**Clostridium tetani** Growth and Toxin Production in the Intestines of Germfree Rats

CAROL L. WELLS† and EDWARD BALISH

Departments of Surgery and Medical Microbiology, University of Wisconsin, Madison, Wisconsin 53706

Received 4 February 1983/Accepted 6 May 1983

Germfree rats were challenged orally and intrarectally with spores of *Clostridium tetani*. Although *C. tetani* spores remained viable in the intestinal tract, they were unable to germinate. Germfree rats were then challenged orally with vegetative cells of *C. tetani*. Vegetative cells were able to colonize the intestinal tract, replicate, and produce toxin. Tetanus antitoxin, but no tetanoxin, was detected in the sera of monoassociated rats.

The clostridia are anaerobic spore-forming bacteria that occur worldwide in the environment. *Clostridium botulinum* and *Clostridium tetani* secrete potent neurotoxins that cause the classic diseases botulism and tetanus, respectively. Botulism has most often been associated with ingestion of preformed botulinum toxin in contaminated food. *C. botulinum* has been thought incapable of replicating and producing toxin in the intestinal tract; however, recent studies show that *C. botulinum* can replicate and produce toxin in the intestinal tracts of neonates (7, 10, 12) and of adult mice recently given broad-spectrum antibiotic therapy (4). In human neonates this newly recognized form of botulism has been implicated as one of the causes of sudden infant death (3). Since *C. botulinum* spores can germinate in the intestinal tract and cause disease, we speculated that *C. tetani* spores could cause an intestinal toxicoinfection. Although soil is the main reservoir of *C. tetani*, the organism has also been isolated from the feces of humans and animals (9). Thus, *C. tetani* might be capable of causing a heretofore unrecognized disease syndrome by replicating and secreting toxin in the intestinal tract under conditions of limited microbial competition. To test this hypothesis, adult germfree (GF) rats were orally challenged with either spores or vegetative cells of *C. tetani*. This animal model was chosen because adult GF animals have been used to demonstrate that, in the absence of a competing microflora, *C. botulinum* can replicate in the intestinal tract and contribute to a fatal toxicoinfection (6, 7, 11).

GF male, 60- to 90-day-old Sprague-Dawley rats were reared in plastic isolators at the Gnotobiotic Laboratory, University of Wisconsin, Madison. All animals received food and water ad libitum. Weekly bacterial cultures (aerobic and anaerobic) demonstrated that the animals remained free of extraneous microbial contamination for the duration of this study.

*C. tetani* C-3-6, a clinical isolate, was obtained from the stock culture collection of the Wisconsin State Laboratory of Hygiene, Madison. Unless otherwise stated, all manipulations of the organism were done by using recommended anaerobic techniques (2, 5). The GF rats were inoculated with cultures containing either *C. tetani* spores or *C. tetani* vegetative cells. To obtain a suspension of bacterial spores, *C. tetani* was inoculated into a prereduced medium consisting of 5% polypeptone (BBL Microbiology Systems, Cockeysville, Md.) and 0.1% sodium thioglycolate (Difco Laboratories, Detroit, Mich.). The culture was incubated at 35°C for 4 days. Spores were aerobically washed three times with sterile saline, heat shocked at 80°C for 10 min, immediately cooled on ice, and then stored at 4°C until used. This spore suspension contained approximately 10⁸ viable spores per ml and was diluted 1 to 10 with sterile saline immediately before inoculation into GF rats. Oral and rectal routes of inoculation, described later, were used to administer *C. tetani* spores to GF rats. To obtain cultures of vegetative cells, *C. tetani* was inoculated into chopped meat-glucose broth (5) and incubated for 24 to 48 h at 35°C. These cultures contained 10⁹ to 10¹⁰ viable cells per ml. The oral cavity of each rat was swabbed with cotton that had been dipped into the broth culture of viable *C. tetani* cells.

To quantitate the number of viable *C. tetani* cells per milliliter of medium, the broth culture was serially diluted in anaerobic dilution fluid (5), plated on supplemented brain heart infusion agar (5), and incubated for 24 to 48 h at 35°C. Viable *C. tetani* cells in the intestinal tracts of
monoassociated rats were quantitated as follows. The monoassociated rats were sacrificed, and intestinal tissues (with contents) were aseptically excised and immediately transferred to an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). The tissues were then placed in phosphate-buffered gelatin (10) and homogenized in blenders (Waring; Arthur C. Thomas Co., Philadelphia, Pa.). The homogenates were serially diluted in anaerobic dilution fluid and plated as described above to assess the numbers of viable organisms per gram (dry weight) of tissue. Intestinal tissue segments cultured included the ileum (a 10-cm portion proximal to the ileocecal valve), the cecum, and the colon.

To detect tetanus toxin in the intestinal tract, tissue segments (with contents) were homogenized in phosphate-buffered gelatin, centrifuged for 30 min at 10,000 × g at 4°C, and serially diluted (phosphate-buffered gelatin); 0.1 ml of each dilution was injected intramuscularly into the right flank of each animal in four-member groups of conventionally reared adult HA/ICR mice (Sprague-Dawley). The mice were observed for 4 days and the 50% lethal dose (LD50) for each tissue segment was calculated by the method of Reed and Muench (8). Gastrointestinal segments assayed for toxin included the stomach, the duodenum (a 10-cm portion adjacent to the stomach), the ileum (a 10-cm portion adjacent to the ileocecal junction), the cecum, and the colon. The presence of tetanus toxin was confirmed by the mouse protection assay with tetanus immune globulin (human) (Hyper-tet; Cutter Laboratories, Berkeley, Calif.) as the antiserum; 30 min before the injection of toxin, 0.5 ml of antiserum, containing 2.5 IU, was injected intraperitoneally into each mouse used in the protection assay.

Sera of C. tetani-monoassociated rats were tested for tetanus toxin, tetanus antitoxin, or both. The monoassociated animals were anesthetized with ether and bled by cardiac puncture. Serum was removed and stored at −40°C until tested. Serum toxin was assayed by the mouse inoculation method described above. Serum antitoxin was assayed in mice by intramuscular injection of 0.1 ml of C. tetani broth culture that had been diluted in phosphate-buffered gelatin to contain one LD100 of tetanus toxin. The presence of antitoxin was demonstrated by the survival of all mice given 0.5 ml of C. tetani-monoassociated rat serum contrasted with the death of all control mice that were injected intraperitoneally with 0.5 ml of GF rat serum before the injection of C. tetani broth culture.

In the first attempt to monoassociate GF rats, nine GF rats were swabbed perorally with a C. tetani spore suspension, and 9 ml of the spore suspension (ca. 10⁶ spores) was added to 150 ml of drinking water. Fresh water was given (the following morning) after this 150 ml was consumed. During the next 7 days, no C. tetani cells were cultured from rat feces. On day 7 postinoculation, three rats were sacrificed, and their ilea, ceca, and colons were individually homogenized, serially diluted, and plated to determine the number of viable C. tetani cells in the intestinal tracts. Only occasional dilutions of the homogenized segments (for example, a 1:10 or a 1:100 dilution tube in a series) contained C. tetani cells. This sporadic recovery of C. tetani cells from serial dilutions of intestinal contents indicated that C. tetani spores remained viable in the intestinal tract for the duration of the experiment. Since rodents are coprophagic, some of these C. tetani spores were probably ingested during the observation period, yet germination did not seem to occur either in the intestinal tract or in the shed fecal pellets. The hearts, lungs, livers, spleens, kidneys, and mesenteric lymph nodes were sterile. No gross pathology was evident at necropsy, and no tetanus toxin could be detected in the ceca of these animals. On day 10, the remaining six rats were anesthetized with phenobarbital (3.5 ml/100 g [body weight]), and 0.5 ml of the spore suspension was administered (orogastric tube) both orally and rectally (total dose, 10⁷ spores per rat). Subsequently collected fecal samples were sterile. Ten days later (day 20 of the experiment), three rats were sacrificed and intestinal segments were assayed as outlined above. Again, bacterial cultures were negative, and intestinal tetanus toxin was not detected. Vegetative cells were then administered by oral swab to the remaining three rats in this isolator. Within 48 h, Gram stains of fecal pellets showed high numbers of gram-positive rods, and fecal cultures consistently yielded toxigenic C. tetani cells. One of these three rats died. This animal appeared listless before death, but classic symptoms of tetanus were not observed. Therefore, this death was attributed to the oral and rectal manipulations; no deaths occurred in subsequent experiments with rats. On day 7 after monoassociation, the two surviving rats appeared healthy, and the experiment was terminated. These rats were sacrificed, and the intestinal tracts were analyzed for viable C. tetani cells and for tetanus toxin. C. tetani cells replicated to high numbers (10¹⁰ per g of cecal tissue) in the lower intestinal tract and produced large amounts (>100,000 LD50 per cecum) of tetanus toxin (Table 1). The hearts, lungs, livers, spleens, kidneys, and mesenteric lymph nodes of these animals were sterile, and no gross pathology was observed at necropsy. The above experiment was repeated twice.
using two separate isolators that housed six and three GF rats, respectively. Again, *C. tetani* spores failed to germinate in the intestinal tract, whereas vegetative cells replicated to high numbers and produced intestinal tetanus toxin in amounts ranging from 20,000 to 100,000 LD₅₀ per rat cecum. All rats appeared healthy. Three monoassociated rats remained healthy for 2.5 months, at which time the experiment was terminated.

To rule out the possibility that rats are unusually resistant to colonization with *C. tetani* cells, adult GF HA/ICR mice were placed in each of three isolators housing GF rats. Each isolator contained male and female mice housed in separate cages. Repeated attempts to monoassociate these GF mice with *C. tetani* spores as the inoculum failed. However, *C. tetani* vegetative cells quickly multiplied in the intestinal tract. In contrast to the lack of mortality in monoassociated rats, deaths were observed in monoassociated mice. In one typical experiment, all eight male mice died by day 14, and only one of eight female mice died during that time period. The reason for these deaths was unclear, since we did not study the relationship between coprophagy and death and since male mice frequently fight when housed together and wound tetanus could not be ruled out as a cause of death. However, it is interesting that classic symptoms of tetanus were not observed in any of the monoassociated mice.

Sera from *C. tetani*-monoassociated rats were tested for the presence of tetanus toxin and tetanus antitoxin. Tetanus toxin was not detected in sera from *C. tetani*-monoassociated rats. No antitoxin was detected in the sera of rats (three rats per group) sacrificed either 12 days after oral inoculation (oral swab and drinking water) with spores, 10 days after oral and rectal administration of spores, or 7 days after monoassociation with vegetative cells. However, tetanus antitoxin was detected in the sera of all rats sacrificed 14 days after monoassociation with vegetative cells. The detection of antitoxin in the sera of monoassociated rats was of interest since tetanus antitoxin has not been detected in the blood of patients who recover from tetanus (1). The presence of tetanus antitoxin in the sera of monoassociated rats indicated that the systemic immune system responded to toxin that was formed in the intestinal tract.

At the beginning of these experiments, we were fairly certain that spores would germinate and that vegetative cells would replicate and produce intestinal toxin and perhaps reveal a new mechanism for the pathogenesis of tetanus. This sequence did not transpire. The most significant findings from this study are that *C. tetani* spores did not germinate in the intestine and that intestinal colonization with vegetative *C. tetani* cells resulted not only in the production of intestinal tetanus toxin but also in the production of serum antitoxin. Further work is needed to more clearly define the ecology, pathogenesis, and immunology of intestinal tetanus.

### LITERATURE CITED


