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Effect of L-Cysteine on the Activity of Penicillin Antibiotics Against *Clostridium difficile*

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We observed elevated MICs of penicillin antibiotics while performing agar dilution susceptibility testing of strains of *Clostridium difficile* on supplemented brain heart infusion agar, an effect which was completely eliminated by the exclusion of L-cysteine from the medium. L-Cysteine antagonizes the activity of penicillins against *C. difficile*, most likely by direct inactivation of the antibiotic.

While performing antimicrobial susceptibility testing of a group of strains of *Clostridium difficile* on various media, we observed a marked medium-dependent increase in the MICs of several penicillin antibiotics, a finding in contrast to previous reports describing almost universal susceptibility of *C. difficile* to the penicillins (3, 4, 6). In the present report we describe the results of susceptibility testing of *C. difficile* and our attempts to define the mechanism(s) of the resistance to the penicillin antibiotics.

Fifteen strains of *C. difficile* were studied. All were maintained anaerobically in chopped meat broth medium at room temperature and periodically streaked on solid medium to confirm purity. β-Lactamase production was determined by the chromogenic cephalosporin method (8). All antibiotics (penicillin G, ampicillin, carbenicillin, ticarcillin, cephalothin, cefoxitin, cefotaxime, clindamycin, erythromycin, tetracycline, doxycycline, vancomycin, metronidazole, and chloramphenicol) were supplied as laboratory standard powders. Appropriate solutions of antibiotics were prepared with sterile water and frozen at −20°C until used. The following working media were utilized: brain heart infusion agar (BHIA; BBL Microbiology Systems, Cockeysville, Md.) with (BHIA-S) or without supplementation (yeast extract, 0.5%; vitamin K_1, 1 μg/ml; hemin, 5 μg/ml; and L-cysteine hydrochloride [Sigma Chemical Co., St. Louis, Mo.]), 0.1%; Mueller-Hinton agar (MHA; Difco Laboratories, Detroit, Mich.) with vitamin K_1 and hemin; tryptic soy agar (TSA; Difco) with vitamin K_1 and hemin; brucella agar (BA) with vitamin K_1 and hemin; and Wilkens-Chalgren agar (WCA) as described previously (12). With the exceptions described below, all media except WCA were used with the addition of 5% sterile laked sheep blood.

The antibiotic susceptibilities of *C. difficile* were determined in triplicate by agar dilution (9) with the various media containing twofold dilutions of antibiotic. Duplicate plates with and without antibiotics served as sterility and growth controls, respectively. The MIC for each organism was defined as the lowest concentration of the antibiotic yielding no visible growth on agar after 48 h. Agar dilution susceptibility testing was repeated on each medium under each of the following conditions where appropriate: without the addition of 5% sheep blood; without hemin; without vitamin K_1; without L-cysteine (BHIA-S only); with L-cysteine (all media except BHIA-S); with glutathione, 0.1% (Sigma) or dithiothreitol, 0.1% (Sigma) (all media except BHIA-S); 2-unit increments of pH from 6.4 to 7.4; and with an inoculum of 10^3 or 10^5 organisms.

A radial agar diffusion assay on BHIA was used with a strain of *Sarcina lutea* as the indicator organism. Mean zone diameters for selected concentrations of penicillin G, ampicillin, carbenicillin, ticarcillin, and cephalothin were determined in triplicate. Bioassays for each antibiotic were repeated after incubation of each antibiotic solution with an equal volume of a 0.1% solution of L-cysteine in sterile water. Bioassays were done after 0, 2, 6, and 24 h of incubation with L-cysteine. Finally, zone diameters were determined separately with L-cysteine in sterile water.

Susceptibility testing of the strains of *C. difficile* to various antibiotics, as determined by agar dilution on BHIA-S, revealed the MICs of the penicillins to be universally greater than 800 μg of antibiotic per ml, including the MIC for strain ATCC 9689. These MICs were 2- to >32-fold higher than the MICs of the cephalosporin and cephamycin antibiotics. All were negative for β-lactamase production.

The susceptibilities of the strains of *C. difficile* to penicillin and cephalosporin antibiotics were compared on different media. The results with penicillin G are shown in Table 1. All strains were resistant to penicillin G on BHIA-S, with a geometric mean MIC of >800 μg/ml. In contrast, all strains were noted to be susceptible to penicillin G when tested on other media, with geometric mean MICs ranging from 2.84 μg/ml on WCA to 4.32 μg/ml on TSA. This medium-dependent variation in MICs for *C. difficile* extended to ampicillin, carbenicillin, and ticarcillin, but not to other antibiotics listed above. The following were systematically excluded from BHIA-S without effect on the MICs of the penicillin: vitamin K_1, hemin solution, and sheep blood. The MICs of penicillin G (Table 1) and all other penicillins (data not shown) fell dramatically with the omission of L-cysteine from BHIA-S. Alterations in pH, temperature, atmosphere, and inoculum size had little effect on the MICs of the penicillins on the various media. A marked increase in the MICs of penicillin G was noted when testing was done on L-cysteine-containing BHIA, MHA, and TSA. A lesser effect was noted on BA; and virtually none was noted on WCA. The addition of L-glutathione or dithiothreitol instead of L-cysteine failed to produce similar effects consistently.

Antibiotic bioassays were done for penicillin G, ampicillin, carbenicillin, ticarcillin, and cephalothin and demonstrated that when penicillin G was preincubated with 0.1%

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TABLE 1. Comparative agar dilution susceptibilities to penicillin G of 15 strains of *C. difficile* on various anaerobic media

Medium	MIC of penicillin G for the following strain no.:															Geometric mean MIC (μg/ml)
	2	3	4	5	6	7	8	9	10	11	12	13	14	15[a]		
BHIA-S	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800		>800
BHIA	1.6	6.25	1.6	6.25	3.12	1.6	12.5	12.5	3.12	3.12	1.6	6.25	1.6	3.12		3.65
WCA	8.0	3.12	6.25	3.12	6.25	3.12	6.25	6.25	1.6	0.8	3.12	3.12	1.6	0.8		2.84
BA	1.6	3.12	1.6	0.8	3.12	1.6	12.5	3.12	1.6	1.6	3.12	1.6	3.12	1.6		2.23
MHA	1.6	6.25	6.25	1.6	6.25	3.12	25	25	1.6	1.6	3.12	6.25	1.6	1.6		4.04
TSA	1.6	6.25	3.12	1.6	6.25	3.12	12.5	12.5	1.6	3.12	6.25	3.12	NG[b]	3.12		4.32

[a] ATCC 9689.
[b] NG, No growth.

L-cysteine, zones of inhibition were either abolished (at concentrations of penicillin G of 0.1 and 0.2 μg/ml) or markedly diminished (at 0.4 μg/ml). This apparent inactivation of penicillin G was independent of the duration of preincubation with L-cysteine. L-Cysteine was noted to have no intrinsic antibiotic activity. Similar results were obtained with ampicillin, carbenicillin, and ticarcillin, with and without L-cysteine. In contrast, L-cysteine had little effect on the activity of cephalothin. In addition, we failed to get reproducible effects with either glutathione or dithiothreitol.

The antimicrobial susceptibility pattern of *C. difficile* has been determined in several studies and indicates almost universal susceptibility to the penicillin antibiotics (3, 4, 6). In contrast, using a standard method for susceptibility testing of anaerobic bacteria, we found persistent and reproducible non-β-lactamase-mediated, high-level resistance to penicillin G, ampicillin, carbenicillin, and ticarcillin, but not to other groups of antibiotics. High-level resistance to the penicillins was seen on BHIA-S, but on none of five other media employed. Systematic elimination of various constituents of BHIA-S clearly revealed that such resistance correlated with the presence of small amounts of L-cysteine in the media. Indeed, the addition of L-cysteine to BA, MHA, and TSA in similar amounts resulted in a 4- to >128-fold increase in the average MICs for 15 strains of *C. difficile*. For reasons not entirely clear, no such increase was observed with WCA or when other reducing agents, such as glutathione or dithiothreitol, were used instead. No significant alterations in MICs were noted with systematic manipulation of cultural conditions.

The observation that L-cysteine antagonizes the antimicrobial activity of penicillin is not a new one. Atkinson and Stanley reported in 1943 that cysteine and related sulfhydryl-containing compounds inactivated penicillin G (1). They and others observed that such penicillin inactivation was accomplished by the opening of the β-lactam ring due to breakage of the amide linkage (1, 7). The practical implications of these observations have received scant attention over the years. Blazevic and Matsen reported that 28% of 69 strains of *Bacteroides* sp. (including 17 strains of *B. fragilis*) were resistant to 100 μg of carbenicillin per ml (2), whereas only 40% of 162 anaerobic bacteria tested by Tally et al. were inhibited by 128 μg of carbenicillin per ml (11). Penicillin G inhibited 90% of *B. fragilis* at a concentration of 32 μg/ml in the latter study (11). Sutter and Finegold found much lower MICs of carbenicillin against many anaerobes, but similar activity for penicillin G when compared with the previous studies, attributing the discrepancy in carbenicillin activity to the use by the other investigators of prereduced media containing cysteine (10). Our studies extend these observations to penicillin G, ampicillin, and ticarcillin, albeit against but one species of anaerobic bacteria. More recently Kahan et al. have described the inactivation of thienamycin by cysteine and other reducing agents (5). Whereas the mechanism of inactivation of certain penicillins by L-cysteine appears secondary to opening of the β-lactam ring, we observed that such inactivation occurred rapidly and completely at lower concentrations of antibiotic, but only partially at higher concentrations. Similar effects were seen with all the penicillins tested (ampicillin, carbenicillin, and ticarcillin).

Our observations may have some practical significance. First, care should be taken to insure that antibiotic susceptibility testing of anaerobic bacteria with penicillins is performed on media free of reducing substances such as L-cysteine. Second, in bioassays of various antibiotics one could

take advantage of the ability of L-cysteine to inactivate penicillins; L-cysteine could be used to "quench" the β-lactam and thus remove its contribution to the antibiotic effect.

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