ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2005, p. 3529–3532 Vol. 49, No. 8
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.8.3529–3532.2005

Effect of Antibiotic Treatment on Growth of and Toxin Production by *Clostridium difficile* in the Cecal Contents of Mice

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Received 29 December 2004/Returned for modification 28 February 2005/Accepted 3 May 2005

In mice, subcutaneous administration of antibiotics that disrupt the anaerobic microflora (i.e., clindamycin, piperacillin-tazobactam, and ceftriaxone) facilitated in vitro growth of and toxin production by *Clostridium difficile* in cecal contents, whereas antibiotics that cause minimal disruption of the anaerobic microflora (i.e., levofloxacin, cefepime, and aztreonam) did not.

Antibiotics play a crucial role in the pathogenesis of *Clostridium difficile*-associated diarrhea (CDAD), presumably by disrupting the indigenous microflora of the colon, thereby allowing *C. difficile* to grow to high concentrations with the production of toxin (2, 7). Antibiotics that have inhibitory activity against *C. difficile* may suppress the organism during treatment; however, *C. difficile* overgrowth and disease may develop after completion of therapy during the period of recovery of the indigenous microflora (2, 4, 7). Antibiotics that disrupt the anaerobic component of the microflora may be particularly likely to cause CDAD (1, 2, 21). However, antibiotics which cause relatively little disruption of the anaerobic microflora, such as ciprofloxacin and trimethoprim-sulfamethoxazole, have also been associated with CDAD (7, 23). In this study, we used an in vitro mouse model to examine the effect of antibiotic treatment on growth of *C. difficile* in cecal contents. We hypothesized that antibiotics that disrupt the anaerobic microflora would facilitate growth and toxin production by *C. difficile*, whereas antibiotics that do not disrupt the anaerobic microflora would not.

Three strains of *C. difficile* were studied. Strain 1 was ATCC 9689, strain 2 was a clinical isolate from Cleveland, and strain 3 was ATCC 43593. Strains 1 and 2 produced toxin, whereas strain 3 did not. Broth dilution MICs of the test antibiotics for the three strains were determined using standard methods for susceptibility testing of anaerobic bacteria (11).

The in vitro model that was used was adapted from the in vitro model of colonization resistance to *C. difficile* infection developed by Borriello et al. (1). These investigators demonstrated that antibiotics that promoted in vitro growth and toxin production by *C. difficile* in cecal emulsions of hamsters also caused *C. difficile* disease in hamsters, whereas antibiotics that did not promote in vitro growth and toxin production did not cause disease (1). The experimental protocol was approved by the Animal Care Committee of the Cleveland Veterans Affairs Medical Center.

Female CF-1 mice weighing 25 to 30 g (Harlan Sprague-Dawley, Indianapolis, Indiana) were housed in individual cages with plastic filter tops to prevent cross-contamination among animals. We studied three antibiotics that we have previously shown to cause marked disruption of the anaerobic stool microflora of mice (clindamycin, piperacillin-tazobactam, and ceftriaxone), and three antibiotics that inhibit facultative gram-negative bacilli but cause minimal disruption of the anaerobic microflora (levofloxacin, cefepime, and aztreonam) (12, 13, 16). Mice received daily subcutaneous injections (0.2-ml total volume) of saline, levofloxacin (0.375 mg/day), cefepime (2.0 mg/day), aztreonam (3.0 mg/day), ceftriaxone (2.0 mg/day), clindamycin (1.4 mg/day), or piperacillin-tazobactam (8 mg/day) for 4 days. Three days after the final antibiotic dose, mice were euthanized by CO_2 asphyxiation. To evaluate the potential for piperacillin-tazobactam to inhibit growth of *C. difficile* during the course of treatment, one group of mice received daily treatment with this agent as described above but were euthanized 2 h after administration of the final antibiotic dose. The doses of antibiotics were equal to the usual human doses administered over a 24-hour period (milligrams of antibiotic per gram of body weight).

After the mice were euthanized, the cecum was removed and opened longitudinally. Cecal contents were collected and transferred to an anaerobic chamber (Coy Laboratories, Grass Lake, Michigan) within 5 min. The cecal contents were diluted threefold (volume/volume) in sterile prereduced phosphate-buffered saline (PBS). A final concentration of 10^4 CFU/ml of each strain was added to separate aliquots of the cecal contents of individual mice. The *C. difficile* strains were prepared for inoculation by serially diluting 72-hour broth cultures in sterile prereduced PBS. After incubation for 24 h, the samples were diluted in sterile PBS and plated on prereduced cefoxitin-cycloserine-fructose agar containing 1% taurocholate to quantify *C. difficile*. To determine toxin production, a commercially available kit for detection of *C. difficile* toxin (Diagnostic Hybrids, Inc., Athens, Ohio) was utilized as recommended by the manufacturer. The cecal content supernatants were serially diluted 10-fold in specimen diluent. Following dilution, samples were added to microtiter plates containing human fibroblast cells and observed by bright-field microscopy, at 24 and 48 h, for evidence of *C. difficile* toxin cytopathic effect. The concentrations of antibiotics in cecal contents were determined by an agar diffusion assay with strains of *Clostridium perfringens*

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TABLE 1. MICs for the three *C. difficile* test strains used in this study

Antibiotic	MIC (μg/ml) for strain[a]:		
	1	2	3
Aztreonam	>128	>128	>128
Levofloxacin	8	4	4
Cefepime	>128	>128	>128
Ceftriaxone	8	16	64
Piperacillin-tazobactam	1	2	8
Clindamycin	64	128	>128

[a] MICs were determined by broth dilution.

(for clindamycin, piperacillin-tazobactam, and ceftriaxone) or *Escherichia coli* (for cefepime, levofloxacin, and aztreonam) as the indicator strains (14). Experiments were performed twice with 6 total mice per group.

Because levofloxacin may be administered orally or parenterally, we performed an additional set of experiments to compare the effects of oral versus subcutaneous levofloxacin. Mice (4 per group) received daily levofloxacin (0.375 mg/day) by subcutaneous injection (0.2-ml total volume) or by orogastric inoculation (0.5-ml total volume) for 4 days. Orogastric inoculation was performed by use of a stainless steel feeding tube (Perfektum; Popper & Sons). The remainder of the experiment was performed as described above.

One-way analysis of variance was performed to compare *C. difficile* densities and toxin production among the treatment groups. *P* values were adjusted for multiple comparisons using the Scheffe correction. Computations were performed with the use of Stata software (version 5.0; Stata, College Station, Texas). $P < 0.05$ was considered significant.

The broth dilution MICs of the antibiotics for the three strains are shown in Table 1. Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) criteria for interpreting susceptibilities are not available for *C. difficile*. None of the cecal contents had detectable concentrations of *C. difficile* prior to inoculation of the test strains (level of detection, ~2 $\log_{10}$ CFU/ml). Figure 1A demonstrates the effect of antibiotic treatment on growth of the *C. difficile* strains in cecal contents. The cecal contents of mice treated with saline, levofloxacin, cefepime, or aztreonam were inhibitory to growth of *C. difficile* and consistently yielded a ≥1-log decrease in density in comparison to density at the time of inoculation (4 $\log_{10}$ CFU/ml). Cecal contents of mice treated with orogastric levofloxacin inhibited *C. difficile* to the same degree as cecal contents of mice receiving subcutaneous levofloxacin (i.e., a ≥1-log decrease in density of *C. difficile* was observed in cecal contents of all mice receiving oral levofloxacin) (data not shown). The cecal contents of mice that received prior treatment with ceftriaxone, clindamycin, and piperacillin-tazobactam supported growth of *C. difficile* ($P < 0.001$ for each group in comparison to saline controls). Ceftriaxone, clindamycin, and piperacillin-tazobactam were detectable in cecal contents 3 days after the final dose (mean ± standard deviation, 3.3 ± 0.6 μg/ml, 22.9 ± 3.2 μg/ml, and 2 ± 0.8 μg/ml, respectively), whereas levofloxacin, cefepime, and aztreonam were not detectable at that time (limit of detection, 1 μg/ml). Cecal contents prepared during the course of piperacillin-tazobactam treatment had higher concentrations of piperacillin-tazobactam (mean ± standard deviation, 31.2 ± 9.7 μg/ml) and were inhibitory to growth of *C. difficile*.

Figure 1B shows the toxin levels of the two toxin-producing *C. difficile* strains (strains 1 and 2). High levels of toxin were produced in cecal contents of mice that received prior treatment with ceftriaxone, clindamycin, and piperacillin-tazobactam, whereas minimal or no toxin was detectable in the saline, levofloxacin, and aztreonam groups ($P < 0.01$). In addition, minimal or no toxin was detectable in cecal contents prepared during the course of piperacillin-tazobactam treatment.

Our findings provide support for the hypothesis that disruption of the anaerobic microflora facilitates overgrowth and toxin production by *C. difficile*. As noted previously, we have shown that clindamycin, ceftriaxone, and piperacillin-tazobactam cause significant disruption of the anaerobic microflora of mice, whereas levofloxacin, aztreonam, and cefepime do not (12, 13, 16). The fact that clindamycin, ceftriaxone, and piperacillin-tazobactam remained detectable in cecal contents 3 days after discontinuation of treatment suggests that these agents were excreted in relatively high concentrations into the intestinal tract. In addition, the persistent detection of these agents could be attributable in part to the fact that inhibition of the anaerobic microflora may cause significant reductions in intestinal motility (20).

Although clindamycin and expanded-spectrum cephalosporins have frequently been associated with CDAD, clinical studies suggest that β-lactam and β-lactamase inhibitors such as piperacillin-tazobactam are a relatively infrequent cause of CDAD (5, 15). Our data suggest the possibility that agents such as piperacillin-tazobactam may be relatively infrequently associated with CDAD because they possess inhibitory activity against many *C. difficile* strains (i.e., *C. difficile* may be inhibited during the course of treatment with piperacillin-tazobactam but not cephalosporins or clindamycin). Alternatively, it has been suggested that piperacillin-tazobactam could be infrequently associated with CDAD because it may cause relatively modest disruption of the intestinal microflora of humans in comparison to expanded-spectrum cephalosporins and may stimulate less toxin production than agents such as cefotaxime (5, 15, 18, 19).

Although aztreonam, cefepime, and levofloxacin do not disrupt anaerobic microflora in the stool of mice, we have previously shown that they inhibit total aerobic and facultative gram-negative bacilli (13). These data suggest that a portion of each of these antibiotics is excreted into the intestinal tract of mice. These agents were not detected in cecal contents in the present study; however, the levels were not measured until 3 days after completion of treatment. Our findings in mice are consistent with previous studies in humans (17). Aztreonam, cefepime, and levofloxacin inhibit facultative gram-negative bacilli in the stool of humans but cause minimal disruption of anaerobes (9, 17). The failure of aztreonam to inhibit anaerobes is attributable to the fact that it has no appreciable in vitro activity against anaerobic bacteria (9). Cefepime is excreted in minimal concentrations in bile and has relatively little activity against intestinal anaerobes (2, 17). Because aztreonam and cefepime had minimal in vitro activity against the *C. difficile* test strains (Table 1) and were not detectable in cecal contents of mice, the absence of growth in cecal contents was unlikely to

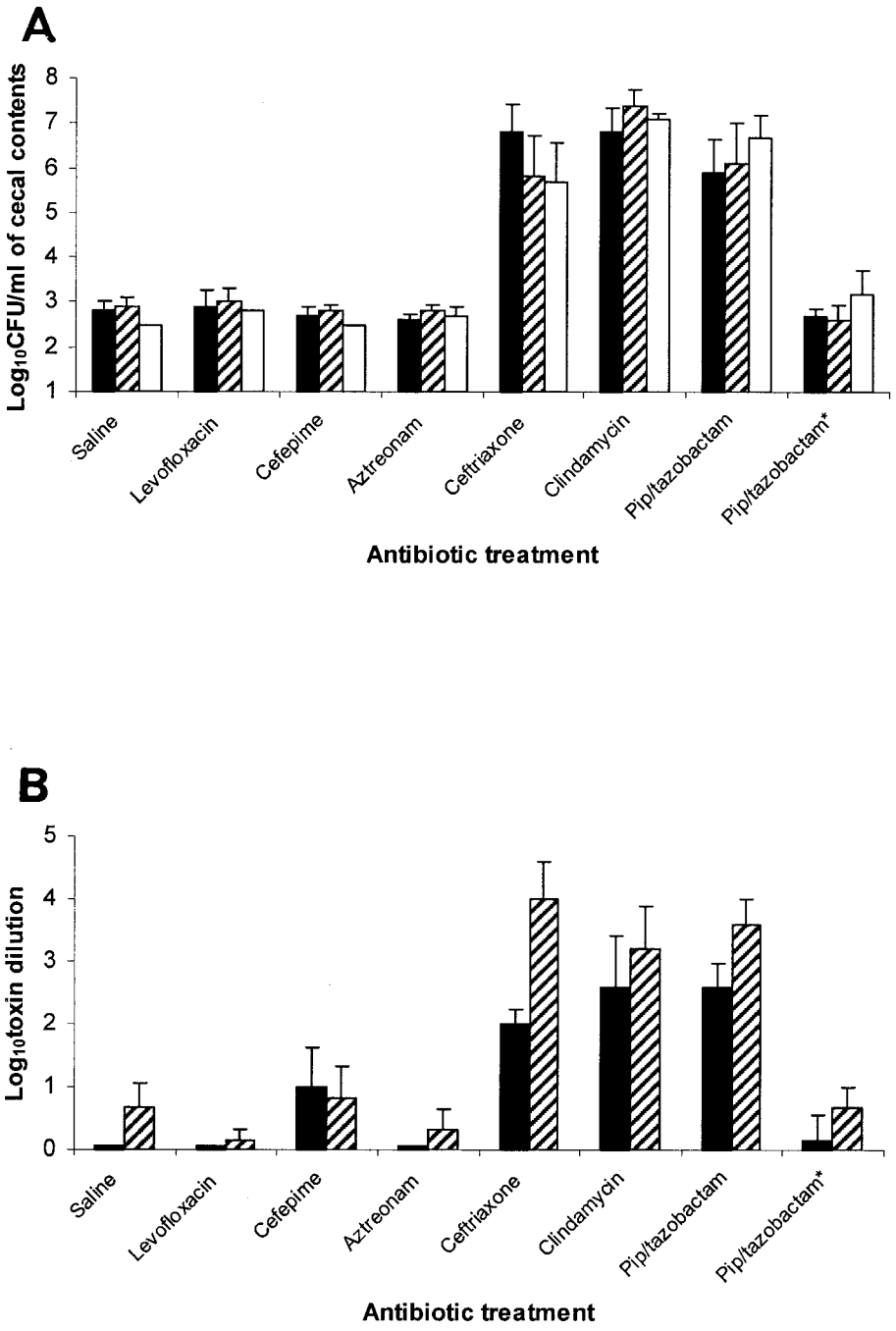

FIG. 1. Effect of antibiotic treatment on growth of (A) and toxin production by (B) *Clostridium difficile* in the cecal contents of mice. Mice received daily subcutaneous antibiotic treatment for 4 days. Three days after the final antibiotic dose, cecal contents were collected and inoculated with 10^4 CFU/ml of *C. difficile* strain 1 (solid bar), strain 2 (cross-hatched bar), or strain 3 (open bar); an additional piperacillin-tazobactam-treated group (*) had cecal contents collected 2 h after the final antibiotic dose. Samples were incubated anaerobically for 24 h, and then serial dilutions were plated onto selective media for quantification of *C. difficile* and assayed for toxin production. Mean toxin titers are expressed as the reciprocal of the highest serial 10-fold dilution that gave positive results. Pip, piperacillin. Error bars represent standard deviation.

be due to inhibitory activity of these antibiotics. Rather, the inhibitory activity of the cecal contents is presumably due to the fact that the anaerobic microflora that suppress *C. difficile* were preserved.

Fluoroquinolone antibiotics such as levofloxacin achieve relatively high concentrations in the stool of humans after oral or intravenous administration (17). The minimal impact of these agents on intestinal anaerobes of humans has been attributed to high degrees of reversible binding of these agents to fecal matter, reduced susceptibility of anaerobes to fluoroquinolones under strictly anaerobic conditions, and an inoculum effect for inhibition of anaerobes (3). Some recent clinical

studies have shown an association between fluoroquinolones such as ciprofloxacin and levofloxacin and CDAD (10, 23). In contrast, other studies have suggested that monotherapy with these agents is rarely associated with CDAD (8, 22). Our findings are consistent with the hypothesis that fluoroquinolones such as ciprofloxacin and levofloxacin are likely to present a relatively low risk for CDAD when administered as monotherapy. Additional research is needed to clarify whether fluoroquinolones with enhanced antianaerobic activity (e.g., gatifloxacin and moxifloxacin) are associated with an increased risk of CDAD in comparison to fluoroquinolones with less antianaerobic activity. Results of a recent study suggest that gatifloxacin may present a higher risk for CDAD than levofloxacin (6).

This work was supported by a grant from Ortho-McNeil Pharmaceutical and by an Advanced Research Career Development Award from the Department of Veterans Affairs to C.J.D.

REFERENCES

1. **Borriello, S. P., F. E. Barclay, and A. R. Welch.** 1988. Evaluation of the predictive capability of an in-vitro model of colonization resistance to *Clostridium difficile* infection. Microb. Ecol. Health Dis. **1:**61–64.
2. **Donskey, C. J.** 2004. The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. Clin. Infect. Dis. **39:**219–226.
3. **Donskey, C. J., M. S. Helfand, N. J. Pultz, and L. B. Rice.** 2004. Effect of parenteral fluoroquinolone administration on persistence of vancomycin-resistant *Enterococcus faecium* in the mouse gastrointestinal tract. Antimicrob. Agents Chemother. **48:**326–328.
4. **Fekety, R., J. Silva, R. Toshniwal, M. Allo, J. Armstrong, R. Browne, J. Ebright, and G. Rifkin.** 1979. Antibiotic-associated colitis: effects of antibiotics on *Clostridium difficile* and the disease in hamsters. Rev. Infect. Dis. **1:**386–396.
5. **Freeman, J., F. J. O'Neill, and M. H. Wilcox.** 2003. Effects of cefotaxime and desacetylcefotaxime upon *Clostridium difficile* proliferation and toxin production in a triple-stage chemostat model of the human gut. J. Antimicrob. Chemother. **52:**96–102.
6. **Gaynes, R., D. Rimland, E. Killum, K. Lowery, T. M. Johnson, G. Kilgore, and F. C. Tenover.** 2004. Outbreak of *Clostridium difficile* infection in a long-term care facility: association with gatifloxacin use. Clin. Infect. Dis. **28:**640–645.
7. **Gerding, D. N.** 2004. Clindamycin, cephalosporins, fluoroquinolones, and *Clostridium difficile*-associated diarrhea: This is an antimicrobial resistance problem. Clin. Infect. Dis. **38:**646–648.
8. **Golledge, C. L., C. F. Carson, G. L. O'Neill, R. A. Bowman, and T. V. Riley.** 1992. Ciprofloxacin and *Clostridium difficile*-associated diarrhea. J. Antimicrob. Chemother. **30:**141–147.
9. **Kager, L., B. Brismar, A. S. Malmborg, and C. E. Nord.** 1985. Effect of aztreonam on the colon microflora in patients undergoing colorectal surgery. Infection **13:**111–114.
10. **McCusker, M. E., A. D. Harris, E. Perencevich, and M. C. Roghmann.** 2003. Fluoroquinolone use and *Clostridium difficile*-associated diarrhea. Emerg. Infect. Dis. **9:**730–733.
11. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow anaerobically, 5th ed. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
12. **Pultz, N. J., A. Hujer, and C. J. Donskey.** 2004. Denaturing gradient gel electrophoresis as a tool to analyze the stool microbiota of laboratory mice, abstr. N-109, p. 136. Abstr. 104th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.
13. **Pultz, N. J., U. Stiefel, M. Ghannoum, M. S. Helfand, and C. J. Donskey.** 2005. Effect of parenteral antibiotic administration on establishment of intestinal colonization by *Candida glabrata* in adult mice. Antimicrob. Agents Chemother. **49:**438–440.
14. **Rolfe, R. D., and S. M. Finegold.** 1983. Intestinal β-lactamase activity in ampicillin-induced, Clostridium difficile-associated ileocecitis. J. Infect. Dis. **147:**227–235.
15. **Settle, C. D., M. H. Wilcox, W. N. Fawley, O. J. Corrado, and P. M. Hawkey.** 1998. Prospective study of the risk of *Clostridium difficile* diarrhea in elderly patients following treatment with cefotaxime or piperacillin-tazobactam. Aliment. Pharmacol. Ther. **12:**1217–1223.
16. **Stiefel, U., N. J. Pultz, M. S. Helfand, and C. J. Donskey.** 2003. Increased susceptibility to vancomycin-resistant *Enterococcus* intestinal colonization persists after completion of anti-anaerobic antibiotic treatment in mice. Infect. Control Hosp. Epidemiol. **25:**373–379.
17. **Sullivan, A., C. Edlund, and C. E. Nord.** 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect. Dis. **1:**101–114.
18. **Wilcox, M. H., S. Baines, J. Freeman, S. Qamer, and F. O'Neill.** 2003. Piperacillin-tazobactam does not induce *Clostridium difficile* toxin production in a human gut model, abstr. K-728, p. 366. Program Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, D.C.
19. **Wilcox, M. H., J. Freeman, W. Fawley, S. MacKinlay, A. Brown, K. Donaldson, and O. Corrado.** 2004. Long-term surveillance of cefotaxime and piperacillin-tazobactam prescribing and incidence of *Clostridium difficile* diarrhea. J. Antimicrob. Chemother. **54:**168–172.
20. **Wilson, K. H., J. N. Sheagren, and R. Freter.** 1985. Population dynamics of ingested *Clostridium difficile* in the gastrointestinal tract of the Syrian hamster. J. Infect. Dis. **151:**355–361.
21. **Wilson, K. H.** 1993. The microecology of *Clostridium difficile*. Clin. Infect. Dis. **16**(Suppl. 4)**:**S214–S218.
22. **Wistrom, J., S. R. Norrby, E. B. Myhre, S. Eriksson, G. Granstrom, L. Lagergren, G. Englund, C. E. Nord, and B. Svenungsson.** 2001. Frequency of antibiotic-associated diarrhea in 2462 antibiotic-treated hospitalized patients: a prospective study. J. Antimicrob. Chemother. **47:**43–50.
23. **Yip, C., M. Loeb, S. Salama, L. Moss, and J. Olde.** 2001. Quinolone use as a risk factor for nosocomial *Clostridium difficile*-associated diarrhea. Infect. Control Hosp. Epidemiol. **22:**572–575.