ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1993, p. 1061–1064
0066-4804/93/051061-04$02.00/0

Vol. 37, No. 5

Resistance to Cefoperazone-Sulbactam in *Klebsiella pneumoniae*: Evidence for Enhanced Resistance Resulting from the Coexistence of Two Different Resistance Mechanisms

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Received 9 December 1992/Accepted 9 March 1993

We investigated the in vitro activity and the in vivo efficacy of the β-lactam–β-lactamase inhibitor combination cefoperazone-sulbactam against an isogenic series of *Klebsiella pneumoniae* strains. Both cefoperazone and cefoperazone-sulbactam were active in vitro against a susceptible clinical strain, and the combination was highly effective in the treatment of rat intra-abdominal abscesses. Loss of expression of a 39-kDa outer membrane protein resulted in at least a fourfold increase in the MICs of cefoperazone and cefoperazone-sulbactam but did not appreciably affect the in vivo efficacy of either regimen. Introduction of plasmid RP4, which encodes the TEM-2 β-lactamase, into the susceptible strain resulted in the loss of in vitro activity and in vivo efficacy for cefoperazone. The in vitro activity of cefoperazone-sulbactam against this strain was diminished, but the antibiotic combination remained highly active in vivo. Introduction of RP4 into the strain lacking the 39-kDa outer membrane protein resulted in a fourfold increase in the in vitro MIC of cefoperazone-sulbactam in comparison with the β-lactamase-producing susceptible strain and resulted in a loss of in vivo efficacy against infections caused by this strain. These results suggest that the combination of different resistance mechanisms, neither of which alone results in substantially diminished cefoperazone-sulbactam efficacy in vivo, can cause in vivo resistance to the β-lactam–β-lactamase inhibitor combination in *K. pneumoniae*.

Bacterial resistance to combinations of β-lactams with β-lactamase inhibitors has been reported to be associated with a number of different mechanisms. The most common mechanism appears to be production of high levels of a β-lactamase normally susceptible to the inhibitor (10, 11, 16, 20). Increased resistance has also been associated with the loss of expression of certain porin proteins in *Escherichia coli* (6, 19). Other reported mechanisms include the production of a β-lactamase normally resistant to the inhibitor, such as the AmpC-type enzymes (3, 15), or mutations in a normally inhibited enzyme rendering it resistant to inhibition (3). In a recent survey of clinical isolates resistant to cefoperazone-sulbactam, we discovered all classes except strains producing inhibitor-resistant enzymes resulting from spontaneous mutation (18). A number of strains exhibited production of high levels of β-lactamase in addition to alterations in the outer membrane proteins (OMPs). A 39-kDa OMP has previously been identified as a porin in *Klebsiella pneumoniae* (5). Loss of this porin in addition to that of a 41-kDa OMP has been associated with the emergence of resistance to cephamycins in a TEM-3-producing *K. pneumoniae* clinical isolate (12). The goal of the experiments reported here was to quantify, in an isogenic series of strains, the effect of loss of expression of the 39-kDa porin protein of *K. pneumoniae* on resistance to cefoperazone-sulbactam in the presence or absence of high levels of expression of the TEM-2 β-lactamase. We attempted to correlate our in vitro results with those obtained with a rat intra-abdominal abscess model.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and susceptibility testing. *K. pneumoniae* 44 was a clinical isolate obtained from the clinical microbiology laboratory of the Department of Veterans Affairs Medical Center, Cleveland, Ohio, and identified as being susceptible to cefoperazone by disk diffusion testing (National Committee for Clinical Laboratory Standards). Further susceptibility testing was performed by a broth macrodilution technique in Luria-Bertani broth with an inoculum of ca. 5×10^5 CFU/ml (7). When sulbactam was used, the concentration was 0.5× the concentration of cefoperazone. *K. pneumoniae* 44 was grown overnight, and 100 μl was plated on Mueller-Hinton agar plus 100 μg of rifampin per ml. Single colonies were purified, and one was picked and used to derive a mutant on Mueller-Hinton agar plus 100 μg of nalidixic acid per ml. This strain was named 44NR. Plasmid RP4, which produces high levels of the TEM-2 β-lactamase (9), was introduced into 44NR by 4-h broth mating between 44NR and *E. coli* HB101(RP4) (4). Transconjugants were selected on agar containing 100 μg of piperacillin per ml, 100 μg of rifampin per ml, and 100 μg of nalidixic acid per ml. *K. pneumoniae* 44NRF was selected by plating 100 μl of an overnight culture of strain 44NR on Mueller-Hinton agar containing cefoxitin (16 μg/ml). Plasmid RP4 was introduced into 44NRF by conjugation as described above.

Characterization of β-lactamase. β-Lactamase was extracted from bacteria by disrupting the cells either by sonication or by agitation in the presence of glass beads, followed by centrifugation to remove unbroken cells, debris, and cell envelopes. These procedures have been previously described (17). Protein in extracts was determined with a commercially available kit (Bio-Rad, Richmond, Calif.).

TABLE 1. MICs of test antibiotics against experimental *K. pneumoniae* strains

Strain	MIC (μg/ml) of antibiotic:			Sp act of β-lactamase[a]
	Cefoperazone	Cefoperazone-sulbactam (2:1 ratio)	Imipenem	
44	≤0.25	≤0.25	≤0.125	0.241
44NRF	2	2	≤0.125	0.346
44NR(RP4)	>2,056	64	≤0.125	87.204
44NRF(RP4)	>2,056	256	0.25	49.217

[a] Micromoles of cephaloridine hydrolyzed per minute per milligram of protein.

β-Lactamase-specific activity was determined by measuring the rate of hydrolysis, in micromoles per minute, of cephaloridine per milligram of protein extract (17).

Characterization of OMPs. These methods have been described previously (5, 8, 12). Briefly, cells were disrupted as described for the β-lactamase extraction above, and membranes were separated by ultracentrifugation at 100,000 × *g* for 45 min at 4°C. Outer membranes were prepared after solubilizing the inner membranes with Sarkosyl and recentrifuging as described above.

Intra-abdominal abscess model. Intra-abdominal abscesses were induced in male Sprague-Dawley rats (150 to 200 g) as described previously (14) with a mixture of sterile rat cecal contents, heat-killed *Bacteroides fragilis* ATCC 25285 (encapsulated strain), and a fresh sample of overnight growth of the test organism. Overnight incubations were in antibiotic-free Luria-Bertani medium, which was diluted in fresh Luria-Bertaini medium to achieve an inoculum at the time of implantation of ca. 10^5 CFU. Antibiotic therapy was begun approximately 2 h after abscess implantation by continuous intravenous infusion via the internal jugular vein and continued for a period of 3 days. Cefoperazone was administered in a dose of 600 mg/kg of body weight per day either with or without sulbactam (300 mg/kg/day). Untreated control animals were included with each group. After 24 h of therapy, blood was sampled and serum antimicrobial agent concentrations were measured. Cefoperazone concentrations in serum were measured by bioassay (1) with a *Bacillus subtilis* spore suspension (Difco, Detroit, Mich.). Sulbactam concentrations were measured by high-pressure liquid chromatography (2). After 3 days of therapy, animals were sacrificed at 2 h after discontinuation of therapy, and the abscesses were excised in a sterile fashion, weighed, serially diluted, and aliquoted for colony counting onto antibiotic-free Luria-Bertani plates. Final counts were expressed as $\log_{10}$ CFU/gram of abscess. Results for each group were compared with Student's *t* test for comparing independent variables.

RESULTS

Susceptibility and β-lactamase production of strains. As shown in Table 1, strain 44 was fully susceptible to cefoperazone, cefoperazone-sulbactam, and imipenem and produced no β-lactamase. Strain 44NR had susceptibilities identical to those of strain 44 for cefoperazone and cefoperazone-sulbactam as well as for multiple other β-lactam antibiotics (data not shown). Strain 44NRF was somewhat more resistant to cefoperazone and cefoperazone-sulbactam than its parent, strain 44. The presence of the TEM-2 β-lactamase encoded on the plasmid RP4 resulted in increased levels of resistance to cefoperazone which was only partially overcome by the addition of sulbactam at 0.5× the concentration of cefoperazone. Strain 44NRF(RP4) was fourfold more resistant to cefoperazone-sulbactam than was the 44NR(RP4) strain.

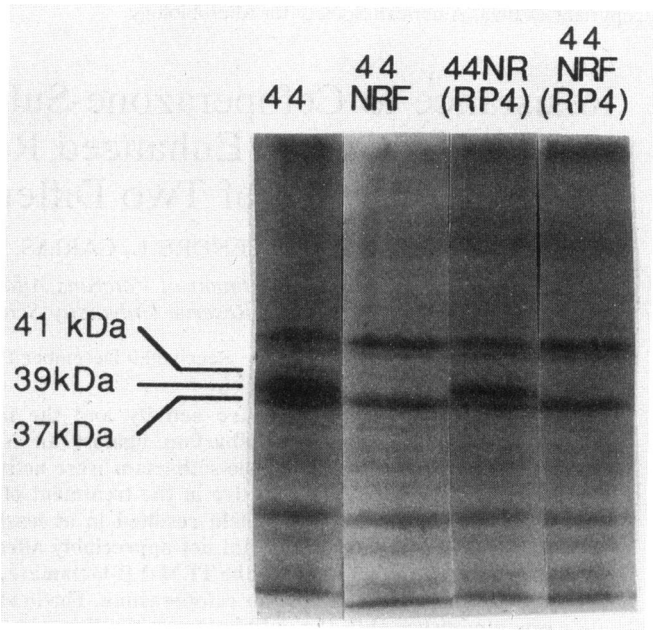

FIG. 1. OMPs of strains used in these studies. Loss of the 41-kDa OMP [seen in 44 but not in 44NR(RP4)] is not associated with a change in the MICs of β-lactam antibiotics tested in this study. Loss of the 39-kDa OMP (present in the 44 and 44NR strains but absent in the 44NRF strains) is associated with at least a fourfold increase in the MICs of cefoperazone and the cefoperazone-sulbactam combination.

OMPs. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMPs extracted from the *Klebsiella* strains are shown in Fig. 1. Compared with strain 44, 44NR(RP4) is missing the 41-kDa OMP. The identical MICs of numerous antibiotics for 44 and 44NR (see above) suggest that the loss of this 41-kDa OMP is not associated with resistance to these antimicrobial agents. Strains 44NRF and 44NRF(RP4) are missing both the 41- and the 39-kDa OMPs, which are thought to be porins in *K. pneumoniae* (5, 12). This is consistent with the slightly increased resistance of strains 44NRF and 44NRF(RP4) compared with that of their parents (Table 1).

Experimental infection. Results of treatment of intra-abdominal abscess infections with cefoperazone and cefoper-

TABLE 2. Mean antimicrobial agent concentrations in serum of treated animals

Infection group	Concn (μg/ml) of drug in serum (± SD)	
	Cefoperazone[a]	Sulbactam
44	19.9 (± 6.9)	10.4 (± 2.1)
44NRF	22.7 (± 5.7)	8.6 (± 3.8)
44NR(RP4)	22.4 (± 5.8)	9.1 (± 5.8)
44NRF(RP4)	31.6 (± 8.2)	12.2 (± 0.74)

[a] There were no significant differences in serum cefoperazone concentrations between animals treated with cefoperazone alone and those treated with cefoperazone-sulbactam for any infection group.

TABLE 3. Intra-abdominal abscess treatment outcomes

Treatment group	No. of rats/CFU of strain per g of abscess (SD)[a]			
	44	44NRF	44NR(RP4)	44NRF(RP4)
No treatment	7/8.46 (0.95)	6/7.3 (1.5)	10/8.36 (1.03)	5/8.16 (1.07)
Cefoperazone	ND	6/3.9 (0.58)*	8/7.8 (1.5)	ND
Cefoperazone-sulbactam	17/4.12 (0.58)*	12/3.4 (0.68)*	13/5.2 (1.2)**	14/7.68 (2.16)

[a] *, $P < 0.05$ in comparison with untreated controls; **, $P < 0.05$ in comparison with untreated controls and cefoperazone-treated group; ND, not done.

azone-sulbactam are shown in Tables 2 and 3. Cefoperazone concentrations in serum ranged from 20 to 30 μg/ml, and sulbactam concentrations averaged 9.9 μg/ml (Table 2). As expected, cefoperazone-sulbactam was very effective in treating abscesses caused by susceptible strain 44 (Table 3). The efficacy of cefoperazone alone against strain 44 was eliminated by the introduction of plasmid RP4. The efficacy of cefoperazone-sulbactam against strain 44NR(RP4), while somewhat diminished in comparison with the efficacy against 44, resulted in bacterial levels 2.5 to 3 $\log_{10}$ CFU/g of abscess lower than those of untreated controls and the cefoperazone-only group ($P < 0.05$). The efficacies of both cefoperazone and cefoperazone-sulbactam against 44NRF were essentially identical to that seen against strain 44, suggesting that the loss of the 41- and 39-kDa OMPs alone is not of clinical significance in this model. However, when the loss of the 39-kDa OMP and the presence of plasmid RP4 were combined, the in vivo efficacy of cefoperazone-sulbactam was eliminated.

DISCUSSION

The fact that most *E. coli* strains exhibiting resistance to the combination of β-lactams and β-lactamase inhibitors have been found to elaborate increased amounts of β-lactamase suggests that the susceptibility of organisms to these combinations is highly dependent upon the relative amounts of enzyme and inhibitor in the periplasmic space. In this scenario, an alteration in the permeability of the outer membrane to the β-lactam or the inhibitor in the presence of a stable level of β-lactamase production would be expected to result in an elevation of the in vitro MIC. Such a phenomenon has been described for *E. coli* in association with the loss of certain porin proteins (6, 19).

The results presented in this paper provide evidence that a similar combination of resistance mechanisms in *K. pneumoniae* can result in a substantial loss of in vivo efficacy of a β-lactam–β-lactamase inhibitor combination. A previous study suggested that loss of expression of both the 41- and 39-kDa OMPs of *K. pneumoniae* in the presence of β-lactamase resulted in increased resistance to β-lactam–β-lactamase inhibitor combinations as well as to cephamycins (12). Our results, showing an increase of fourfold or more in the MICs of both cefoperazone and cefoperazone-sulbactam associated with the loss of these proteins, support the view that they are important in β-lactam resistance, although we were unable to detect any clinical effect resulting from the loss of these OMPs alone. Our in vitro data suggest that the 41-kDa OMP plays only a minor role, if any, in transport of β-lactams across the outer membrane of *K. pneumoniae*. In association with the production of high levels of the TEM-2 β-lactamase, however, the loss of both of these OMPs results in an increase of the cefoperazone-sulbactam MIC from 64 to 256 μg/ml, an increase which results in the elimination of the clinical efficacy of the combination against this strain.

It is interesting that cefoperazone-sulbactam demonstrates significant (although diminished in comparison with that against the non-β-lactamase-producing strain) efficacy against a strain [44NR(RP4)] which has an in vitro MIC substantially in excess of the mean levels in serum of the antibiotic combination. We have also observed this phenomenon in rat intra-abdominal abscesses caused by β-lactamase-producing strains of *E. coli* treated with ampicillin-sulbactam (13). We have no data at the present time to explain the greater-than-expected efficacy of sulbactam-containing combinations against β-lactamase-producing strains of gram-negative bacilli.

These results serve to emphasize the resourcefulness of bacterial species in thwarting our attempts to address the challenges posed by different mechanisms of antimicrobial resistance. The widespread elaboration of TEM- and SHV-type β-lactamases by *Klebsiella* strains, in addition to the relative ease with which clinical strains decrease expression of the 41- and 39-kDa OMPs, suggests that we may expect to see more of this combination type of resistance as β-lactam–β-lactamase inhibitors gain more widespread clinical use.

ACKNOWLEDGMENTS

We are indebted to Roger Bawdon for his assistance in determining serum sulbactam concentrations.

These studies were supported by a grant from Pfizer, Inc. L. B. Rice is supported by a Department of Veterans Affairs Research Associate Career Development Award. D. M. Shlaes is supported by a Department of Veterans Affairs Clinical Investigator Career Development Award.

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