity of *B. botulinus*. J. Infect. Dis. **31**:622–649.
5. **Dack, G. M., and D. Hoskins.** 1942. Absorption of botulinum toxin from the colon of *Macaca mulatta*. J. Infect. Dis. **71**:260–263.
6. **Keppie, J.** 1951. The pathogenicity of the spores of *Clostridium botulinum*. J. Hyg. **49**:36–45.
7. **Litchfield, J. T., and F. Wilcoxon.** 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. **96**:99–113.
8. **May, A. J., and B. C. Whaler.** 1958. The absorption of *Clostridium botulinum* type A toxin from the alimentary canal. Br. J. Exp. Pathol. **39**:307–316.
9. **Midura, T. F., and S. S. Arnon.** 1976. Infant botulism: identification of *Clostridium botulinum* and its toxins in faeces. Lancet **ii**:934–936.
10. **Minervin, S. M.** 1966. On the parenteral-enteral method of administering serum in cases of botulism, p. 336–345. *In* M. Ingram and T. A. Roberts (ed.), Botulism 1966. Chapman and Hall Ltd., London.
11. **Orr, P.** 1922. The pathogenicity of *Bacillus botulinus*. J. Infect. Dis. **30**:118–127.
12. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31**:107–133.
13. **Shvedov, L. M.** 1959. Neutralization of toxin formed in the alimentary tract in experimental toxico-infection with *Clostridium botulinum* type A by parenteral administration of antiserum. Zh. Mikrobiol. Epidemiol. Immunobiol. **30**:72–77.
14. **Smith, L. DS.** 1975. Common mesophilic anaerobes, including *Clostridium botulinum* and *Clostridium tetani*, in 21 soil specimens. Appl. Microbiol. **29**:590–594.
15. **Smith, L. DS.** 1977. Botulism: the organism, its toxins, the disease. Charles C Thomas, Publisher, Springfield, Ill.
16. **Starin, W. A., and G. M. Dack.** 1925. Pathogenicity of *Clostridium botulinum*. J. Infect. Dis. **36**:383–412.
17. **Sugii, S., I. Ohishi, and G. Sakaguchi.** 1977. Intestinal absorption of botulinum toxins of different molecular sizes in rats. Infect. Immun. **17**:491–496.
18. **Sugiyama, H., and K. H. Yang.** 1975. Growth potential of *Clostridium botulinum* in fresh mushrooms packaged in semipermeable plastic film. Appl. Microbiol. **30**:964–969.
19. **Suzuki, J. B., R. R. Booth, and N. Grecz.** 1970. Pathogenesis of *Clostridium botulinum* type A: release of toxin from *C. botulinum* spores in vitro by leucocytes. Res. Commun. Chem. Pathol. Pharmacol. **1**:691–711.
20. **Turnbull, P. C. B., A. A. Ghosh, R. J. Gilbert, and J. Melling.** 1976. Botulism and sudden infant death. Lancet **ii**:1410–1411.