NOTES

Lyophilized Airborne Clostridium botulinum Spores as Inocula That Intestinally Colonize Antimicrobially Pretreated Adult Mice

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Adult mice, made susceptible to Clostridium botulinum by feedings of metronidazole, were immobilized with an anesthetic and held for 30 min in isolators in which a fine powder of lyophilized pathogen spores was made airborne. Exposed mice were surface decontaminated before being kept for 2 days in holding isolators. Mice were intestinally colonized by the pathogen. Colonization rates were related to spore numbers (10^4 to 10^7 type A or B) seeded into isolators.

Since the toxin responsible for infant botulism is produced by Clostridium botulinum infecting the intestinal tract (1, 3), pathogen-containing foods are obvious vehicles for transmitting the pathogen. Honey is one food known to be important in this respect (2); corn syrup is a possibility (6), but its practical importance remains to be shown. However, the botulism occurring among strictly breast-fed babies (1) is not likely to be transmitted by foods.

When conventional infant and metronidazole-pretreated adult mice are intestinally colonized by C. botulinum, they excrete as many as 10^9 pathogen CFU/g of feces. If such mice are used as pathogen donors in animal isolators, the pathogen being excreted becomes airborne and cross-colonizes other mice (recipients) in other cages. The cross colonizations occur during the first few days when the isolator is not disturbed (8).

These experiments kept mice in the same cages throughout the exposures since the primary concern was avoiding the transfer of pathogen from donors to recipients during housekeeping. Thus, the inocula causing cross colonizations could have been organisms which were ingested after settling on fur, food, or bedding. The present communication describes the colonization of recipient mice which are unable to ingest the pathogen during the time they are being exposed to lyophilized, airborne C. botulinum spores.

Test animals were HA/ICR strain conventional female mice (Sprague Dawley, Madison, Wis.) weighing 20 to 23 g. They were pretreated during a 4-day period by twice daily feedings of 6.1 mg of metronidazole (Sigma Chemical Co., St. Louis, Mo.), an antimicrobial agent active only against obligate anaerobes. They were used 18 h after the last pretreatment dose when most susceptible to peroral challenges with pathogen spores (4). These mice were exposed to the pathogen while distributed as equally as possible in four screen-floor cages placed in a sterilized isolator (180 by 75 cm) (model 6MP-A; Germfree Laboratories, Miami, Fla.). One cage was in each corner of this exposure isolator.

Spores of type A strain 62 and type B strain Okra (10) were lyophilized in skim milk so that known viable numbers were in the vials. Mice ready for testing were put in the exposure isolator and anesthetized by intraperitoneal injection of 6.0 mg of sodium pentobarbital (Nembutal; Abbott Laboratories, Chicago, Ill.) per 100 g of body weight. The ventilating fan of the isolator was turned off, and as rapidly as possible the dried product in one vial was ground into a fine powder with a mortar and pestle and then manually fanned for a few seconds until all was airborne (8). The isolator fan was immediately turned on for 10 s to distribute the spores through the isolator and then turned off for the remaining 30-min exposure period.

Mice were removed as rapidly as possible after exposure and temporarily placed in sterile, filter-lid, screen-floor cages (10). The mice were then dipped feet first up to the neck in rapid succession in two baths of chlorine dioxide (An-Fa-Cide; Pharmcal Research Laboratory, Greenwich, Conn.), and the head part was swabbed with the solution. The wet mice were then held for 1 h in sterilized cages and then given another treatment with chlorine dioxide. They were immediately put into a sterilized holding isolator (91 by 77 by 47 cm) and distributed among four cages (screen floor and filter lid; provisioned with sterilized water and food). A second anesthetic dose was given to any mouse starting to regain consciousness before it was put in the holding isolator.

Test mice were sacrificed on postexposure day 2, and their large bowels were removed. The digesta in the excised gut was gently scraped out and titrated for C. botulinum toxin (8). The pathogen CFUs were also enumerated by plating dilutions of the digesta on an agar medium which is selective for the pathogen (5). The finding of toxin or ≥10^5 CFU/g was taken as an indication that the mouse was intestinally colonized by C. botulinum.

The pathogen was usually recovered from digesta containing toxin but the reverse correlation was not as high (Table 1). The CFUs ranged from 10^2 to 10^7 per g of digesta; toxin ranged from 10^2 to 10^5 intraperitoneal 50% lethal doses per g. Colonization rates, based on either toxin or CFU, were higher when isolators were seeded with more spores of the same type. When numbers were high enough, colonized mice were in all cages instead of just one or two cages. The postexposure holding was limited to 2 days since maximal toxin and CFU levels usually occur at this time after mice are perorally challenged with pathogen spores (4). This period would be shorter than required for toxin to be formed in vivo and would then cause a systemic effect that would be
recognized as botulism (10). Infant mice could not be studied since some died from the decontamination treatment and survivors were not readily accepted back by their mother.

A petri plate containing _Clostridium botulinum_-selective agar was left uncovered next to each of the four cages during the time mice were being exposed to pathogen. The plates were anaerobically incubated at 37°C for 4 days, and the pathogen colonies on the four plates were counted and averaged. When the inoculum was 10^7 spores, overcrowding of plates by colonies was avoided by substituting a set of three plates for the normally used single plate. Plates of each set were sequentially exposed at different times during the 30-min spore exposure period, and the total CFU that was recovered by a set was used as the number that would have been collected by a single plate. Average CFU per plate (Table 1) confirmed that only a small fraction of the spores introduced into an isolate reached any one mouse.

All experiments were done so that up to three mice would be left over after test mice were put into the holding isolator. These extra mice were held up to 1.0 h after the last decontamination treatment and then rinsed successively in two 25-ml volumes of sterile water. The rinse water was filtered through the Iso-Grid system (QA Laboratories, Toronto, Canada), and the filter membrane was washed by passing it through 25 ml of clean water. It was then incubated 4 days on pathogen-selective agar in petri plates.

Viable pathogen was either not recovered from the extra mice or was less than one mouse colonizing dose (Table 1). In a different test not reported in Table 1, an average of 300 viable pathogen cells was recovered from three mice which were exposed in an isolator seeded with 10^7 type B spores but not given the decontamination treatment. The effectiveness of the decontamination indicated that the observed colonizations were not started by organisms that mice carried into the holding isolator on their fur. Moreover, actively ingested spores could not have been the colonizing incula since mice were immobilized with an anesthetic until they had received the full decontamination treatment.

The results can be explained if the colonizations were started by spores inhaled in the exposure isolator; such spores would not be affected by the surface decontamination procedure. The powder resulting from grinding lyophilized spore preparations would contain mostly particles larger than aerosol size. When larger than aerosol particles are inhaled, many would be caught in the mouth and nasopharynx, be carried by secretions into the stomach, and thereby reach the large bowel, where _Clostridium botulinum_ colonization occurs (3).

The present and earlier observations (8) indicate that _C. botulinum_ causing infant botulism need not be ingested in food; they suggest ways which strictly breast-fed infants could acquire the toxicoinfection. The pathogen could become airborne from its soil habitat and settle on a surface that an infant licks. Alternatively, the pathogen could be ingested while airborne. These suggestions agree with the epidemiologically based conclusions that infant botulism is more common in geographic areas where soils have relatively high pathogen concentrations. In such areas, the pathogen is more likely to be made airborne by construction and agricultural activities (9) and even be carried into homes on clothes if farming is an important industry (7).

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**LITERATURE CITED**