Isolation of Enterotoxin-producing *Clostridium sporogenes* from Cefmetazole-associated Diarrheic Rabbit

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It is well known that *Clostridium difficile* is a major pathogenic agent of antibiotic-associated diarrhea in man and animals, and especially of pseudomembranous colitis [1, 8, 9]. However, *C. difficile* is neither isolated nor the toxin is detected in some cases of acute hemorrhagic colitis in man. It has been reported that *Klebsiella oxytoca* is predominantly isolated from man with antibiotic-associated acute hemorrhagic colitis, but toxin production has not been detected in that case [11]. On the other hand, *Clostridium sporogenes* was isolated from some infectious diseases, but direct association of the bacterium as an etiological agent has not been proven. The cytotoxic production by *C. sporogenes* in the pseudomembranous colitis induced by antibiotics was previously reported [10], but its diarrheic toxicity has not been reported so far. In the present communication, we report isolation of enterotoxin-producing *C. sporogenes* from an antibiotic-associated diarrheic rabbit.

All antibiotic preparations for injection are subject to the pyrogen test specified in the pharmacopeias [7, 12]. For the test, rabbits are used and they receive a single intravenous injection of antibiotics. Following the injection, the rectal temperature is monitored for several hours for detecting pyrogen contamination. We have experienced that some rabbits injected with antibiotics, especially cephem antibiotics, suffer from diarrhea after the test is completed, and some of them die in 7 to 10 days post injection. We examined the cecum contents of diarrheic rabbits, and isolated *C. difficile* and detected the toxin in some cases of diarrhea [6]. However, in many cases of diarrhea *C. difficile* toxin was neither detected nor the bacterium was isolated. In most cases of no detection of *C. difficile* toxin, rabbits suffered from severe hemorrhagic colitis, and the ceca were filled with fluid. We therefore examined the cecum contents of rabbits where the toxin was not detected with a C. D. check D-1 kit (Mitsubishi Chemical Industries, Tokyo). Cecum contents of a rabbit having diarrhea caused by the injection of the cepham antibiotic, cefmetazol (50 mg/ml/kg), were diluted to 10⁻³ with saline and the dilutions were cultured in cooked meat medium (Difco, Detoriot, Mich.) at 37°C for five days. The cultured broth was inoculated on EG agar (Nissui Co., Tokyo), which was incubated at 37°C for 2 days in an anaerobic steel-wool jar filled with 100% CO₂. Then spore forms were isolated and cultured in GAM semisolid medium (Nissui Co., Tokyo) at 37°C for 18 hr. The cultured media were centrifuged at 12,000 × g for 20 min at 4°C and the supernatants were sterilized by passing through 0.45 μm Millipore filters. The cell-free supernatants were examined by several toxicity tests including rabbit ligated ileal loop test, rabbit skin vascular permeability test, mouse lethality test and HeLa S3 cells toxicity test.

The isolated bacterium was identified by the methods outlined in the Berg's Manual of Systematic Bacteriology [3]. The isolate produced acid from maltose, but did not from fructose and mannitol. The isolate also produced lipase and could digest meat. These biochemical and metabolic characteristics were different from those of *C. difficile*. These and other biochemical and metabolic characteristics of the isolate were similar to those of *C. sporogenes* and *Clostridium botulinum* type A, B and F except for acid production from galactose. The two species may be distinguished by the lethality of *C. botulinum* strains to mice. Our isolate was not lethal by a single intraperitoneal injection to mice. Thus the isolate was identified as *C. sporogenes*. This identification was ascertained with the type strain of *C. sporogenes* (JCM 1416).

The loop test was performed according to the methods of Evans et al. [5] and Burrows et al. [2] with minor modification, employing female Japanese white rabbits of 3 to 4 kg in weight. The cultured supernatants (1 ml) were injected to the loops of 4-cm length. The rabbits were sacrificed 7 hr post injection and the loops were separated. The loop fluid was withdrawn and the volume was determined. The loop tissues were weighed and the fluid secretion/tissue weight (ml/g) were estimated. Five loops were used for each sample, and the data were analyzed for statistical significance by Student's t test. As shown in Fig. 1, the cultured medium of *C. sporogenes* isolate induced a 2.5-fold accumulation of fluid compared with the control GAM semisolid medium, and the increase was comparable to the fluid accumulation induced by our *C. difficile* cultured medium similarly treated. The loops were fixed in 10% formalin and then embedded in paraffin. Sections were stained with hematoxylin and eosin. Marked hemorrhage in the mucosa, and edema and hemorrhage in the submucosal intestinal space were observed (Fig. 2). The cultured supernatants were tested also for rabbit skin vascular permeability by the method of Craig et al. [4]. Samples (0.1 ml) were injected subcutaneously in the back and lateral abdominal skin, and after 18 hr the size of hemorrhagic patch was measured as two perpendicular diameters (mm). The cultured medium of *C. sporogenes*
To examine the cultured medium of *C. sporogenes* isolate for the cytotoxic effect, HeLa S3 cells were cultivated in 24-well plastic plates with Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum for 24 hr at 37°C in a CO₂-incubator. Sample solution (0.1 ml) of serial 3-fold dilutions of cultured medium were added to the cells in 0.5 ml of growth medium, and the cells were incubated for 48 hr at 37°C followed by microscopic examination and cell counting with a cell counter (Coulter electronics, Inc.). The cells were morphologically degenerated extensively with undiluted cultured medium, and its growth was inhibited 85 and 50% with undiluted and 3-fold diluted cultured medium, respectively. The cultured medium of *C. difficile* had a much stronger cytotoxic effect against HeLa cells; degeneration of the cells was evident with 10⁻³-fold diluted medium.

These results demonstrated that *C. sporogenes* isolated from the cecum contents of cemetazol-associated diarrheic rabbit produced an enterotoxic substance in GAM semisolid medium. It significantly increased fluid secretion in ligated ideal loops and induced marked hemorrhage in the mucosa and also showed positive results in both the skin vascular permeability test and the cytotoxic test against cultured cells with less potencies than those of *C. difficile* toxin.

REFERENCES