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Activity of Cefoperazone and Two β-Lactamase Inhibitors, Sulbactam and Clavulanic Acid, Against *Bacteroides* spp. Correlated with β-Lactamase Production

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A total of 102 isolates of *Bacteroides* spp. were studied for β-lactamase production and susceptibility to cefoperazone alone or in combination with either of the β-lactamase inhibitors sulbactam and clavulanic acid. The geometric mean minimal inhibitory concentration of cefoperazone alone was 31.5 μg/ml and when combined with 10 μg of sulbactam per ml or 2 μg of clavulanic acid per ml was reduced to 5.4 and 9.2 μg/ml, respectively. When bacterial suspensions were tested for β-lactamase production with nitrocefin, 91 (89.2%) of these isolates produced the enzyme. The geometric mean minimal inhibitory concentrations of cefoperazone rose only slightly for isolates with low or intermediate enzyme activity but rose significantly for those with high activity. The addition of EDTA to cefoperazone significantly more frequently enhanced the activity of cefoperazone against β-lactamase-negative as opposed to β-lactamase-positive isolates. Furthermore, EDTA resulted in synergistic activity of the cefoperazone-sulbactam combination on β-lactamase-positive isolates for which the combination had previously not shown a synergistic effect. This study demonstrates the relationship between β-lactamase production and the resistance of *Bacteroides* spp. to cefoperazone and shows that inhibition of these enzymes can reverse this resistance.

The production of β-lactamase by members of the *Bacteroides fragilis* group, especially those having cephalosporinase activity, is well documented (5, 7, 16). A number of studies have correlated enzyme production with resistance to a variety of antimicrobial agents, including cephalosporins (5, 7, 19, 21). However, investigators have also reported the resistance of β-lactamase-negative isolates of *Bacteroides* spp. to β-lactams (16, 18–20, 27). The resistance of β-lactamase-negative bacteria to β-lactams is believed to be due in part to a permeability barrier to the antibiotic (23).

Recently, β-lactamase inhibitors have been utilized in conjunction with β-lactam antibiotics to reverse the resistance resulting from the production of β-lactamase (6). Sulbactam (CP-45,899), a penicillanic acid sulfone (8), and clavulanic acid, a naturally occurring β-lactam isolated from *Streptomyces clavuligerus* (22), are examples of two such inhibitors.

The present study was carried out to determine (i) the efficacy of cefoperazone against various species of *Bacteroides*, (ii) the correlation of resistance with β-lactamase production, (iii) the ability of sulbactam and clavulanic acid to reverse this resistance, and (iv) the effect of EDTA, an agent known to increase the permeability of gram-negative cell walls (13, 14, 28), on the resistance of some of these *Bacteroides* isolates.

MATERIALS AND METHODS

Drugs. Cefoperazone and sulbactam were gifts from Pfizer Inc., New York, N.Y. Clavulanic acid was a gift from Beecham Laboratories, Bristol, Tenn. Appropriate dilutions of all of these compounds in Sorensen phosphate buffer (pH 7.0, 66 mM) were performed on the day that they were used to ensure that they were optimally active. Clavulanic acid solutions were used within 1 h after they were prepared.

Bacterial isolates. One hundred *B. fragilis* group isolates and two *B. melaninogenicus* isolates were used in these studies. In the *B. fragilis* group the following species were represented: *B. fragilis*, 42 isolates; *B. thetaiotaomicron*, 27 isolates; *B. distasonis*, 12 isolates; *B. ovatus*, 10 isolates; and *B. vulgatus*, 9 isolates. Isolates were obtained from the following sources: 25 from Nancy Hodinka, Anaerobic Laboratory of Analytab Products, Plainview, N.Y.; 7 from Andrew Onderdonk, Tufts School of Veterinary

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Medicine, Boston, Mass.; and 3 from Arthur E. Girard, Pfizer Inc. The remaining 67 were isolated in our laboratory or the clinical laboratory of the Medical Center Hospital of Vermont, Burlington. Isolates were identified by the criteria of the *Wadsworth Anaerobic Bacteriology Manual* (26) and the Virginia Polytechnic Institute and State University *Anaerobe Laboratory Manual* (10).

Susceptibility testing. All agar dilution minimal inhibitory concentrations (MICs) were performed by the proposed method of the National Committee for Clinical Laboratory Standards, Villanova, Pa., for susceptibility testing of anaerobes (15). The MIC for inhibition of an isolate was considered to be the minimum concentration of drug which completely inhibited the growth of the isolate. *B. fragilis* ATCC 23745 was included in each susceptibility run, and generally the MIC required for the inhibition of this isolate was 12.5 μg/ml, with a range of 6.3 to 25 μg/ml. To achieve a final bacterial inoculum of 10^4 colony-forming units, the inoculum was adjusted to equal the density of one-half the density of a McFarland no. 1 standard (2). Adjusting the density of an overnight culture to equal that of the above McFarland standard yielded a cell density of 10^7 colony-forming units per ml, which would yield a final inoculum of 10^4 colony-forming units, since a Steers replicator (25) deposits approximately 0.001 ml of inoculum on the agar surface. Inoculation of plates was performed in room atmosphere, whereas incubations were performed in an anaerobic glove box at 35°C with an atmosphere of 5% CO_2–10% H_2–85% N_2. All plates were read after 48 h of incubation.

Cefoperazone was tested in doubling dilutions at concentrations from 0.39 to 800 μg/ml and combined with sulbactam at the following concentrations: 0.5, 1.0, 5.0, and 10.0 μg/ml. Sulbactam was also tested alone at concentrations of 5, 10, 20, and 40 μg/ml. Clavulanic acid was tested alone at concentrations of 0.5, 1, and 2 μg/ml and combined with cefoperazone at these same concentrations. Only 29 of the 102 isolates were tested against the combination of cefoperazone and clavulanic acid. These were randomly selected from β-lactamase-producing organisms. Synergism between sulbactam and cefoperazone or cefoperazone and clavulanic acid was deemed to have occurred when the cefoperazone MIC for inhibition of an organism was reduced fourfold by the addition of the highest concentration of the β-lactamase inhibitor that was tested. The ratio of the cefoperazone MIC to the cefoperazone MIC plus the β-lactamase inhibitor was defined as the potentiation ratio, and any ratio of 4 or greater was considered to represent synergism.

For the experiments with EDTA, four randomly selected *Bacteroides* isolates were tested against increasing concentrations of EDTA, and the minimum concentration (2 mM) of EDTA which did not inhibit the growth of any of the isolates was determined. In all subsequent experiments with EDTA, this concentration of EDTA was used and was not found to be inhibitory for any of the other *Bacteroides* isolates used.

β-Lactamase detection. β-Lactamase production was determined on all suspensions by a slight modification of the technique used by Kammer et al. (12). The chromogenic cephalosporin nitrocefin (17) was used as the indicator substrate. Nitrocefin was a gift from Group Research Ltd., Glaxo, Greenford, Middlesex, England. Four brucella agar plates supplemented with vitamin K and hemin were inoculated to yield confluent growth. The bacteria were scraped from the surface after 18 h of incubation and suspended in 5.0 ml of sterile phosphate-buffered saline (pH 7.0). A 0.05-ml amount of this suspension was placed in a microtiter well along with 0.05 ml of nitrocefin. Each set of determinations included a negative control, which consisted of only buffer and nitrocefin. The plate was covered with Parafilm and incubated at room temperature (22 to 25°C). Readings for color change were performed at 30 min and 18 h. A modification of the semiquantitative rating scheme of Olsson et al. (18) was used to divide the organisms into one of four categories based on enzyme production: 0, no color change at 18 h; 1, orange at 18 h; 2, red at 18 h; and 3, red at 30 min. β-Lactamase-negative cell suspensions, as determined by the above method, were subjected to ultrasonic disruption with a Branson W185D Sonifier equipped with a microtip (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The power was set at 75 W and delivered in three bursts each of 30-s duration with 15-s cooling intervals, and sonification was carried out in a slush ice bath. The enzyme activity of β-lactamase-producing isolates was not altered by identical sonication conditions. Also, β-lactamase induction was attempted by growing β-lactamase-negative isolates on brucella agar plates containing subinhibitory concentrations of cefoperazone. β-Lactamase determinations were then carried out as described above.

RESULTS

A total of 91 (89.2%) of 102 *Bacteroides* isolates tested by the nitrocefin technique produced β-lactamase (Table 1). However, the majority of these isolates produced only moderate amounts of enzyme. Thus, 81 (79.4%) of the isolates fell into categories 1 and 2 of β-lactamase production, and only 10 (9.8%) fell into category 3. Of the 11 β-lactamase-negative isolates, none could be shown to elaborate enzyme after either induction or sonication. Of 12 *B. distasonis* isolates, 5 (41.7%) were negative for β-lactamase, which was the highest percentage of any of the six species. Also shown in Table 1 is the susceptibility of these six species of *Bacteroides* to cefoperazone.

Table 2 shows the cefoperazone geometric mean MICs by species, as well as the effect of increasing concentrations of sulbactam on the activity of cefoperazone. *B. fragilis* isolates were the most susceptible (geometric mean MIC, 23.0 μg/ml) and were the only group whose geometric mean MIC of cefoperazone for inhibition of growth was below that of all 102 isolates (geometric mean MIC, 31.5 μg/ml). It is evident that sulbactam acted to reduce the MICs of cefoperazone for inhibition of all species but was least effective for the *B. distasonis* and *B. melaninogenicus* isolates. The MIC of sulbactam at which 90% of the isolates were inhibited

TABLE 1. Production of β-lactamase and susceptibility of *Bacteroides* spp. to cefoperazone

Species	No. of isolates	β-Lactamase production[a] (no. of isolates)				Susceptibility to cefoperazone (μg/ml)		
		0	1	2	3	MIC_{50}	MIC_{90}	Range
B. fragilis	42	5	11	21	5	25	50	3.12–800
B. thetaiotaomicron	27	0	10	15	2	50	50	6.25–100
B. distasonis	12	5	2	4	1	25	50	12.5–50
B. ovatus	10	1	0	8	1	50	100	25–100
B. vulgatus	9	0	4	5	0	25	50	25–100
B. melaninogenicus	2	0	0	1	1	50	100	50–100

[a] Categories defined in the text.

(MIC_{90}) was 40 μg/ml. No isolates were susceptible to this compound at 10 μg/ml.

Table 3 shows the geometric mean MICs of cefoperazone for inhibition of all 102 *Bacteroides* isolates in relation to β-lactamase production. The lowest geometric mean MIC (20.7 μg/ml) of cefoperazone was seen in β-lactamase-negative organisms, slightly higher geometric mean MICs were seen in organisms which produced intermediate amounts of β-lactamase, and the highest geometric mean MIC (81.3 μg/ml) was seen in the organisms which produced the most β-lactamase. The geometric mean MIC (81.3 μg/ml) of cefoperazone for inhibition of the category 3 organisms was significantly ($F[3,98] = 10.2$; $P < 0.001$) higher than the geometric mean MIC of cefoperazone for inhibition of the other three groups.

Table 3 also shows the geometric mean MICs of cefoperazone when combined with four different concentrations (0.5, 1, 5, and 10 μg/ml) of sulbactam. It is evident that increasing the concentration of the β-lactamase inhibitor progressively lowered the geometric mean MICs. The most dramatic reduction in geometric mean MICs was seen with organisms belonging to the category 3 β-lactamase producers; the geometric mean MIC of cefoperazone was reduced from 81.3 to 4.7 μg/ml by the addition of 10 μg of sulbactam per ml. The comparable figures for those isolates not producing β-lactamase were 20.7 and 7.5 μg/ml, respectively. Surprisingly, sulbactam significantly potentiated the activity of cefoperazone against five isolates not producing any detectable β-lactamase.

Table 4 shows the effect of increasing concentrations of clavulanic acid on the activity of cefoperazone against 29 isolates of the *B. fragilis* group. Marked potentiation of the activity of cefoperazone was noted regardless of the amount of β-lactamase produced by these isolates. Clavulanic acid was not inhibitory when tested alone at the concentrations used in the synergy study.

Figure 1 depicts the cumulative percentage of inhibition of all 102 *Bacteroides* isolates to increasing concentrations of cefoperazone alone, as well as combined with three concentrations of sulbactam. Again, it was evident that the highest concentration (10 μg/ml) of sulbactam most effectively potentiated the activity of cefoperazone.

Figure 2 shows the effect of three concentrations, 0.5, 1.0, and 2 μg/ml, of clavulanic acid in potentiating the effect of cefoperazone on 29 *Bacteroides* isolates. It was evident that all three concentrations of clavulanic acid enhanced the activity of cefoperazone and that generally 2.0 μg/ml was the most effective concentration. Not shown in Fig. 2 was the fact that of these 29

TABLE 2. Geometric mean MICs of cefoperazone alone and combined with increasing concentrations of sulbactam

Species	No. of isolates	Geometric mean MIC (μg/ml) with the following sulbactam concn (μg/ml):				
		0	0.5	1.0	5.0	10.0
B. fragilis	42	23.0	8.3	5.4	4.8	2.9
B. thetaiotaomicron	27	41.8	22.5	21.4	14.6	8.3
B. distasonis	12	33.3	25.0	22.3	18.7	14.0
B. ovatus	10	43.6	15.4	14.4	13.4	4.7
B. vulgatus	9	31.5	25.0	13.5	15.7	9.2
B. melaninogenicus	2	70.6	70.6	70.6	70.6	35.3
All isolates	102	31.5	14.2	11.7	9.3	5.4

TABLE 3. Geometric mean MICs for β-lactamase production categories 0 to 3 of cefoperazone alone and combined with increasing concentrations of sulbactam

β-Lactamase production category[a]	No. of isolates	Geometric mean MIC (μg/ml) with the following sulbactam concn (μg/ml):				
		0	0.5	1.0	5.0	10
0	11	20.7	13.3	13.3	10.3	7.5
1	27	32.3	13.5	12.5	11.6	7.5
2	54	28.4	13.5	10.7	8.3	4.4
3	10	81.3	23.3	13.4	8.8	4.7
All isolates	102	31.5	14.2	11.7	9.3	5.4

[a] Categories defined in the text.

isolates, the addition of 10 μg of sulbactam to cefoperazone resulted in synergism for 19 (65.5%) isolates. The comparable figure for the same 29 isolates with 2 μg of clavulanic acid per ml was 21 (72.4%). Curiously, only the combination of sulbactam and cefoperazone was synergistic for four isolates, and only the combination of clavulanic acid and cefoperazone was synergistic for six other isolates.

To test the hypothesis that the basis for the resistance of some of the β-lactamase-negative isolates to cefoperazone was due to a permeability barrier to the antimicrobial agent, the combination of 2 mM EDTA and cefoperazone was tested against 11 β-lactamase-negative and 19 β-lactamase-positive isolates randomly selected from the *B. fragilis* group. A total of 7 (63.6%) of 11 β-lactamase-negative isolates demonstrated a fourfold or greater reduction in cefoperazone MIC with EDTA added as contrasted with only 3 (15.8%) of 19 β-lactamase-positive isolates (Fig. 3). This difference is statistically significant ($\chi^2 = 4.98$; $P < 0.05$).

The effectiveness of cefoperazone against 12 β-lactamase-positive isolates was not enhanced by the addition of sulbactam. This group included 10 isolates belonging to the *B. fragilis* group and the 2 *B. melaninogenicus* isolates. It was theorized that a permeability barrier to cefoperazone or sulbactam or both might exist to explain this lack of synergy. For this reason, EDTA was again employed to increase cell wall permeability. Initial studies showed that neither EDTA alone nor EDTA combined with 10 μg of sulbactam per ml had any effect on these 12 isolates. In the presence of EDTA, the combination of cefoperazone and sulbactam was synergistic for 11 (91.7%) of these 12 isolates (Fig. 4). Two isolates were included for which the combination of cefoperazone and sulbactam was previously shown to be synergistic. The addition of EDTA did not further enhance the activity of cefoperazone and sulbactam against these two isolates.

DISCUSSION

The production of β-lactamase by 89% of the 100 isolates of the *B. fragilis* group in this study is very similar to the figure of 90% determined by Olsson et al. (19) for 231 *Bacteroides* isolates and 82% determined by Brook et al. (4) for 65 similar isolates. In light of the fact that 89% of the *B. fragilis* group of organisms that we studied elaborated a cephalosporinase, it is not surprising that cefoperazone alone was not particularly active against this group of anaerobes. Our results are similar to those of Jacobus et al. (11), who observed an MIC_{90} of 32 μg of cefoperazone per ml for inhibition of 86 isolates of the *B. fragilis* group as compared with our figure of 50 μg/ml. Brown et al. (5) observed an even higher MIC_{90} (256 μg/ml) of cefoperazone for inhibition of 100 isolates of *B. fragilis*.

TABLE 4. Geometric mean MICs for β-lactamase production categories 0 to 3 of cefoperazone alone and combined with increasing concentrations of clavulanic acid against 29 isolates of the *B. fragilis* group

β-Lactamase production category[a]	No. of isolates	Geometric mean MIC (μg/ml) with the following clavulanic acid concn (μg/ml):			
		0	0.5	1.0	2.0
1	11	25.5	8.5	13.3	9.1
2	13	27.8	10.1	9.0	8.2
3	5	100.0	12.4	14.4	12.4
All isolates	29	36.6	12.2	11.4	9.2

[a] Categories defined in the text.

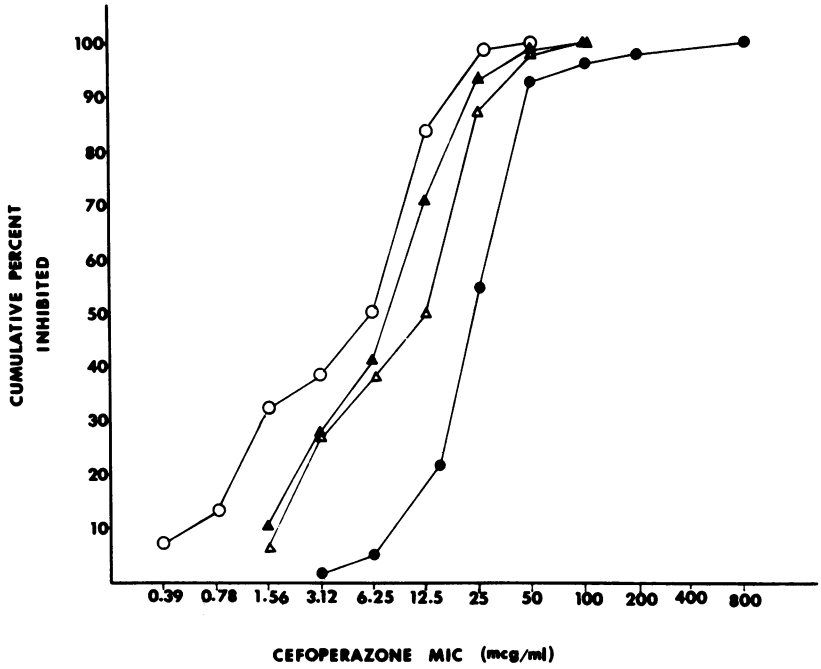

FIG. 1. Cumulative percent of 102 *Bacteroides* isolates inhibited by cefoperazone (●), cefoperazone and 1.0 μg of sulbactam per ml (△), cefoperazone and 5.0 μg of sulbactam per ml (▲), and cefoperazone and 10 μg of sulbactam per ml (○). Not shown are the data for 0.5 μg of sulbactam per ml since this curve was so similar to that obtained with a concentration of 1 μg of sulbactam per ml.

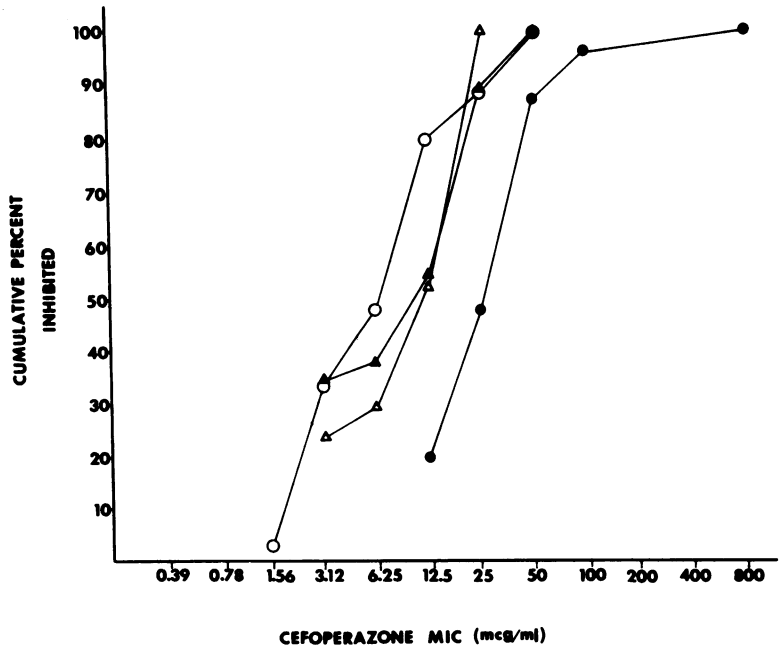

FIG. 2. Cumulative percent of 29 *Bacteroides* isolates inhibited by cefoperazone (●), cefoperazone and 0.5 μg of clavulanic acid per ml (△), cefoperazone and 1.0 μg of clavulanic acid per ml (▲), and cefoperazone and 2.0 μg of clavulanic acid per ml (○).

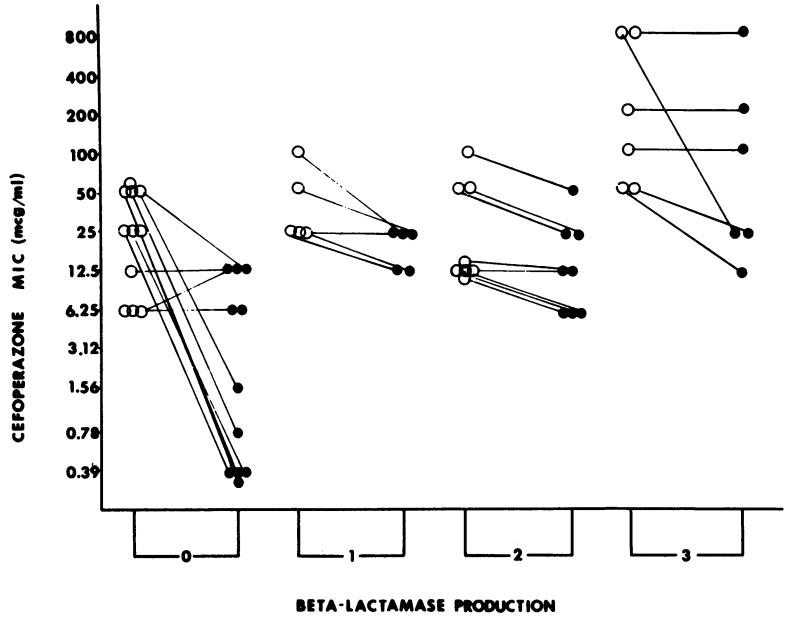

FIG. 3. Reduction of cefoperazone MICs with EDTA for organisms belonging to all four categories of β-lactamase production. These categories are defined in the text. Shown are cefoperazone MICs without EDTA (○) and MICs with EDTA (●).

The relationship of β-lactamase production and resistance of these isolates to cefoperazone was made apparent by the marked increase in the geometric mean MIC (81.3 μg/ml) of cefoperazone for inhibition of isolates producing large amounts of β-lactamase compared with the geometric mean MIC (20.7 μg/ml) of cefoperazone for inhibition of β-lactamase-negative isolates. Similarly, Brown et al. (5) related enzyme production to resistance to cefoperazone, and Sato et al. (24) showed that β-lactamase obtained from a *B. fragilis* isolate readily hydrolyzed cefoperazone.

Our data indicate that the β-lactamase elaborated by the *B. fragilis* group of organisms is adequately inhibited by either of the β-lactamase inhibitors which we studied. These findings are consistent with the data of English et al. (8), who showed that sulbactam at a concentration of 4 μg/ml inhibited 94% of the activity of the cephalosporinase derived from an isolate of *B. fragilis*. With concentrations of either sulbactam or clavulanic acid that can readily be obtained in the serum of humans, our data indicated that 90% or more of the 102 *Bacteroides* isolates which we tested were susceptible to 25 μg of cefoperazone per ml; without the β-lactamase inhibitors, the MIC_{90} was 50 μg/ml. It would thus appear that the activity of cephalosporins with modest activity against β-lactamase-producing *Bacteroides* spp. could be greatly enhanced by the addition of agents which inhibit these hydrolyzing enzymes. Furthermore, both sulbactam and clavulanic acid appeared to function similarly, although one inhibitor or the other was uniquely effective for some isolates (10 [34.5%] of 29 isolates tested with both inhibitors). The differential efficacy of these inhibitors raised the following questions. (i) Are there a variety of β-lactamases produced which are inhibited to a different degree by the two inhibitors? (ii) Are the factors which govern the permeability of substances across the cell wall selective for one or the other of the inhibitors?

It is puzzling why there was a modest degree of synergy demonstrated by the cefoperazone-sulbactam combination for 5 of the 11 enzyme-negative isolates. Aswopokee and Neu (1) reported similar findings; they found that ampicillin and sulbactam were synergistic against some strains of apparently β-lactamase-negative *B. fragilis*. However, they might not have detected β-lactamase production by some of their isolates due to a short incubation time (5 min) with nitrocefin. An incubation period of 30 min detected enzyme production in only 10 (9.8%) of our 102 isolates.

Yokota et al. (Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 602, 1980) showed that sulbactam bound to a penicillin-binding protein of *Escherichia coli*. If this is also true of *Bacteroides* spp., it may be that in certain instances sulbactam may synergize with cefoperazone by

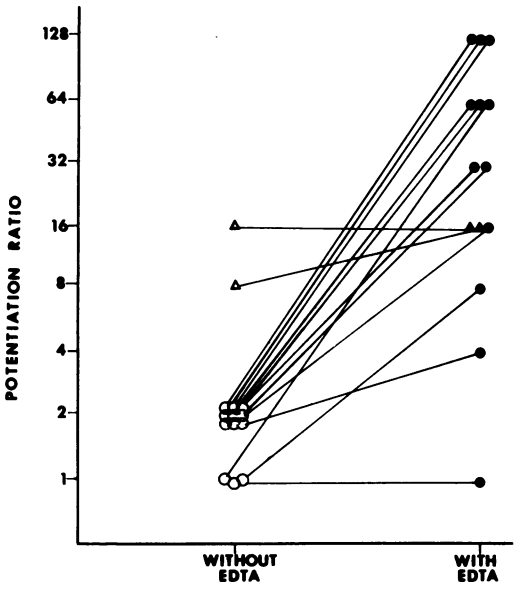

FIG. 4. Effect of EDTA on the action of cefoperazone and sulbactam for 12 isolates (○, without EDTA; ●, with EDTA) which produced β-lactamase but for which the cefoperazone-sulbactam combination was not synergistic. The potentiation ratio is calculated by dividing the MIC obtained with cefoperazone alone by the MIC obtained when cefoperazone was combined with 10 μg of sulbactam per ml. A value of 4 or greater was considered to represent synergism. Also included are two isolates (△, without EDTA; ▲, with EDTA) for which the cefoperazone-sulbactam combination was synergistic and which were included as controls.

potentiating the activity of the cephalosporin in interfering with cell wall synthesis. The fact that higher concentrations of sulbactam alone were inhibitory for some of these *Bacteroides* isolates is consistent with the hypothesis that sulbactam may also interfere with cell wall formation. Such a mechanism could explain the synergism that we observed in the case of isolates which could not be demonstrated to produce β-lactamase.

EDTA was used to increase the permeability of the cell wall in an attempt to assess the importance of impermeability of this wall in terms of antimicrobial resistance. Leive (13, 14) and Voll and Leive (28) have shown that exposure of bacteria to EDTA results in a loss of lipopolysaccharide, probably due to chelation of Mg^{2+}, from the cell wall and a resultant increase in the permeability of a variety of substances, including antibiotics (3, 29). The fact that EDTA was synergistic for a significantly greater proportion of β-lactamase-negative than β-lactamase-positive isolates and that the degree of potentiation was much greater in the case of the β-lactamase-negative as contrasted with the β-lactamase-positive isolates seems to indicate that impermeability of the cell is a major mechanism of resistance to cefoperazone of the β-lactamase-negative isolates. Finally, the fact that the addition of EDTA to cefoperazone and sulbactam resulted in synergism against isolates not previously affected in this manner by the combination again suggests a permeability block, but in this instance to sulbactam.

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