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Microbial Ecological Basis of Infant Botulism as Studied with Germfree Mice

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The possible role of the indigenous intestinal microflora in the toxicoinfection of human infant botulism was studied with adult germfree mice. Intraintestinal botulinum monoassociation was consistently produced when mice were fed 10 *C. botulinum* type A spores. Control germfree mice became enterically infected when placed in the same isolator with, but separated from, animals that had been fed spores. When transferred into a room holding a colony of normal mice, the highly susceptible gnotobiotes became resistant to challenge of 10^5 spores after about 3 days of the conventionalizing exposure. The findings are interpreted as evidence that enteric botulinum infection occurs in human infants whose intestinal tract has not yet been colonized by bacteria which are indigenous to adults and prevent growth of *C. botulinum*. Intestinal monoassociation could not be developed in germfree infant mice younger than 7 days.

The botulinum etiology of what is now known as infant botulism was not recognized until 1976 (1, 10), but the 98 cases distributed in 21 states of the United States and the single ones in England and Australia (3) show the widespread occurrence of the disease. Additionally, a fulminating form may be one cause of the sudden infant death syndrome (2).

Infant botulism has affected children up to about 8 months of age (3). It is apparently a strict toxicoinfection such that the responsible neurotoxin is only that produced in vivo during growth of *Clostridium botulinum* in the intestinal tract (1, 10). The classical food poisoning botulism of older individuals differs in that the important toxin is formed in a food. The persistence of *C. botulinum* toxin, or organisms, or both in the gut of some food poisoning cases (9) indicates that enteric botulinum infection may not be restricted to infants. However, such an infection not involving toxin in a food has been demonstrated only with infants.

A comparable age-related susceptibility to intraintestinal botulinum infection has been demonstrated in conventionally reared infant mice (16). The gut was colonized by *C. botulinum* when its spores were administered intragastrically to 7- to 13-day-old mice but not when given to younger or older animals. Infection rates up to 100% resulted when 8- to 11-day-old mice were each challenged with 10^5 spores, and 700 spores constituted the 50% infective dose. Infected mice did not become overtly ill so that the criterion of infection was the production of botulinum toxin in the colon. Optimum post-challenge time for testing for the in vivo-formed toxin was 2 to 3 days; toxin could not be detected by day 8.

The differing susceptibilities of the several age groups to enteric botulinum infection could be the consequences of the different microorganisms that colonize the intestinal tract of a mouse during its first postnatal weeks (13). Should the intestinal tract be intrinsically unsuitable for growth of *C. botulinum*, the period of susceptibility could be that transient stage when a microorganism(s) facilitating growth of the inoculum is present. Or, the infection could be possible because the indigenous intestinal microflora does not include a competitor(s) capable of preventing growth of *C. botulinum*. These possibilities were examined with germfree adult mice.

MATERIALS AND METHODS

Germfree mice. Axenic mice (HA/ICR strain) of 6 to 8 weeks of age were purchased (Charles River Breeding Laboratory, Wilmington, Mass.) and maintained in the Gnotobiote Laboratory of the University of Wisconsin. They were kept in Trexler-type plastic isolators with autoclave-sterilized Purina Laboratory Chow 5010 (Ralston Purina Co., St. Louis, Mo.) and water always available. Axenic infant mice were bred from purchased animal stocks. Samples obtained not more than 3 days before starting a test showed the animals and isolators to be used were germfree (8).

Germfree adult mice were conventionalized by transferring from the sterile environment into a room in which a colony of normal mice was maintained.

Their food was that brought from the Gnotobiote Laboratory and exposed to contamination of the mouse room.

Spores. The spore suspension (*C. botulinum* type A, strain 62A) was that used in the previous study (16). Most-probable-number enumerations were done periodically and showed that the viable count did not change during the course of the present work.

Spores in 0.5-ml volumes were administered intragastrically to adult mice with a 20-gauge feeding needle (Popper and Sons, New Hyde Park, N.Y.). Infants were given intragastric challenges by injecting 0.05 ml of spore suspension by the percutaneous method (16). Residual toxin was inactivated by heating the spore suspension at 80°C for 20 min.

Tests for enteric infection. The details of the tests for enteric infection have been published elsewhere (16). Mice given spores were observed at least twice daily for illness. They were sacrificed when advanced illness was noted; those not afflicted were sacrificed at the indicated times. Observation periods are times elapsing from spore challenge to sacrifice, e.g., 3 days would mean 72 ± 2 h.

The intestines of all test animals were examined for type A botulinum toxin. When the lower ileum, cecum, and colon were to be tested separately for toxin, the intestinal segments were isolated with hemostat clamps before being removed. The procedure was used to minimize mixing between contents of adjoining segments.

Toxin was titrated as the 50% lethal dose for conventional adult mice (LD_{50}). Homogenates of the intestines were diluted in serial twofold increments, and the dilutions were injected intraperitoneally into separate groups of four mice.

RESULTS

Germfree adult mice in an isolator were separated into groups of four or five in a cage. Animals in a given cage were each fed the same dose of *C. botulinum* spores and were observed for 3 days (Table 1). All test mice had botulinum toxin in their intestines at the end of the period. At least one mouse of each test group showed signs of botulism. Toxin levels of 2,300 to 215,000 LD_{50} were titrated in the intestinal tracts of these ill mice.

Controls not fed spores were also in the isolator but were kept in a cage set apart from the test animals. None of these controls was ill on day 3 after administration of spores to the other mice. However, two controls had become enterically infected since type A botulinum toxin was present in their intestines.

Since findings with the controls suggested that airborne transmission of infective doses could have influenced the results, repeat tests at different times were done to confirm the high susceptibility of adult germfree mice to enteric infection from intragastrically administered botulinum spores. Axenic mice were each fed 10 spores and watched for 3 to 4 days (Table 2). All mice became ill by postchallenge day 4, except for the one (experiment 1) which was held for only 3 days.

TABLE 1. *Intraintestinal colonization after feeding differing C. botulinum spore doses to germfree adult mice*

Spores/mouse	No. infected[a]/no. tested	LD_{50} in intestine[b]
10^4	4/4	146,000
10^3	5/5	17,000
10^2	5/5	215,000
10^1	5/5	2,300
Control[c]	2/5	1,300

[a] Type A botulinum toxin in gut 3 days after spore challenge.
[b] Titrated in a representative of the group.
[c] Not fed spores, but placed in same isolator holding the test mice.

TABLE 2. *Development of enteric botulinum infection in germfree adult mice fed 10 C. botulinum spores*

Expt[a]	No. infected/no. tested	LD_{50} in intestine[b]	Days to botulism[c]
1	3/3	3,000–33,000	—, 3, 3
2	4/4	1,700–22,000	3, 4, 4, 4
3	2/2	NT–112,000	4, 4

[a] Separate isolator for each experiment.
[b] Total in lower ileum, cecum, and colon; NT, Not tested since found dead in morning.
[c] —, Not ill when sacrificed on day 3.

Botulinum toxin was found in the gut of all animals. Although relative amounts in the colon and cecum of a mouse varied, toxin in one was never more than 10 times the quantity of that in the other. Except for two mice, toxin was also found in the terminal ileum in an amount generally less than 1% of the total intestinal toxin.

The colon and cecum of all animals showed numerous gram-positive rods, with some having endospores; the lower ileum had much fewer organisms. Preliminary examinations with the scanning electron microscopic technique (5) did not find large numbers of bacteria associated with the mucosal surface.

The isolator for experiment 2 (Table 2) was used further after all mice, but not their cages, were removed. Three individually caged germfree mice were introduced into the isolator and held for 1 week. When none became ill, one of the three was taken from the isolator, and its intestine was examined for botulinum toxin. The second mouse was transferred into a cage which had held botulinum-monoassociated mice; the third was left in the cage in which it had entered the isolator.

Toxin was not found in the mouse that was

taken from the isolator after 7 days. The mouse transferred into the contaminated cage became ill within 3 days. Its activities resulted in airborne dispersal of *C. botulinum*, since the third mouse was ill on the next day. When sacrificed at the time that illness was first noticed, the gut of the second mouse contained 9,000 LD_{50}, whereas that of the third had 8,600 LD_{50}.

Axenic mice were made ex-germfree by transferring them into a room holding normal mice. A few mice died of undetermined causes during this conventionalizing exposure. Those that were apparently healthy at the time were fed 10^5 spores at different intervals of the conventionalizing treatment (Table 3).

The enteric botulinum infection developed only in those mice which were fed spores at time of removal from isolators (day 0). One of these seven showed botulism on day 3 postchallenge. Test for botulinum toxin in some gut homogenates had to be done with a dilution at which a nonbotulinum, mouse lethal agent(s) was no longer toxic. If the original extract contained only a few lethal doses of botulinum toxin, the test dilution could have an undetectable concentration of the toxin. However, every test group gave at least two intestinal homogenates which could be used without dilution. The data permit the conclusion that germfree mice become refractory to enteric botulinum infection within a few days of exposure to conventional mice. The acquired intestinal bacteria were not identified, but a variety of morphologically different ones were seen in freshly passed feces of ex-germfree mice (3 days of conventionalizing exposure).

Since intestinal infection could not be produced by intragastric administration of 10^5 spores to conventional infants less than 7 days of age (16), the susceptibility of 2- to 10-day-old axenic mice was examined. A separate isolator was used for each litter, and 10^5 spores were given to each animal. Testing for intraintestinal toxin was done at 3 days postchallenge (Table 4).

Based on the failure to find either botulinum toxin or bacteria in their gut, infant mice 2 or 3 days of age at time of challenge were not receptive to intestinal colonization by the inoculum. All mothers, including those of infants which did not become infected, acquired *C. botulinum*, probably with the feces of the infants. Adult intestines had 9,000 to 100,000 LD_{50} of toxin, and botulism was apparent in nearly all adults by the end of the test period.

Toxin was found in about one-half of the infants which were challenged when 4 days old but did not exceed 30 LD_{50}/mouse. In situ production was unlikely since bacteria were not present in the gut; the assayed toxin probably came in the milk of the sick mothers.

TABLE 3. *Resistance to intestinal botulinum infection when germfree adult mice are conventionalized by exposure to normal mice for different periods*

Days[a]	No. tested	LD_{50} in intestine[b]
0	7	300–9,900[c]
3	6	Nil[d]
6	3	Nil
9	4	Nil[e]
12	3	Nil

[a] Days in room with normal mice before receiving spore challenge.

[b] Three days after intragastric dose of 10^5 spores.

[c] One with 300 LD_{50}; remaining six with 1,000 LD_{50} or more.

[d] Three homogenates tested with 1:25 to 1:625 dilutions.

[e] Two homogenates tested with 1:25 dilution.

TABLE 4. *Influence of age of germfree infant mice on susceptibility to intestinal botulinum infection*

Age at challenge[a]	No. with toxin in intestine[b]/no. tested
2	0/8
2	0/9
3	0/8
3	0/9
4	4/7[c]
7	10/10
10	9/9

[a] Age in days when given 10^5 spores intragastrically; see text for results of a litter of 2-day-old mice.

[b] Three days postchallenge; denominators are numbers of infants in litter.

[c] Gut of all mice devoid of bacteria.

Toxin in absence of organisms occurred more frequently than indicated by the tabulated data. The results with another litter were not entered in the table so that the trend of results would not be confused. The nine infants of this litter were 2 days old when challenged. On day 3 postchallenge, seven had low toxin levels in the gut, but microscopic examinations showed that the intestines had not been colonized.

An infection rate of 100% resulted when the spores were given to 7- and 10-day-old mice. Smears of intestinal materials showed good growth of a clostridium-like organism, although the animals did not develop overt botulism during the holding period. Representatives of the test groups had 200 to 4,000 LD_{50}/intestine.

DISCUSSION

Germfree adult mice could be consistently infected with *C. botulinum*, although the conventional adult is highly resistant to the challenge (4, 12, 15, 16). Axenic adults were more easily infected than conventional infant mice of

the most susceptible age; 10 spores infected all axenic adults tested, whereas about 700 spores of the same suspension constituted the 50% infective dose for conventional infant mice (16).

The ease of monoassociating axenic adult mice indicates that the intestinal physiology of the adult is compatible with multiplication of *C. botulinum*. The resistance of the conventional adult mouse can be attributed, therefore, to its intestinal microflora. A microorganism(s) which prevents growth of *C. botulinum* probably colonizes the intestinal tract at the age when the susceptible infant becomes resistant to the challenge and then persists as part of the adult's climax microbial community. In the situation being discussed, the susceptible stage of conventional infants would not require the presence of organism(s) which makes possible the growth of the challenge organism.

That the sequential susceptible and resistant stages of conventional infant mice has an ecological basis is supported by the tolerance developed by germfree adults as they are exposed to conventional mice. In the transition to the ex-germfree status, mice would acquire competitors of *C. botulinum* which are part of the indigenous intestinal microflora of conventional mice. One such bacterial species would suffice as a barrier to botulinum infection, but several could be acting independently or in concert to prevent the enteric infection (6, 7, 13, 17).

Human infant botulism can be suggested to have a similar etiology. The illness develops when *C. botulinum* is swallowed at a time when the competitor(s) is absent or its antibotulinum potential is suppressed. The inhibitory organism may not be as well established in infants as in older individuals, so that a disturbance of the gut such as constipation or entry of a bacterium not encountered previously supplants or suppresses the antibotulinum potential of the inhibitor for a critical period. If this should be the case, there could be circumstances in which adults develop a toxicoinfection counterpart of infant botulism.

The infection started in germfree mice had severe consequences, whereas the infected conventional infant mice are asymptomatic (16). The coprophagic propensity of adult mice may account for the difference; when ingested, as with feces, toxin would likely be absorbed into the systemic circulation. However, the different distributions of in vivo-formed toxin in adult and infant mice could be important. In the botulinum-monoassociated adult, toxin was present not only in the colon but also in the cecum and to some extent in the terminal ileum. This contrasts with the colonic localization in the infant mouse, probably because the infant cecum is very small. Although *C. botulinum* type C is quite unlike type A in cultural properties (14), the importance of the cecum in type C botulism of chickens has been reported (11).

Similar to conventional infant mice (16), axenic infants of less than 1 week of age were not enterically infected when administered 10^5 spores by the intragastric route. The gut of the very young infants seems to be unsuitable for the growth of *C. botulinum* by a mechanism not involving bacteria.

The results reported here suggest the usefulness of the germfree adult mouse for studying the microbial interactions which are important in the pathogenesis of botulism in human infants.

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