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Susceptibility to Enteric Botulinum Colonization of Antibiotic-Treated Adult Mice

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The relationship between the indigenous intestinal microflora of adults and their resistance to the enteric botulinum infection of infant botulism was studied. Orogastric challenges of 10^5 type A *Clostridium botulinum* spores were given to adult mice whose gut flora had been altered by feedings of a mixture of erythromycin and kanamycin sulfate. From 80 to 100% of mice became infected when challenged 15 to 60 h after antibiotic administration. The mean infective dose of 2×10^4 spores per mouse for challenges given 23 h after antibiotic administration contrasted with the failure of 10^6 spores to infect control mice. Botulinum-colonized mice remained asymptomatic, although colonization lasted up to 5 days, and total botulinum toxin in the gut on days 3 and 4 postchallenge averaged 3,400 and 2,200 mouse intraperitoneal mean lethal doses. The mean infective dose for inocula placed in the colon of antibiotic-treated mice was 10^3 spores per mouse, and *C. botulinum* multiplied in the cecum as well as in the colon.

Since the recognition of infant botulism in 1976, nearly 200 cases have been reported in the United States (4), and the suggestion has been made that it is one cause of sudden infant death syndrome (2). The pathogenesis differs from the classically known food poisoning botulism, acquired by eating contaminated food, in that the causative toxin is acquired when *Clostridium botulinum* infects the intestinal tract (1).

Mice (12) and rats (9) have an age-determined susceptibility to enteric botulinum colonization comparable to the restricted age distribution of the human disease. When *C. botulinum* spores are administered intragastrically to these rodents of differing ages, the organism multiplies in the guts of only 7- to 13-day-old animals. Overt botulism does not occur, but infection is indicated by the botulinum toxin produced in the gut.

Conventional adult mice and rats are resistant to orogastric challenges with 10^6 or more *C. botulinum* spores, but their germ-free counterparts are infected by 10 spores. The highly susceptible axenic adults become resistant to challenges of more than 10^5 spores when they acquire a complex intestinal microflora during 3 days of exposure to conventional animals (8, 9).

These observations indicate (i) that the natural resistance of conventional adult mice depends on members of their indigenous intestinal flora acting as barriers to the multiplication of the pathogen, and (ii) that adult mice could be made susceptible by properly modifying their gut flora. This communication reports that the resistance of conventional adult mice is reduced significantly when their gut microbiota is changed by feeding an erythromycin-kanamycin sulfate combination.

MATERIALS AND METHODS

Treatment with antibiotics. Test animals were 25- to 30-g HA/ICR strain mice housed 10 in a cage. A mixture of 5 mg of erythromycin (erythromycin lactobionate; Abbott Laboratories) and 4 mg of kanamycin sulfate (Kantrex; Bristol Laboratories) in 0.5 ml of water was fed with a 20-gauge feeding needle (Popper & Sons, New Hyde Park, N.Y.). A total of five doses were given at 10- to 12-h intervals. After each dose, the animals were transferred to a freshly sterilized, filter-lid cage (Scientific Products, Inc., Chicago, Ill.) in which Laboratory Chow 5010 (Ralston Purina, St. Louis, Mo.) had been autoclaved. Drinking water during the drug treatment had 1.0 mg of erythromycin and 0.8 mg of kanamycin sulfate per ml and was changed daily to a freshly prepared solution. Plain tap water was used after the treatment period.

Inoculum. The stock spore suspension of *C. botulinum* type A strain 62 was that used in previous work (12). Viable spores were enumerated as the average of triplicate five-tube most-probable-number tests. Working suspensions in water were heated for 15 min at 80°C to inactivate free toxin and were fed in 0.5-ml volumes.

Colonization. In vivo growth of the challenge organism was monitored by determining whether type A botulinum toxin had been produced in the gut, usually on day 3 postchallenge. The procedure (12) was modi-

fied by homogenizing excised cecum-colon segments in 3.5 ml of diluent and by holding the homogenates overnight at 4°C before centrifuging to obtain the extract to be tested for toxin.

Type A antitoxin was used to identify the mouse lethal agent. If one unit of antitoxin did not protect mice challenged with 0.5 ml of undiluted extract, the neutralization test was done with dilutions of the extract on the possibility that the interfering agent(s) could be diluted below lethal concentration and permit detection of botulinum toxin. When the toxin was not found in the lowest dilution that did not cause "nonspecific" deaths, the extract was considered negative, although botulinum toxin also could have been diluted to a nonlethal level. When dilutions were necessary, the lowest extract dilution suitable for botulinum toxin testing are included with the results. Adding several antibiotics to extracts did not prevent nonspecific deaths.

Mean lethal doses (LD_{50}) were estimated by using two mice for each of the serial fivefold dilutions made of the extract. Differing colonization rates of mouse groups were evaluated statistically in 2 × 2 contingency tests (10).

Enumeration of organism. Cecum and colon segments were removed aseptically and transferred immediately into a glove box (Coy Manufacturing Co., Ann Arbor, Mich.) having an atmosphere of 80% N_2–10% H_2–10% CO_2. The segments and their contents were homogenized, and 10-fold serial dilutions were made in a reduced salts–0.2% gelatin solution (5). The dilutions (0.05 ml) were spread on plates of egg yolk agar (Difco Laboratories, Detroit, Mich.), and the inoculated plates were incubated for 4 days at 37°C. *C. botulinum* colony-forming units (CFU) were counts of colonies with an iridescent sheen on the surface and a halo of precipitate in the medium. Subcultures of representatives of such colonies in cooked meat medium (Difco) always produced type A botulinum toxin.

Exposure to normal mice. After 10 treated mice were fed the last dose of the antibiotic schedule, they were transferred into each of five cages with an untreated mouse. All of the mice in each cage were held for the same selected time period before being fed 10^5 *C. botulinum* spores. Mice were tested separately for botulinum toxin 3 days later.

Spore challenge in colon. Spores in 0.5 ml of water were deposited in the colon lumen of mice 23 h after the last dose of antibiotics. Inoculation was via a flexible Tygon tube (0.05-mm outside diameter), which was passed through the anus until about 4 cm of tubing had been inserted. Leakage of part of the inoculum occurred in some cases.

RESULTS

Period of susceptibility. Mice were challenged orogastrically with 10^5 *C. botulinum* spores at different times after the last dose of antibiotics, and their guts were examined for botulinum toxin 3 days later (Table 1). Almost all of the animals were colonized by challenges given between 14 to 60 h after discontinuing antibiotics; challenges after 60 h gave progressively lower colonization rates. Although extracts of guts from several animals challenged 144 to 168 h after antibiotic administration had to be tested at dilutions of 1/5 or 1/25 to avoid nonspecific deaths, those that could be used without diluting were negative for botulinum toxin. Control mice not given antibiotics were resistant to challenges of 10^6 spores.

TABLE 1. Intraintestinal botulinum colonization rates among mice fed 10^5 *C. botulinum* spores at different times after last dose of erythromycin-kanamycin sulfate mixture

Time after antibiotic administration (h)	No. colonized among 12 tested[a]
1	0
7	2
15	11
23	12
36	12
60	10
83	6
116	1
144	1[b]
168	0[c]

[a] Type A botulinum toxin in gut 3 days after challenge.

[b] Toxin in one extract tested at 1/5 dilution; toxin was not found in five tested undiluted or in six tested at 1/25 dilution (see text for explanation of dilutions).

[c] Toxin not found in six extracts tested undiluted and in six tested at 1/5 dilution.

Challenges given 1 and 7 h after antibiotic administration had no or low infectivity, probably because of the antibiotics in the gut. In agreement with published data (13), the minimum inhibitory concentration of erythromycin for the culture strain of the present study was 1 μg/ml; that of kanamycin sulfate was 128 μg/ml. The concentration of one or both antibiotics in the gut within 7 h after antibiotic administration could exceed these levels.

Microscopic examinations of feces passed at the time of the last antibiotic dose showed that the drug schedule had greatly reduced the varieties and total population of bacteria in the gut. Repopulation occurred within a few days; many bacteria were present by day 4 or 5 after antibiotic administration.

Toxin amounts and distribution. When 10^5 spores were fed 23 h after antibiotic administration, nearly all mice had toxin in their cecum-colon on days 3 and 4 postchallenge, when the total LD_{50} was highest (Table 2). One of 10 mice had detectable toxin amount within 24 h. All extracts of gut segments obtained 5 and 6 days postchallenge caused nonspecific deaths and had to be diluted for the botulinum toxin test. However, even if masked by an interfering agent, botulinum toxin concentration was lower than on day 4. Extracts of cecum-colon taken on day 6 postchallenge from three other mice used

TABLE 2. Botulinum toxin in the cecum-colon on different days after orogastric challenges of 10^5 *C. botulinum* spores to mice 23 h after antibiotic administration

Time postchallenge (days)	No. of mice with toxin among 10 tested	LD_{50}/gut[a]
0.75	0	0
1	1	22
2	5	65 ± 50
3	10	3,400 ± 3,000
4	9	2,200 ± 1,900
5	0[b]	0[b]
6	0[b]	0[b]

[a] Mean of all toxin-positive specimens ± standard deviation.

[b] All extracts tested at 1/25 to 1/625 dilution (see text for explanation of dilutions).

for a different test and which could be used undiluted did not have botulinum toxin.

Despite the botulinum LD_{50} in the gut during colonization, similarly treated mice did not develop botulism during 2 weeks of observation. Toxin was rarely found in the small intestine proximal to the cecum. It was about equally distributed in the cecum (1,300 ± 700 [standard deviation] LD_{50}) and the colon (2,000 ± 300 LD_{50}) of three other mice on day 3 postchallenge. *C. botulinum* at this time in two other mice averaged 9.2×10^8 CFU/g (dry weight) of cecum and 3.5×10^8 CFU/g (dry weight) of colon.

ID_{50}. The 50% infective dose (ID_{50}) was determined by feeding different spore doses to separate groups of 15 mice 23 h after antibiotic administration and testing for botulinum toxin 3 days later. The 1×10^5-spore dose colonized all test mice, the 5×10^4-spore dose colonized 10 mice, the 1×10^4-spore dose colonized 7 mice, and the 1×10^3-spore dose colonized none. A plot of spore number versus percentage of animals colonized showed that the ID_{50} for antibiotic-treated mice was 2×10^4 spores per mouse.

Exposure to normal mice. Although mouse lethal agent(s) other than botulinum in some extracts complicated the interpretation, the duration of susceptibility was shortened when antibiotic-treated mice were exposed to normal mice before being challenged (Table 3). Whereas 70% of animals were colonized when 10^5 spores were given 72 h after antibiotic administration (Table 1), none of 10 mice was infected when caged with a normal mouse during the 72 h between the end of antibiotic treatment and challenge ($P < 0.05$). The intestinal microflora of these resistant animals at the time of challenge was microscopically indistinguishable from that of mice not treated with antibiotics.

TABLE 3. Development of resistance to intraintestinal botulinum colonization among antibiotic-treated mice exposed to a normal mouse for different periods preceding challenge with 10^5 *C. botulinum* spores

Time after antibiotic administration (h)	No. colonized among 10 tested
6	0
18	4[a]
24	7[b]
48	8[c]
72	0[d]

[a] Toxin in four of five extracts tested at 1/10 dilution; toxin was not found in five tested at 1/10 to 1/600 dilutions (see text for explanation of dilutions).

[b] Toxin in one of three extracts tested undiluted and six tested at 1/10 dilution; toxin was not found in extract tested at 1/200 dilution.

[c] Toxin in eight of nine extracts tested undiluted; toxin was not found in extract tested at 1/10 dilution.

[d] Eight extracts were tested undiluted, one was tested at 1/10 dilution, and one was tested at 1/200 dilution.

Challenge in colon lumen. Spores were more infective when inoculated in the lumen of the colon than by the orogastric route. The 70% colonization rate resulting from 10^5 spores inoculated in the colon (Table 4) was not significantly different ($P > 0.05$) from the 100% obtained by feeding the same dose (Table 1), but 10^3 spores in the colon colonized 5 of 10 mice versus 0 of 10 by the orogastric route ($P = 0.025$). The average CFU in the gut on day 3 after challenge with 10^3 spores was lower than that with 10^5 spores, but botulinum colonization of the cecum occurred with both doses

DISCUSSION

Erythromycin and kanamycin sulfate in combination inhibit a broad spectrum of bacteria (6, 7). When fed in relatively large amounts they reduced, but did not eliminate, the natural resistance of conventional adult mice to enteric botulinum colonization. The coincident change in intestinal microbiota and the development of

TABLE 4. Enteric botulinum colonization of mice challenged 23 h after antibiotic administration with spores inoculated in lumen of colon[a]

Spore dose	No. infected among 10 tested	CFU[b]		Toxin[b,c]	
		Cecum	Colon	Cecum	Colon
10^5	7	8.8	8.9	2.6	2.6
10^3	5	7.7	7.7	1.6	1.7

[a] Analysis was done on day 3 postchallenge.

[b] Logarithm (base 10) per gram dry weight; averages from four randomly chosen, toxin-positive mice.

[c] Total LD_{50} values are lower than normal because extracts were frozen before testing.

susceptibility to the enteric botulinum infection are similar to experimental intestinal infections with *Salmonella enteritidis* (3), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (14, 15). The enterocecitis caused by *Clostridium difficile* is usually a consequence of administering antibiotics (11).

The explanation of the residual resistance to botulinum colonization depends on whether natural resistance is due to one or more than one barrier. If the former, the antimicrobial agents did not completely suppress the single barrier; if the latter (C. Wells and H. Sugiyama, unpublished data), an important barrier could have been suppressed, but at least one remained functional.

The contents of the colon come from the ileum and cecum and become concentrated as water is absorbed. Since treatment with the antibiotics caused enlargement of the cecum, the colonic and cecal contents were about equal in amounts. These facts suggest that CFU and toxin levels should be higher in the colon than the cecum if *C. botulinum* multiplied to the same extent in the two parts of the gut. Such was not the case; since they were higher in the cecum or equal, the cecum appears to be an important site of multiplication of the pathogen. This suggestion is supported by the *C. botulinum* CFU in the ceca of mice challenged by the colonic route. However, some growth in the colon must occur; the inoculum can reach the cecum only by contiguous spread during multiplication unless the force of injection or retrograde transport is the reason.

The results support the proposition that infant botulism depends on the host lacking the anti-*C. botulinum* organisms indigenous to the gut of older individuals. They also suggest the possibility that some adult botulism cases of "undetermined classification" (4) may be due to the opportunistic growth of *C. botulinum* in the gut which is predisposed to the infection by a change in microflora.

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