

Cecal Toxin(s) from Guinea Pigs with Clindamycin-Associated Colitis, Neutralized by *Clostridium sordellii* Antitoxin

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The cecal contents of guinea pigs with clindamycin-associated colitis contained a heat-labile toxin. This toxin was lethal for guinea pigs and mice, produced vascular permeability in the skin of rabbits, and was cytotoxic in tissue culture. The lethality in mice, vascular permeability in rabbit skin, and cytotoxicity in tissue culture monolayers were neutralized by *Clostridium sordellii* antitoxin.

Antibiotic-associated colitis in guinea pigs has been associated with a variety of antimicrobial agents (10, 11, 13, 14, 15). Several investigators have implicated *Clostridium difficile* toxins in clindamycin-associated colitis in humans (17, 22) and hamsters (7, 23). More recently, *Clostridium perfringens* type E toxin has been implicated in clindamycin-associated colitis in rabbits (16), and *Clostridium histolyticum* toxin has been similarly implicated in guinea pigs (15). The purpose of this study was to investigate the association of a bacterial toxin in the pathogenesis of clindamycin-associated colitis in guinea pigs. Knoop (15) demonstrated a toxin in the feces of clindamycin-treated guinea pigs which was lethal for guinea pigs. *C. histolyticum* antisera neutralized the cytotoxic effect in the Y-1 adrenal cell assay, but neutralization of the toxin's lethal effect was not tested. In the present study, a lethal heat-labile toxin was demonstrated in the cecal contents of guinea pigs with clindamycin-associated colitis. The lethality in mice and vascular permeability in the rabbit skin assay were neutralized by *C. sordellii* antitoxin.

MATERIALS AND METHODS

Experimental animals. Hartley strain guinea pigs of both sexes, weighing 275 to 325 g, were obtained from West Jersey Biological Supply (Wenonah, N.J.). They were housed in solid-bottom cages (20 by 22 by 9 inches, ca. 51 by 56 by 23 cm), six per cage, fed Wayne guinea pig diet fortified with vitamin C, and permitted tap water ad libitum. CD-1 mice weighing 20 to 25 g were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). They were housed in solid polycarbonate cages, fed Wayne Lab-Blox diet, and permitted tap water ad libitum.

Preparation of cecal filtrates. Eighteen guinea pigs received a single 50-mg/kg dose of clindamycin (Cleocin; Upjohn Co., Kalamazoo, Mich.) subcutaneously. Six control animals received a single injection of 0.85% NaCl (saline) subcutaneously. Cecal contents were collected immediately after death from clinda-

mycin-treated guinea pigs and immediately after ether euthanasia of saline-treated animals. From each antibiotic-treated animal approximately 30 to 50 ml of cecal contents was obtained, pooled, and clarified by centrifugation at $10,000 \times g$ for 30 min at 4°C. The crude cecal supernatant fluids were filtered through a 0.45- μ m membrane (Millipore Corp., Bedford, Mass.). Cecal filtrate samples of clindamycin-treated animals (CL-FIL) were stored at -70°C and thawed just before individual experiments. Cecal contents from saline-treated animals (CON-FIL) were processed the same as those of clindamycin-treated animals. Bacteriological sterility of the filtrates was confirmed by: (i) inoculating 1 ml of filtrate from each sample into 10 ml of thioglycollate broth and incubating aerobically at 37°C and (ii) culturing a sample of filtrate on blood agar under aerobic and anaerobic conditions. All samples were checked daily for 2 weeks for evidence of bacterial growth. At the end of 2 weeks, a sample from each tube was cultured on blood agar under both aerobic and anaerobic conditions. Protein concentrations were determined by the method of Lowry et al. (19).

Lethal toxin assays. The lethality of the filtrates from clindamycin-treated guinea pigs (CL-FIL) was determined by intraperitoneal injection of guinea pigs and mice. Toxicity in mice was measured by determining the 50% lethal dose (LD_{50}) at 24 h. The LD_{50} was calculated by the Reed and Muench method (20).

Vascular permeability activity assay in rabbit skin. New Zealand white rabbits (Sunny Acres, Tyler, Tex.) weighing 2 kg were depilated and, 24 h later, injected intradermally with 100 μ l of twofold serial dilutions of CL-FIL and appropriate control solutions (CON-FIL, cholera toxin, or saline). Twenty-two hours later, 40 mg of 2% Evans Blue solution per kg was injected intravenously; diameters of edema (induration) and increased vascular permeability (evident by blueing due to dye leakage) were measured 2 h later according to the method of Evans et al. (9). Each dilution was tested eight times in a minimum of four animals. The amount of CL-FIL that produced a 7-mm mean diameter of blueing is the equivalent of one blueing dose.

Tissue culture cytotoxicity assay. Filtrates were assayed for cytotoxicity in WI-38 (human embryonic lung fibroblasts) cell cultures by John G. Bartlett (4),

Veterans Administration Hospital, Boston, Mass. Samples (0.1 ml) of undiluted and a 1:10 dilution of CL-FIL and CON-FIL were inoculated into WI-38 cell cultures. The cultures were examined at 4, 24, and 48 h.

Heat sensitivity assay. Samples tested for heat sensitivity were incubated at 60°C for 30 min and cooled to room temperature before intradermal or intraperitoneal injection of test animals. Control samples were incubated at room temperature.

Toxin neutralization. Toxin neutralization tests were performed by testing the ability of *C. sordellii* (lot 4761G; 100 U/ml), *C. histolyticum* (lot 3647G; 800 U/ml), *C. novyi* type A (lot 5451G; 250 U/ml), *C. septicum* (lot 7035G-100; 85 U/ml), *C. perfringens* type A (lot 3973G; 115 U/ml) (all from American Cyanamid Co., Lederle Laboratories Div., Pearl River, N.J.), and *C. perfringens* type E (lot K6292; Burroughs Wellcome Research Laboratories, Bechenham, England) antitoxins to neutralize CL-FIL lethality in mice, vascular permeability in the rabbit skin assay, and cytotoxicity in WI-38 cell cultures. Three CL-FIL 24-h mouse LD₅₀ units were incubated with 0.5-ml twofold serial dilutions of each of the specific antitoxins in a final volume of 1 ml for 1 h at 25°C before intraperitoneal inoculation.

A 100- μ l sample of a 1:6 dilution of CL-FIL was incubated for 1 h at 25°C with an equal volume of twofold serial dilutions of each of the six specific antitoxins before intradermal injections. A 100- μ l dose of the toxin-antitoxin mixture was injected intradermally; each dilution was tested eight times in a minimum of four rabbits. CL-FIL was also incubated with mycoplasma-free horse serum (control no. E051412) (GIBCO Laboratories, Grand Island, N.Y.) or 0.1 M phosphate-buffered saline (pH 7.2) for 1 h at 25 or 37°C before testing in all three bioassays.

Cytotoxic-positive CL-FIL was retested in the WI-38 cell culture assay with an equal volume of a 1:10 dilution of monovalent clostridial antitoxin or horse serum by John G. Bartlett (4). Cecal filtrates from control animals and the six antitoxins were also tested for cytotoxicity.

RESULTS

Death after subcutaneous injection of clindamycin. Thirteen of the 18 guinea pigs died during the 14-day observation period after subcutaneous administration of clindamycin. All 13 deaths occurred between days 3 and 9 after clindamycin administration. Grossly, the animals had fluid-filled distended ceca with varying degrees of hyperemia. The ceca of eight animals autopsied immediately upon death were examined microscopically. These ceca showed capillary congestion, polymorphonuclear infiltration of the lamina propria and submucosa, and occasional focal erosion of the mucosal epithelium.

Toxicity of CL-FIL for guinea pigs and mice. Six guinea pigs died within 24 h of intraperitoneal injection of 1 ml of CL-FIL. None of the six control guinea pigs died within the 7-day observation period after injection of 1 ml of CON-FIL or saline intraperitoneally. A 24-h

mouse LD₅₀ was calculated to be 100 μ l of CL-FIL, which was equivalent to 800 μ g of protein. A 7-day mouse LD₅₀ was calculated to be 70 μ l of CL-FIL, which was equivalent to 560 μ g of protein. Six mice inoculated with 200 μ l of heat-treated CL-FIL lived for 2 weeks, whereas six mice inoculated with 200 μ l of unheated CL-FIL died within 24 h.

Assay of vascular permeability activity in rabbit skin. When rabbits were injected intradermally with CL-FIL, localized induration and vascular permeability (blueing) increased in size with increasing doses of filtrate. Zones of induration were disproportionately larger than the zones of blueing. The maximum diameter of blueing was approximately 9.1 mm, whereas the maximum diameter of induration was approximately 49.5 mm. This disproportionate size of blueing and induration may be due to the increasing pressure of extracellular fluid at maximum induration which prevented the movement of dye into the surrounding tissue. Intradermal hemorrhage was also associated with areas of induration larger than 30 mm in diameter. One milliliter of CL-FIL was calculated to have 125 blueing units.

Cytotoxicity of CL-FIL in tissue culture. The undiluted and 1:10 dilution of CL-FIL were positive within 24 h for cytotoxic changes characterized by cellular rounding with loss of cell processes. CON-FIL did not produce cytotoxic changes.

Neutralization of CL-FIL activity by clostridial antitoxins. *C. sordellii* antitoxin diluted 1:64 was the lowest concentration that protected mice for 24 h, whereas 7-day protection was achieved only with dilutions of 1:16 or lower. *C. histolyticum*, *C. novyi*, *C. septicum*, and *C. perfringens* type A or type E antitoxins and control horse serum did not protect mice from the lethal effects of CL-FIL (Table 1).

C. sordellii antitoxin prevented the blueing effect of induration at 1:64 dilution or lower. Blueing and induration produced by CL-FIL were not prevented by incubation with normal horse serum or antisera of *C. histolyticum*, *C. novyi*, *C. septicum*, and *C. perfringens* type A or E (Table 2).

C. sordellii antitoxin neutralized the cytotoxic effect of CL-FIL on tissue culture monolayers of WI-38 cells. Neither *C. histolyticum*, *C. novyi*, *C. septicum*, or *C. perfringens* type A or type E antitoxin, nor control horse serum, neutralized the cytotoxic effect of CL-FIL.

DISCUSSION

Cecal filtrates from guinea pigs inoculated with clindamycin contain a heat-labile toxin which is lethal for guinea pigs and mice when

TABLE 1. Effect of clostridial antitoxins on the lethality of CL-FIL in mice^a

Test solution dilution	Antitoxins						Saline	Horse serum
	<i>C. sordellii</i>	<i>C. histolyticum</i>	<i>C. novyi</i>	<i>C. septicum</i>	<i>C. perfringens</i> type A	<i>C. perfringens</i> type E		
1:2	0	3	3	3	3	4	3	4
1:4	0	NT	NT	NT	NT	NT	NT	NT
1:8	0	4	4	3	4	3	NT	NT
1:16	0	NT	NT	NT	NT	NT	NT	NT
1:32	0	3	3	4	4	3	NT	NT
1:64	0	NT	NT	NT	NT	NT	NT	NT
1:128	3	4	4	3	4	4	NT	NT
1:250	4	NT	NT	NT	NT	NT	NT	NT
1:512	4	4	4	3	3	3	NT	NT

^a Four mice were injected intraperitoneally with 0.5 ml of twofold dilutions of various solutions incubated for 1 h with 0.45- μ m filtrates (3 LD₅₀) from clindamycin-treated guinea pigs in a total volume of 1 ml. Data are the number of mice to die in 24 h after challenge with test solution. NT, Not tested.

TABLE 2. Clostridial antisera neutralization of CL-FIL-induced vascular permeability in rabbit skin^a

Antitoxin	Antisera dilution											
	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
<i>C. sordellii</i>	-	-	-	-	-	-	-	±	+	+	+	+
<i>C. histolyticum</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. septicum</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. novyi</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. perfringens</i> type A	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. perfringens</i> type E	+	+	+	+	+	+	+	+	+	+	+	+

^a One-tenth milliliter of the indicated clostridial dilution was incubated with an equal volume of a 1:6 dilution of CL-FIL; 0.1 ml of this incubation mixture, with one blueing dose, was injected intradermally. -, 0-mm diameter of blueing; +, >6-mm average diameter of blueing.

injected intraperitoneally. These filtrates produced edema, hemorrhage, and increased vascular permeability in rabbit skin. They also produced cytotoxic changes in cell cultures. These toxic preparations of the cecal filtrates were neutralized by in vitro incubation with *C. sordellii* antitoxin but not with other clostridial antitoxins. The lethal effect of the guinea pig CL-FIL, its vascular permeability effect in rabbit skin, its cytotoxicity in cell cultures, and its heat lability resemble the properties of *C. sordellii* toxins (1, 2). The morphological alterations in the ceca of guinea pigs that died after subcutaneous administration of clindamycin were similar to those of hamsters (6) and guinea pigs (15) with clindamycin-associated colitis.

Toxigenic *C. difficile* is presumed to be the cause of clindamycin-associated colitis in hamsters (7, 23) and humans (8, 17). The toxins derived from clindamycin-treated hamsters (23) and humans (17, 18) or from culture filtrates of *C. difficile* are reported to be neutralized by *C. sordellii* antitoxins (4, 7, 23). These studies suggest that *C. sordellii* and *C. difficile* produce a common toxin or share a toxic component, or that *C. sordellii* antitoxin available for these

studies was not a monovalent antitoxin. Recent studies employing partially purified *C. difficile* and *C. sordellii* toxins demonstrated no cross-reactivity with antisera specific for these toxins (3). The cross-reactivity with *C. difficile* toxin and U.S. Standard *C. sordellii* antitoxin has been questioned and attributed to possible impurity of the original strain used in preparation of the antitoxin (3, 8).

Knoop (15) has demonstrated the presence of a lethal toxin in the feces of clindamycin-treated guinea pigs which was neutralized by *C. histolyticum* antitoxin in the Y-1 adrenal cell assay. Our findings suggest that toxigenic *C. sordellii* or an antigenically related toxin is associated with the clindamycin colitis in guinea pigs.

In contrast to hamsters and guinea pigs, in which *C. difficile* and *C. histolyticum* have been associated with clindamycin colitis, LaMont et al. (16) have demonstrated that *C. perfringens* type E antitoxin neutralized a toxic filtrate associated with clindamycin colitis in domestic rabbits. This observation further indicates an association of different toxigenic clostridial organisms with the antibiotic-associated colitis syndrome in different animal species.

Until now, only one toxigenic *Clostridium* sp. has been implicated in any one animal species with clindamycin colitis, but the possibility that multiple toxigenic clostridia occur within one animal species may also account for the difference in our data and Knoop's. Rifkin et al. (21) have isolated both *C. difficile* and *C. sordellii* from hamsters with clindamycin colitis; however, the *C. sordellii* isolates were not toxigenic. The association of multiple toxigenic clostridia within an animal or animal population may account for the variation observed in the passive immunity (1, 5) and antimicrobial therapy studies (6, 10, 11, 14) employed in antibiotic-associated colitis. Antibiotic-associated colitis in humans and animals may not be as simplistic as initially thought.

The role of *Clostridium* spp. other than *C. difficile* in the various animal models and in humans needs to be pursued.

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