Protective Effect of Amdinocillin against Emergence of Resistance to Ceftazidime in Enterobacter cloacae

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Enterobacter cloacae infections have been shown clinically to respond less reliably to monotherapy with broad-spectrum cephalosporins than in the past. Selection of populations producing high levels of β-lactamase has been shown to be the most frequent reason for treatment failure, and the use of these agents with another active antibiotic is recommended. In this study, E. cloacae strains from clinical specimens were examined for ceftazidime-resistant populations by broth dilution and disk tests. In the presence of ceftazidime at 12 μg/ml, the in vitro selection of resistant organisms was demonstrated for 3 of 11 strains. Selection was prevented when amdinocillin was added in combination. A more rapid killing was also demonstrated with this combination. At inocula of 10⁶ CFU/ml, ceftazidime-resistant populations were isolated from 6 of 11 strains in vitro, and the emergence of this resistance was prevented by amdinocillin. The enhanced killing effect noted for amdinocillin with ceftazidime may have resulted in part from complementary activity of the antibiotics on penicillin-binding proteins. The ceftazidime-amdinocillin combination offers an interesting prospect for the therapy of infections caused by E. cloacae strains which are initially susceptible to both antibiotics.

The resistance of Enterobacter cloacae to broad-spectrum cephalosporins is often the result of chromosomally mediated β-lactamase enzymes that are induced by the presence of beta-lactam antibiotics. Mutants with high-level enzyme production may arise or, alternatively, production may be reversibly derepressed in the presence of inducer (2, 18, 23, 26, 28). In contrast, amdinocillin, although not hydrolyzed by these enzymes, induces round forms with a reduced growth rate (15, 22, 30, 32). Most E. cloacae strains isolated from patients who have not received broad-spectrum cephalosporins appear to be susceptible to ceftazidime and amdinocillin by the disk diffusion method.

In this study, we investigated the effect of ceftazidime and amdinocillin at 12 μg/ml alone and in combination on these wild-type strains to define (i) the killing rate in a 10⁶ CFU/ml culture and (ii) the ability of the combination to prevent a selection of ceftazidime-resistant populations from a dense bacterial culture.

MATERIALS AND METHODS

Bacterial strains. Eleven strains of E. cloacae were obtained from clinical specimens (pus and blood cultures from patients who had not received broad-spectrum cephalosporins). These strains were identified by methods recommended by Kelly et al. (12). The strains were numbered 1 to 11.

Antibiotics. The following antibiotics were used: ceftazidime (Glaxo Pharmaceuticals Ltd., Greenford, England) and amdinocillin (Leo Pharmaceutical Products, Ballerup, Denmark).

Antimicrobial susceptibility testing. Agar diffusion susceptibility tests were performed by using the Neo-Sensitabs system (A/S Rosco, Taastorp, Denmark) on Mueller-Hinton agar (Difco Laboratories Inc., Detroit, Mich.) (9). MICs were determined by the microdilution broth method described by Jones et al. (11). The tubes were incubated at 35°C for 24 h.

β-Lactamase detection and characterization. β-Lactamase extracts for isoelectric focusing were prepared from strains grown on nutrient agar slopes (14). Isoelectric points (pIs) of β-lactamases were determined by the method of Matthew et al. (17). β-Lactamase induction was inferred from the disk approximation test with cefoxitin and ceftazidime (30-μg disks) (27).

Killing curves. (i) Preparation of inocula. Single colonies growing on Iso-Sensitest agar (Oxoid Ltd., Basingstoke, England) were suspended in 10 ml of Iso-Sensitest broth in screw-cap tubes. The tubes were placed at 35°C in an incubator on a tube rotator (Test Tube Rotator; Cenco, Breda, The Netherlands), and growth was continued until a density (turbidimeter HF Instrument; Shaban Manufacturing Inc., Fredonia, N.Y.) equivalent to 0.5 McFarland standard was reached; the culture was then diluted 1/100. This culture in the log phase was inoculated into Iso-Sensitest broth medium containing different antibiotic concentrations (amdinocillin and ceftazidime at 10 μg/ml alone and in combination). The initial bacterial density was approximately 10⁸ CFU/ml as determined by the Spiral counting system (see below).

(ii) Incubation. All inoculated tubes (final volume, 2 ml) were incubated in plastic racks in a water bath at 35°C.

(iii) Sampling and counting. Immediately after inoculation and vortexing and then every hour up to 6 and 24 h, a 14-cm-diameter petri dish containing 30 ml of Iso-Sensitest agar medium was inoculated (before-and-after dilutions, 1/10 and 1/100) with 0.05 ml of the culture distributed by the Spiral inoculator system (Spiral System Inc., Cincinnati, Ohio). In this system, a variable cam-activated syringe dispenses the culture from the center to the edge of the plate in a logarithmically decreasing quantity in the form of an Archimedes' spiral (6, 13, 25). A Spiral 500 laser colony counter and a Casba 800 microprocessor (Spiral System Inc.) were used for the CFU count. The lower count determined by Spiral laser was five colonies per plate (10² CFU/ml) even in the presence of antibiotic at 200 times the MIC (34). However, at least 30 colonies (0.05 ml yielding 30 colonies = 600 CFU/ml) per plate was considered the lower
TABLE 1. MICs and β-lactamases of *E. cloacae* wild strains isolated from patients who had not received broad-spectrum cephalosporins

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Ceftazidime (µg/ml)</th>
<th>Amdinocillin (µg/ml)</th>
<th>β-Lactamase*</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.06</td>
<td>0.12</td>
<td>Chr</td>
<td>+ 7.95</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>1.00</td>
<td>Chr</td>
<td>+ 7.8</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>0.25</td>
<td>ND</td>
<td>+ 7.3</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>0.25</td>
<td>Chr</td>
<td>ND 7.3</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>1.00</td>
<td>ND</td>
<td>+ 8.1</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.25</td>
<td>Chr</td>
<td>ND 7.4</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td>0.25</td>
<td>Chr</td>
<td>+ 8.16</td>
</tr>
<tr>
<td>8</td>
<td>0.12</td>
<td>0.25</td>
<td>ND</td>
<td>+/− 8.16</td>
</tr>
<tr>
<td>9</td>
<td>0.12</td>
<td>0.12</td>
<td>Chr</td>
<td>+/− 8.16</td>
</tr>
<tr>
<td>10</td>
<td>0.25</td>
<td>0.50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>0.50</td>
<td>0.06</td>
<td>Chr</td>
<td>ND 8.16</td>
</tr>
</tbody>
</table>

* Chr, Chromosomal; ND, not detected; +/−, weak inducibility.
* a, Not determined.

limit for an accurately detectable count, and we selected 2.7 CFU/ml as the lowest limit for our data presentation.

**Dense and large culture studies.** Single colonies growing on Iso-Sensitist agar were inoculated into Iso-Sensitist broth and incubated to a McFarland 0.5 standard (approximately 10⁶ CFU/ml). Ceftazidime and amdinocillin alone and in combination were added to final concentrations of 10 µg/ml in 10-ml log-phase cultures in 20-ml screw-cap tubes. The tubes were placed at 35°C in an incubator on a tube rotator. At 0, 6, and 24 h, 0.05-ml aliquots of culture were plated on a 14-cm antibiotic-containing agar plate (ceftazidime: 0, 10, and 64 µg/ml; amdinocillin: 0, 10, and 16 µg/ml). A Spiral colony counter was used for the CFU count after 48 h of incubation.

**Statistics.** The statistical significance of differences between groups was determined by the Mann-Whitney test.

**RESULTS**

**Antimicrobial susceptibility testing.** By the disk diffusion method using the Neo-Sensitabs system, all strains were fully susceptible to ceftazidime (zone size, 32 to 35 mm for a break point of 23 mm) and amdinocillin (zone size, 30 to 38 mm for a break point of 26 mm).

Table 1 shows the MICs (10³ CFU/ml) of ceftazidime and amdinocillin and β-lactamase detection for the 11 strains included in this study. β-Lactamase production was low, and in some isolates enzyme was undetectable by isoelectric focusing. None of the strains had derepressed β-lactamase; some showed weak inducibility in the disk test.

**Time-kill studies.** Table 2 shows the killing effect obtained for a 10⁶ CFU/ml inoculum of *E. cloacae* (11 strains) exposed to 10 µg of ceftazidime and amdinocillin per ml alone and in combination. The bactericidal activity of ceftazidime began after 1 h. A regrowth (CFU per milliliter greater than that of the initial inoculum) was observed for three strains (strains 1, 4, and 6). The rate of bactericidal activity of amdinocillin was similar to that of ceftazidime during the first 6 h (P > 0.5), but a regrowth was observed for the 11 strains after 24 h. However, phase-contrast microscopy showed that the bacteria were transformed into round forms. These cells are phenotypically resistant to amdinocillin (7, 10, 15, 30). These microorganisms remained able to revert to the bacillary form and were able to form colonies on antibiotic-free agar medium. The ceftazidime-aminocillin combination was more rapidly bactericidal (P < 0.01) during the first 6 h than either antibiotic used alone. Additionally, no regrowth due to a ceftazidime-resistant population was observed in bacterial cultures exposed to ceftazidime and amdinocillin in combination.

**Antimicrobial effect on dense cultures.** Figure 1 shows the population analysis in 10 ml of a culture with a high initial bacterial density (8.4 ± 0.1 [standard deviation] log₁₀ CFU/ml) of 11 strains of *E. cloacae* exposed to 10 µg of ceftazidime and amdinocillin per ml alone and in combination. At time zero, no resistant colonies were selected by plating 0.05 ml of the cultures on agar containing ceftazidime (Fig. 1A).

After 6 h, the total number of CFU per milliliter isolated on drug-free medium indicated a reduction of 3.4 ± 0.5 log₁₀ CFU/ml. However, at this time four strains harbored bacteria able to form colonies on ceftazidime-containing agar (strains 1, 4, 6, and 8).

After 24 h, these four strains generated a population resistant to ceftazidime (10 µg/ml). For the five other strains, the bactericidal activity was more pronounced, and two additional strains contained ceftazidime-resistant bacteria (strains 5 and 9). The colonies formed on 10-µg/ml ceftazidime agar were also resistant to 64 µg/ml. Among these six strains, only two remained stable after three passages on antibiotic-free medium (strains 4 and 8). The four others returned to initial susceptibility.

After exposure to amdinocillin (Fig. 1B), the number of CFU per milliliter grown on antibiotic-free agar was the same as that of the inoculum. In this high-bacterial-density culture, amdinocillin appeared to be bacteriostatic, and the bactericidal activity shown in Table 2 was not observed. At time zero, few colonies were formed on amdinocillin-containing agar; this number increased slowly after 6 and 24 h. These colonies contained bacillary forms and were resistant to amdinocillin at 16 µg/ml. This resistance remained stable after passage on antibiotic-free medium. It must be stressed that round forms remained in great number in broth containing amdinocillin after 6 and 24 h of incubation.

After 6 h of exposure to ceftazidime and amdinocillin in combination (Fig. 1C), a more rapid killing resulted than with ceftazidime alone (P < 0.00005), with a reduction of 4.4 ± 1.1 log₁₀ CFU/ml. The culture did not harbor ceftazidime-resistant bacteria. An additional study (results not shown) showed that amdinocillin does not select or induce ceftazidime-resistant bacteria. No resistant colonies were detected after exposure for 24 h followed by plating on agar containing ceftazidime.

TABLE 2. Killing effect on the 11 *E. cloacae* strains studied (inoculum, 10⁶ CFU/ml) after exposure to 10 µg of amdinocillin and ceftazidime per ml alone and in combination

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ceftazidime</th>
<th>Amdinocillin</th>
<th>Amdinocillin-ceftazidime combination*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.1</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>2.2</td>
<td>1.8</td>
<td>3.1</td>
</tr>
<tr>
<td>6</td>
<td>2.4</td>
<td>1.9</td>
<td>3.2</td>
</tr>
<tr>
<td>24</td>
<td>2.6,± 0,0</td>
<td>0,11</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* P < 0.01 for the combination with each antibiotic.
* ± 0. The inferior number is the number of strains with the reduction indicated.
* A value of zero indicates that the CFU per milliliter was greater than that of the initial inoculum.
E. cloacae strains were selected by 10 μg of ceftazidime per ml. The selection of ceftazidime-resistant populations was found in 3 of 11 strains in 2 ml of broth at 10⁶ CFU/ml and in 6 strains in 10 ml of broth at 10⁸ CFU/ml.

After exposure to aminocillin, cultures also showed regrowth. However, round forms, which are characteristic for microorganisms which are initially susceptible to aminocillin, were observed under phase-contrast microscopy (7, 10, 20, 27).

The ceftazidime-aminocillin combination (10/10 μg/ml) resulted in a much more rapid bactericidal effect than did the same agents used separately. This more rapid killing rate was described previously (8, 20, 31, 33). The most important observation concerns the absence of regrowth of a ceftazidime-resistant population, even in a 10-ml culture of a 10⁸ CFU/ml inoculum of these wild-type strains. The uninduced strains demonstrated relatively modest production of β-lactamase under basal conditions. Indeed, neither the MIC, the type of β-lactamase, nor the induction procedure seems to be able to predict a regrowth. With a culture of high bacterial density, only two strains produced a stable mutant which remained resistant to 64 μg/ml after three passages on antibiotic-free medium.

The rate of ceftazidime mutation is about 10⁻⁷ to 10⁻⁹ CFU/ml in wild strains. Thus, the regrowing population included mutant strains that were stably resistant to ceftazidime and did not revert to susceptibility when ceftazidime was removed and also strains which were reversibly derepressed. The colony count on ceftazidime-containing agar was not able to differentiate these two populations. It was shown recently that aminocillin may potentiate the activity of other beta-lactam antibiotics not only by binding to complementary penicillin-binding protein but also by causing leakage of β-lactamase from the cell (29). This effect may be related to its ability to bind to penicillin-binding protein 2 and to subsequently produce changes in outer membrane permeability. This phenomenon may contribute to the protective effect of aminocillin against the emergence of ceftazidime-resistant bacteria. The large number of β-lactamase molecules within the periplasmic space may be reduced to a level which is not able to protect the cell against the bactericidal activity of ceftazidime.

The clinical relevance of this observation remains to be demonstrated. Heterogeneous resistance to beta-lactams among the cells within a given E. cloacae population should be taken into consideration to obtain a good in vitro-in vivo correlation (16). The resistant populations are often not detectable by conventional susceptibility tests that use an inoculum of only 10⁴ to 10⁵ CFU/ml. The aminocillin-ceftazidime association is of clinical interest when E. cloacae strains are susceptible to the two drugs by the disk diffusion method. From a clinical point of view, aminocillin would need to be added to ceftazidime at the initiation of the therapy of an infection caused by E. cloacae strains that are susceptible to both antibiotics.

In conclusion, the combination of ceftazidime and aminocillin was more rapidly bactericidal and also prevented the in vitro emergence of E. cloacae strains resistant to ceftazidime. The combination of these two beta-lactams may be of value in the therapy of E. cloacae infection.

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**FIG. 1.** Population analysis of 11 strains of E. cloacae exposed in a 10-ml broth culture to 10 μg of ceftazidime per ml (A), 10 μg of aminocillin per ml (B), and 10 μg of both drugs per ml in combination (C). At 0, 6, and 24 h, 0.05 ml of the broth culture was plated on antibiotic-free agar (C) and antibiotic-containing agar (B). In these dense and large cultures, E. cloacae strains resistant to ceftazidime at 10 μg/ml (also resistant to 64 μg/ml) and to aminocillin at 10 μg/ml (also resistant to 16 μg/ml) generated colonies. Ceftazidime and aminocillin in combination prevented the emergence of ceftazidime-resistant bacteria; no colony was formed on ceftazidime-containing agar.

**DISCUSSION**

It was recently demonstrated that E. cloacae, part of the human endogenous flora, becomes an important pathogen in hospitalized patients when generated by prophylactic antibiotics; it is less often transmitted horizontally (4). The wild strains included in the present study correspond to E. cloacae strains probably selected by narrow-spectrum cephalosporins in patients who had not received a broad-spectrum cephalosporin. In this very common clinical situation, E. cloacae is susceptible by the disk diffusion method to both ceftazidime and aminocillin. However, treatment failures resulting from the selection of a resistant population of E. cloacae have been documented (5, 19, 21, 24). A combination of two drugs is recommended (1, 3).

The time-kill studies showed how these strains produce regrowth in the presence of 10 μg of ceftazidime per ml. Bactericidal activity was observed for the first 6 h, followed by a regrowth of resistant bacteria. Resistant populations were selected by 10 μg of ceftazidime per ml. The selection of ceftazidime-resistant populations was found in 3 of 11 strains in 2 ml of broth at 10⁶ CFU/ml and in 6 strains in 10 ml of broth at 10⁸ CFU/ml.
LITERATURE CITED


