ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1983, p. 936–940
0066-4804/83/120936-05$02.00/0

Vol. 24, No. 6

Cefoxitin Inactivation by *Bacteroides fragilis*

GEORGE J. CUCHURAL, JR., FRANCIS P. TALLY,* NILDA V. JACOBUS, PETER K. MARSH, AND JAMES W. MAYHEW

Infectious Disease Service, Tufts New England Medical Center Hospital, and Department of Internal Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

Received 3 June 1983/Accepted 28 September 1983

We have surveyed the susceptibility of 1,575 clinical isolates of the *Bacteroides fragilis* group of organisms to cefoxitin and eight other antimicrobial agents. Eleven isolates, 0.7% of the total, were highly cefoxitin resistant and had minimum inhibitory concentrations of ≥64 μg/ml. These isolates were also resistant to other beta-lactam antibiotics. Of 11 isolates, 4 were able to inactivate cefoxitin in broth cultures, as measured by microbiological and high-pressure liquid chromatography assays. Two distinct patterns of cefoxitin breakdown products were detected by high-pressure liquid chromatography analysis. The beta-lactamase inhibitors clavulanic acid and sulbactam failed to show synergism with cefoxitin. These data demonstrate that members of the *B. fragilis* group have acquired a novel resistance mechanism enabling them to inactivate cefoxitin.

Cefoxitin possesses excellent in vitro activity against the *Bacteroides fragilis* group of organisms (20) and has been shown to be efficacious in the treatment of mixed aerobic and anaerobic infections involving these pathogens (21). The *B. fragilis* group, which includes: *B. fragilis*, *Bacteroides distasonis*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, and *Bacteroides vulgatus*, is resistant to many commonly used antimicrobial agents including penicillins and cephalosporins. *Bacteroides uniformis* has recently been considered with the *B. fragilis* group because of similar susceptibilities. These organisms are known to possess beta-lactamases which inactivate most of the first and second generation cephalosporins and penicillins (18, 22). Of the currently available beta-lactam antibiotics, cefoxitin has the best in vitro activity (4, 19). The enhanced activity of cefoxitin against these pathogens has been attributed to the resistance of the drug to the beta-lactamases of *B. fragilis* (3, 5, 7, 11, 22). Although there have been rare reports documenting the inactivation of the drug, the most common reported mechanism of cefoxitin resistance in this group of organisms has been the failure of the drug to penetrate through the outer membrane (6).

Our laboratory has been the reference center for a multicenter study of the susceptibility of the *B. fragilis* group of organisms in the United States (19). During 1981 and 1982, we have surveyed the susceptibility of 1,575 clinical isolates from this group; 11 isolates were found to have cefoxitin minimal inhibitory concentrations (MICs) of ≥64 μg/ml. This report documents the ability of four isolates to inactivate cefoxitin.

MATERIALS AND METHODS

Antimicrobial agents. Standard powders were obtained from the following sources: cefoxitin from Merck Sharpe & Dohme, Rahway, N.J.; cefazolin, cephalothin, and moxalactam from Eli Lilly & Co. Indianapolis, Ind.; cefoperazone and sulbactam from Pfizer Pharmaceuticals, New York; cefotaxime from Hoechst Roussel Pharmaceuticals, Sommerville, N.J.; piperacillin from Lederle Laboratories, Pearl River, N.Y.; clindamycin from The UpJohn Co., Kalamazoo Mich.; metronidazole from Searle Laboratories, Chicago, Ill.; tetracycline, penicillin, and chloramphenicol from Sigma Pharmaceuticals, St. Louis, Mo.; and clavulanic acid from Beecham Pharmaceuticals, Bristol, Tenn.

Isolates. The *B. fragilis* strains were obtained from clinical isolates referred to the Tufts Anaerobe Laboratory (TAL) from November 1980 through December 1982. Species identification was performed by standard methods (8). Highly cefoxitin-resistant strains were defined as those isolates with an MIC of ≥64 μg/ml. All highly cefoxitin-resistant isolates possessed beta-lactamase activity as determined by a slide test with nitrocephin (17). Two cefoxitin-susceptible *B. fragilis* (TAL 2497 and JC101) were used as controls.

Media and incubation. The isolates were stored on anaerobic (prereduced) brain heart infusion (BHI) agar slants supplemented with yeast extract (5 mg/ml), vitamin K_1 (0.5 μg/ml), and hemin (5 μg/ml) (Scott Laboratory, Fiskeville, R.I.). Solid growth medium was brucella agar supplemented with defibrinated sheep blood (5%) and vitamin K_1 (1 μg/ml). Liquid growth medium was BHI broth supplemented with yeast extract (5 mg/ml), vitamin K_1 (0.5 μg/ml), and hemin (5 μg/ml) (BHIS). All anaerobic cultures were incubated in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) with an atmosphere consisting of 85% N_2, 10% H_2, and 5% CO_2.

Susceptibility testing. MICs were determined by agar

dilution and broth dilution (microtiter) methods, employing anaerobic chamber techniques as previously described (2, 20). Synergy studies of cefoxitin with clavulanic acid and sulbactam were performed as previously described (9).

Effect of inoculum size. The effect of an increase in the inoculum size on MICs for cefoxitin with TAL strains 2480, 3636, 3998, and 4170 were determined by the microtiter techniques, using BHIS and bacterial inocula of 10^4, 10^6, and 10^8 CFU/ml. The MICs, at the different inocula, were read after 48 h of incubation in an anaerobic chamber. The presence of an inoculum effect was defined as a fourfold or greater rise in the MIC when the inoculum density was raised from 10^4 to 10^8 CFU/ml.

Assay for residual cefoxitin. The isolates were grown overnight in BHIS broth and diluted into prereduced BHIS broth containing 100 μg/ml of cefoxitin to achieve ca. 10^8 CFU/ml. Controls consisted of uninoculated BHIS broth with and without cefoxitin, BHIS broth with cefoxitin inoculated with the two susceptible *B. fragilis*, and inoculated BHIS broth without cefoxitin, incubated under the same conditions. Samples from the test samples and controls were collected at 0 and 24 h. The samples were centrifuged at 8,000 rpm for 20 min to remove cells. The supernatant was collected and kept frozen at −20°C until the cefoxitin assays were performed. The percentage of cefoxitin remaining after 24 h of incubation was determined by both the bioassay and high-pressure liquid chromatography (HPLC) methods.

Microbiological assay of cefoxitin. A two-dimensional agar diffusion microbiological assay was performed with a highly susceptible strain of *Escherichia coli*, kindly supplied by Arnold Damian, Massachusetts Institute of Technology, Cambridge, Mass. The cefoxitin standards were prepared in BHIS broth. Each sample was assayed in triplicate, and care was taken to space out the wells to avoid overlapping zones of inhibition.

HPLC assay of cefoxitin. An Altex high-pressure liquid chromatograph (Beckman Instruments, Inc., Berkeley, Calif.) was used. The instrument was equipped with two model 110A pumps, a Rheodyne model 7125 injector (50-μl load loop), a model 420 Microprocessor System Controller, and a reverse-phase ultrasphere-octadecylsilyl column (15 cm by 4.6 mm inside diameter; 5 μm average particle size). A 3.0-cm C18 precolumn was used (Brownlee Labs, Santa Clara, Calif.). Eluates were monitored by absorbance of UV light at 260 nm for the detection of the beta-lactam ring and 234 nm for the detection of the thienyl-acetyl group present in the side-chain at the 7-beta position of cefoxitin (14). An isocratic mobile phase consisting of 17% methanol (J. T. Baker, Phillipsburg, N.J.) and 83% 0.01 M KH_2PO_4 was operated at 1.4 ml/min. A 50-μl volume of clarified broth culture supernatant was directly chromatographed. The elution volume of unaltered cefoxitin bearing both the 260- and the 234-nm chromophore was 11 ml. The elution volume of the 234-nm chromophore after microbial degradation was 14 ml. Interfering chromatographic bands were not detected at either elution volume. Only those peaks absent in the controls were considered to represent microbial breakdown products of cefoxitin. Cefoxitin concentration was quantified by absorbance (peak height) at 11 ml of elution volume with a wavelength of 260 nm. The correspondence between cefoxitin concentration and peak height had a correlation coefficient of >0.99 with $P < 0.05$.

RESULTS

The antimicrobial susceptibility of the 11 highly cefoxitin-resistant strains to eight other antimicrobial agents was determined (Table 1). Eight strains (TAL 2480, 3507, 3636, 3747, 3943, 3998, 4170, and 4201) were highly resistant to the other beta-lactam antibiotics. There were four isolates which inactivated cefoxitin (see below). High-level beta-lactam resistance was found in the four isolates (TAL 2480, 3636, 3998, and 4170) that could inactivate cefoxitin. An inoculum effect to cefoxitin could also be demonstrated with these inactivating strains. In addition, isolate TAL 2480 showed an inoculum effect to moxalactam and piperacillin (Table 2). Clavulanic acid and sulbactam were not synergistic with cefoxitin against any of these four strains. All 11 isolates were susceptible to chloramphenicol and metronidazole. There was no correlation between tetracycline or clindamycin resistance and beta-lactam resistance.

TABLE 1. Isolate susceptibility MIC (μg/ml)[a]

TAL isolate no.	Species	CFX	MOX	CTX	CPZ	PIP	TET	CLN
2480	*B. fragilis*	128	>128	>128	>128	>128	2.0	>256.0
3507	*B. ovatus*	64	64	64	64	256	0.25	≤0.25
3533	*B. ovatus*	64	128	64	32	8	1.0	0.5
3636	*B. fragilis*	64	256	>256	>256	>128	16.0	>256.0
3706	*B. distasonis*	64	32	4	32	8	0.5	0.5
3747	*B. fragilis*	64	64	128	>128	>128	32.0	≤0.25
3943	*B. uniformis*	64	16	128	64	64	32.0	>256.0
3998	*B. ovatus*	128	>128	>128	64	>128	0.5	≤0.25
4170	*B. fragilis*	64	>128	>128	>128	>128	32.0	≤0.25
4201	*B. fragilis*	64	128	>128	128	>128	≤0.25	≤0.25
4266	*B. fragilis*	64	32	32	16	32	4.0	≤0.25

[a] Performed by the agar dilution method. CFX, Cefoxitin; MOX, moxalactam; CTX, cefotaxime; CPZ, cefoperazone; PIP, piperacillin; TET, tetracycline; CLN, clindamycin.

TABLE 2. Inoculum effect of TAL 2480

Antibiotic	Low (6×10^4)	Medium (6×10^6)	High (6×10^8)
Cefoxitin	64[a]	128	256
Moxalactam	64	128	>256
Piperacillin	64	128	>256

[a] MIC determined by the microtiter method in μg/ml.

When the four strains that could inactivate cefoxitin were incubated with the drug, very low or undetectable levels of the drug remained after 24 h (Table 3). Strains 4201 and 4266 partially inactivated cefoxitin, whereas the remaining five cefoxitin-resistant and two cefoxitin-susceptible isolates did not appear to degrade the compound when compared to uninoculated control broths containing the drug. Approximately 90% of the cefoxitin dose could be recovered from the controls after 24 h of incubation.

The appearance of cefoxitin breakdown products was determined by HPLC (Fig. 1). The left two panels, A and C, are chromatograms of cefoxitin diluted in BHIS broth monitored, respectively, at 260 and 234 nm. Panels B and D are chromatograms of the sample after 24 h of incubation with strain TAL 2480. Only trace amounts of the cefoxitin band could be detected at either wavelength after 24 h. In panel D, a new chromatographic band was detected with an elution volume of 14 ml and with absorbance at 234 but not 260 nm. This suggests the absence of an intact beta-lactam ring structure in the product. Inactivation of cefoxitin with strains TAL 3636 and 4170 gave similar results. Although 3998 readily degrades the drug, no analogous product could be found. This 14-ml eluting product peak was not found in incubations with the remaining cefoxitin-resistant isolates, the susceptible control isolates, or with the drug in uninoculated broth.

TABLE 3. Cefoxitin inactivation

TAL isolate no.	% Cefoxitin remaining after 24 h of incubation with resistant isolates[a]	
	Bioassay[b]	HPLC
2480	<1	<1
3507	100	71
3533	100	96
3636	<1	<1
3706	93	98
3747	93	66
3943	100	86
3998	<1	2
4170	7	17
4201	67	38
4266	68	49
Control	90	93

[a] Initial cefoxitin concentration was 100 μg/ml.
[b] Interassay correlation coefficient = 0.96.

DISCUSSION

Many of the cephalosporin antimicrobial agents can be degraded by a variety of cephalo-

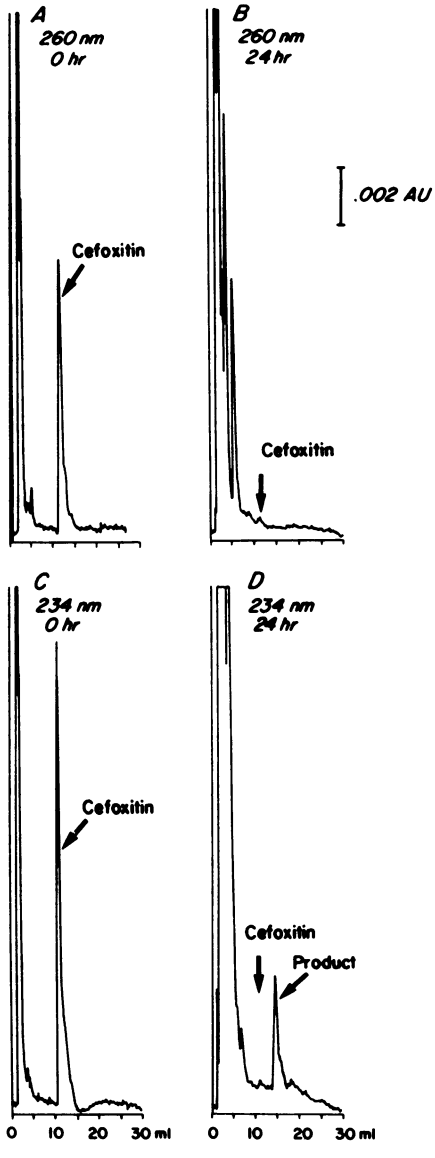

FIG. 1. HPLC of BHIS broth containing cefoxitin and incubated with TAL 2480. (A and C) The chromatograms at the start of incubation; (B and D) chromatograms after 24 h. (A and B) Monitored at 260 nm; (C and D) monitored at 234 nm. AU, Absorbance units.

sporinases elaborated by many species of aerobic and anaerobic bacteria. Cefoxitin, however, has shown high-level resistance to such microbial decomposition by these enzymes. In our studies, we have provided the first demonstration, by both biological and HPLC means, of the inactivation of cefoxitin by four strains of the *B. fragilis* group of organisms. The data suggests a beta-lactamase-mediated mechanism for this inactivation. The HPLC analysis was consistent with the disappearance of the beta-lactam ring and the appearance of a new product with the characteristics of the thienyl ring. Since at least two distinct cefoxitin breakdown patterns were observed, one for strains 2480, 3636, and 4170 and another for 3998, there may be multiple mechanisms for cefoxitin metabolism or product rearrangement with *B. fragilis*. In our chromatographic studies, we limited detection to the UV-absorbing beta-lactam and thienyl rings. Highly polar metabolites which might elute extremely rapidly or fragments without UV-absorbing chromophore would not be detected by these methods.

Multiple breakdown products may be found after enzymatic disruption of the beta-lactam nucleus of cephalosporins (13). In contrast, penicillins are usually converted to a single stable product, i.e., penicilloic acid, by penicillinase (13). More advanced analytical techniques, i.e., mass spectroscopy or nuclear magnetic resonance, will be needed to further define the chemical structure of the cefoxitin breakdown products.

Although we do not know the structure of the 14-ml eluting cefoxitin metabolite, the HPLC and absorbance characteristics suggest certain properties. The product is probably less polar than the parent molecule since it has a longer retention time on the reverse phase column. The presence of the 234-nm chromophore implies that the fragment contains the thienyl ring which in intact cefoxitin is linked to the 7-position of the beta-lactam ring.

Beta-lactamases are the most important mechanism of transferable beta-lactam antibiotic resistance. Our data indicate that, fortunately, the frequency of isolates which inactivate cefoxitin is low, i.e., 4 out of 1,575. However, the potential for an increase in the number of isolates exists through continued selection pressure of cefoxitin use and the efficient genetic system to transfer genes coding for antimicrobial resistance possessed by the *B. fragilis* group. Several of these resistance transfer systems have been described. Clindamycin-erythromycin and tetracycline resistance transfer have been extensively studied (12). The transfer of high-level ampicillin resistance has been previously reported (T. Butler, F. P. Tally, S. L. Gorbach, and M. H. Malamy, 1980. Clin. Res. 28:365A.). They demonstrated that the transfer of this resistance was associated with the acquisition by the recipient of the beta-lactamase from the highly resistant donor. Sato and colleagues were also able to demonstrate transfer of the beta-lactamase which resulted in high-grade penicillin resistance (18). This enzyme had little cephalosporinase activity, whereas it readily inactivated penicillins including piperacillin, azlocillin, and mezlocillin (18). This is uncharacteristic of commonly described beta-lactamases of the *B. fragilis* group which inactivate cephalosporins at higher rates than penicillins and represents the acquisition of a qualitatively different resistance mechanism transfer capability (18, 22). Finally, cefoxitin resistance transfer has been reported in a strain of *B. thetaiotaomicron* by Rashtchian et al. (16). However, the mechanism of the resistance in this isolate was not determined.

The inactivation of cefoxitin, which is more pronounced at high inocula, is clinically important. Anaerobic abscesses typically contain high bacterial density, i.e., 10^7 to 10^8 organisms per ml. Thus, therapeutic levels of the drug may not be achieved in the abscess owing to high levels of beta-lactamase activity. This has previously been demonstrated with compounds such as penicillin, cefazolin, and cephalothin in experimental in vivo models (1, 10, 15). As a case in point, the patient harboring strain 2480 died from the anaerobic infection caused by this organism having failed to respond to treatment with clindamycin and cefoxitin.

Fortunately, metronidazole and chloramphenicol were found to be consistently active against these cefoxitin-resistant strains. However, if one of these agents is not used for therapy, we feel susceptibility testing should be performed on *B. fragilis* isolates obtained from seriously ill patients and from those failing standard therapy. The recognition of this new mechanism of cefoxitin resistance in *B. fragilis* coupled with the recent description of a new penicillinase in *Bacteroides* spp. by Sato et al. (18) indicates that continued surveillance of the *B. fragilis* group of organisms for changes in resistance patterns is warranted.

ACKNOWLEDGMENTS

F.P.T. is the recipient of Research Career Development Award AI00393 from the National Institute of Allergy and Infectious Diseases. We thank Susan Cooper, June Cox, and Joanne Di Pasqua for their excellent technical assistance.

This study was supported in part by a grant from the National Institutes of Allergy and Infectious Diseases AI15389.

LITERATURE CITED

1. **Bartlett, J. G., M. Dezfulian, and K. Joiner.** 1983. Relative efficacy and critical interval of antimicrobial agents in experimental infections involving *Bacteroides fragilis*.

Arch. Surg. 118:181–184.
2. **Barza, M., F. P. Tally, N. V. Jacobus, and S. L. Gorbach.** 1979. In vitro activity of LY 127935. Antimicrob. Agents Chemother. 16:287–292.
3. **Brown, J. E., V. E. Del Bene, and C. D. Collins.** 1981. In vitro activity of *N*-formimidoyl thienamycin, moxalactam, and other new beta-lactam agents against *Bacteroides fragilis*: contribution of beta-lactamase to resistance. Antimicrob. Agents Chemother. 19:248–252.
4. **Cuchural, G. J., Jr., N. V. Jacobus, S. L. Gorbach, and F. P. Tally.** 1981. A survey of *Bacteroides* susceptibility in the United States. J. Antimicrob. Chemother. 8(Suppl. D):27–31.
5. **Darland, G., and J. Birnbaum.** 1977. Cefoxitin resistance to beta-lactamase a major factor for susceptibility of *Bacteroides fragilis* to the antibiotic. Antimicrob. Agents Chemother. 11:725–734.
6. **Dornbusch, K., B. Olsson-Liljequist, and C. E. Nord.** 1980. Antibacterial activity of new beta-lactam antibiotics on cefoxitin-resistant strains of *Bacteroides fragilis*. J. Antimicrob. Chemother. 6:207–216.
7. **Eley, A., and D. Greenwood.** 1981. In vitro activity of ceftizoxime against *Bacteroides fragilis*: comparison with benzylpenicillin, cephalothin, and cefoxitin. Antimicrob. Agents Chemother. 20:332–335.
8. **Holdeman, L. V., E. P. Cato, and W. E. Moore.** 1977. Anaerobic laboratory manual, 4th Edition, Virginia Polytechnic Institute, Blacksburg, Va.
9. **Jacobus, N. V., M. C. Ferreira, and M. Barza.** 1982. In vitro activity of arthreonam, a monobactam antibiotic. Antimicrob. Agents Chemother. 22:832–838.
10. **Joiner, A., B. R. Lowe, J. L. Dzink, and J. G. Bartlett.** 1981. Antibiotic levels in infected and sterile subcutaneous abscesses in mice. J. Infect. Dis. 143:487–494.
11. **Leung, T., and J. D. Williams.** 1978. Beta-lactamases of the subspecies of *Bacteroides fragilis*. J. Antimicrob. Chemother. 4:47–54.
12. **Malamy, M. H., and F. P. Tally.** 1981. Mechanisms of drug resistance transfer in *Bacteroides fragilis*. J. Antimicrob. Chemother, 8(Suppl. D):59–75.
13. **Neu, H., and V. Lorian.** 1980. Antibiotics in laboratory medicine 1980. First edition, The Williams & Wilkins Co., Baltimore.
14. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. Shingler.** 1972. Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. 1:283–288.
15. **O'Keefe, J. P., F. P. Tally, M. Barza, and S. L. Gorbach.** 1978. Inactivation of penicillin G during experimental infection. J. Infect. Dis. 137:437–442.
16. **Rashtchian, A., G. R. Dubes, and S. J. Booth.** 1982. Transferable resistance to cefoxitin in *Bacteroides thetaiotaomicron*. Antimicrob. Agents Chemother. 22:701–703.
17. **Rosenblatt, J. E.** 1980. Antimicrobial susceptibility testing of anaerobes, p. 129. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
18. **Sato, K., Y. Matsuura, M. Inoue, and S. Mitsuhashi.** 1982. Properties of a new penicillinase type produced by *Bacteroides fragilis*. Antimicrob. Agents Chemother. 22:579–584.
19. **Tally, F. P., G. J. Cuchural, N. V. Jacobus, S. L. Gorbach, K. E. Aldridge, T. J. Cleary, S. M. Finegold, G. B. Hill, P. B. Iannini, R. V. Mc Closkey, J. P. O'Keefe, and C. L. Pierson.** 1983. Susceptibility of the *Bacteroides fragilis* group in the United States in 1981. Antimicrob. Agents Chemother. 23:536–540.
20. **Tally, F. P., N. V. Jacobus, J. G. Bartlett, and S. L. Gorbach.** 1975. Susceptibility of anaerobes to cefoxitin and cephalosporins. Antimicrob. Agents Chemother. 7:128–132.
21. **Tally, F. P., K. McGowan, J. M. Kellum, S. L. Gorbach, and T. F. O'Donnell.** 1981. A randomized comparison of cefoxitin with or without amikacin and clindamycin plus amikacin in surgical sepsis. Ann. Surg. 193:318–323.
22. **Tally, F. P., P. O'Keefe, N. M. Sullivan, and S. L. Gorbach.** 1979. Inactivation of cephalosporins by *Bacteroides*. Antimicrob. Agents Chemother. 18:565–571.