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Development of β-Lactam–Resistant *Enterobacter cloacae* in Mice

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We compared the ability of four newer β-lactam compounds to produce resistance in an experimental model of *Enterobacter cloacae* infection. Mice infected intraperitoneally developed resistance depending on antibiotic treatment and the dose given. Percentages of mice in which resistance was observed were as follows: 100% after ceftriaxone (50 mg/kg, two doses); 87% after ceftriaxone (50 mg/kg, one dose); 35% after ceftriaxone (500 mg/kg, one dose); and 21% after carumonam (25 mg/kg, two doses). No resistance occurred after therapy with either BMY 28142 (25 mg/kg, two doses) or Sch 34343 (50 mg/kg, two doses). Heterogeneous resistance to β-lactams among the cells within a given *Enterobacter* population accounted for these differences. The minimal concentration inhibiting the growth of the preexisting resistant variants, together with the antibiotic concentrations obtained in the peritoneal fluid, were associated with further emergence of resistance in the mouse treated with this antibiotic.

Some gram-negative bacteria, although classified as susceptible with the conventional susceptibility testings, become resistant during therapy with newer β-lactam compounds. Bacterial species possessing this capacity include mainly *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Serratia marcescens*, and *Citrobacter freundii* [1]. Such emergence of resistance has been well documented with most of the enzyme-stable β-lactam compounds. Investigators, however, have ignored whether the rate of resistance varies according to the compound considered.

In our laboratory, we have developed a murine model to detect and quantify the resistance that emerges during short-term therapy [2]. We found that ceftriaxone was prone to select resistance in *Enterobacter cloacae* infection. Using the same model, we compared the resistance-producing ability of four β-lactam antibiotics with distinct antibacterial activities. Besides ceftriaxone, which is a third-generation cephalosporin, we tested the following: carumonam, a monobactam with a narrower antibacterial spectrum and a more-potent activity against gram-negative rods [3]; BMY 28142, a newer cephalosporin with exceptionally low MICs for Enterobacteriaceae [4] and low affinity for β-lactamase [5]; and Sch 34343, a new penem [6].

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Materials and Methods

Chemicals. Antibiotic solutions were prepared in 0.1 *M* phosphate buffer from the following diagnostic powders of known potency: ceftriaxone and carumonam (Hoffman La Roche, Basel, Switzerland), BMY 28142 (Bristol-Myers AG, Cham, Switzerland), Sch 34343 (Schering Corp., Bloomfield, NJ), and nitrocefin (Becton-Dickinson AG, Basel, Switzerland).

Bacterial strains. We used four clinical isolates of *E. cloacae.* Strains 218 and 219 were isolated from CSF in patients with meningitis, and strain 895 from a patient with postoperative mediastinitis. Strain 908 was provided by R. Then and P. Angehrn [7]. In spite of their initial susceptibility to the drug administered, strains 218, 219, and 895 were responsible for a therapeutic failure during therapy with a third-generation cephalosporin. Strains were maintained in skim milk at −70 C. When required, the strains were thawed and grown overnight at 37 C in L-broth (10 g of Bacto-tryptone, 10 g of NaCl, and 5 g of yeast extract/liter).

Susceptibility testing. MICs were determined by an agar-dilution method [8]. The inoculum, 10^4–10^5 cfu per spot, was deposited onto Mueller-Hinton agar with a multiple inoculum replicator. Bacterial populations were also analyzed on freshly prepared antibiotic-gradient agar as previously described [9]. Gradients were prepared in 9 × 9-cm petri dishes

and provided antibiotic concentrations ranging from zero to the chosen maximum (6.4, 64, or 640 μg/ml) as a linear function of the distance from the side of the dish where the gradients started. One hundred microliters of an overnight culture (i.e., 2–3 × 10^8 cfu) was plated onto each gradient. After 18 hr of incubation at 37 C, bacterial growth was examined, and when possible, colonies were counted.

β-Lactamase studies. Single colonies were grown overnight in antibiotic-free L-broth. A portion (50 μl) of this culture was mixed with 10 μl of a 1 m*M* nitrocefin solution. The test was considered positive if the mixture turned red within 10 min. Under these conditions, a positive reaction occurred only in strains producing great amounts of β-lactamase. To assess the inducibility of a strain with a negative reaction, we performed the nitrocefin test on bacteria grown in L-broth for 3 hr at 37 C with or without cefoxitin at a final concentration of 10 μg/ml.

Animal model. We used Swiss ICR mice of both sexes with a mean weight of 25 g (Institut für Zuchthygiene, Zürich, Switzerland). Experimental peritonitis was established by an ip injection of 1 ml of a mixture containing 0.5 ml of a bacterial suspension (0.5–3 × 10^8 cfu) and 0.5 ml of sterile talcum (125 mg). Talcum was added to avoid spontaneous cure of the infection. Antibiotics were administered sc 2 hr and eventually 8 hr after challenge: ceftriaxone (one or two doses); carumonam, BMY 28142, and Sch 34343 (two doses). Twenty-four hours after inoculation, animals were killed by hyperanesthesia. Peritoneal fluid was collected for bacterial counts and bacterial population analysis. For the latter, the fluid was grown overnight at 37 C in L-broth, and 100 μl of this culture was plated onto L-agar containing a gradient of the antibiotic used for treatment.

Antibiotic assays in mice. Mice were injected ip with 1 ml of 0.9% NaCl, containing 250 mg of sterile talcum. Two hours later, antibiotics were administered sc. Then, at different times, mice were killed (four animals per time interval), and 10 μl of peritoneal exudate was sampled and deposited on 6.5-mm paper disks. Assays were performed by using a disk-diffusion method, with Mueller-Hinton agar and *E. coli* or *Staphylococcus aureus* as the test organism. Apparent half-lives were estimated from results obtained at three different time intervals.

Results

Bacteriologic studies on initial strains. Before therapy, all strains of *E. cloacae* were susceptible to the four antibiotics tested (table 1). On the gradients, at the lowest antibiotic concentrations, the bacterial growth was confluent and sharply limited by a clear-cut boundary. The antibiotic concentration corresponding to the boundary was similar to the MIC determined by using the agar-dilution method. At higher antibiotic concentrations, single colonies were seen on the gradients prepared with ceftriaxone, carumonam, and BMY 28142, but not on those prepared with Sch 34343. These colonies were resistant clones that were included within the susceptible bacterial population. With all strains, the frequency of the clones was ~10^{-6}–10^{-7}. The MIC for the resistant clones varied with the strain and the compound considered: 25–200 μg/ml with ceftriaxone, 3–20 μg/ml with carumonam, and 0.5–2 μg/ml with BMY 28142 (table 1). When resistant colonies from the gradient were retested by using the agar-dilution method, their MICs were similar to the minimal concentration inhibiting their growth on the gradient plate. The nitrocefin test applied to bacterial colonies sampled from the confluent growth zone on

Table 1. Susceptibility testing using the agar-dilution method or antibiotic-gradient agar.

	MIC (μg/ml) with agar-dilution method				Minimal concentrations (μg/ml) inhibiting the growth of the resistant clones*			
Strain no.	Ceftriaxone	Carumonam	BMY 28142	Sch 34343	Ceftriaxone	Carumonam	BMY 28142	Sch 34343†
218	0.25	0.06	0.03	1	25	3	0.5	–
908	0.25	0.06	0.03	1	30	3	0.5	–
219	0.25	0.06	0.03	1	150	10	2	–
895	0.25	0.06	0.03	1	200	20	2	–

* On antibiotic-gradient agar.

† No resistant colonies were seen on gradients prepared with Sch 34343.

Table 2. Bacterial population analysis from treated mice.

Antibiotic	Dose	Strains	Mice		
			Total treated	No. with acquired resistance (range of fold increase of MIC)*	Mean (range) of $\log_{10}$ cfu decrease in peritoneal fluid*
Ceftriaxone	50 × 1	218	5	5(40–140)	1.96 (1.15–3.05)
		908	5	2 (200)	3.74 (2.0–5.05)
		219	5	5 (400–800)	1.64 (1.0–3.0)
		895	8	8 (800–1,200)	0.98 (0.0–2.0)
Ceftriaxone	50 × 2	Total	22	22 (140–1,200)	-
Ceftriaxone	500 × 1	218	5	0 -	4.15 (4.05–4.3)
		908	5	1 (120)	4.74 (3.3–5.3)
		219	5	3 (800)	4.66 (3.9–5.4)
		895	5	3 (800)	4.49 (4.0–4.9)
Carumonam	25 × 2	218	6	0 -	5.1 (3.5–6.05)
		908	6	0 -	5.96 (5.3–6.9)
		219	6	4 (200)	4.37 (3.0–5.3)
		895	6	1 (400)	4.82 (4.2–5.3)
BMY 28142	25 × 2	Total	19	0 -	5.80 (3.9–6.7)
Sch 34343	25 × 2	Total	20	0 -	2.52 (0.5–5.3)

* Compared with untreated animals. The *MIC* was defined as the concentration on the gradient corresponding to the boundary limiting the confluent growth zone. This concentration was similar to the MIC determined by the agar-dilution method.

antibiotic-gradient agar was negative without induction, but in all cases became immediately positive after induction by cefoxitin. When the nitrocefin test was applied to the resistant clones, it was positive without induction in 40 of 40 colonies from ceftriaxone gradients, in 20 of 20 colonies from carumonam gradients, and in 18 of 20 colonies from BMY 28142 gradients. The nitrocefin test involved overnight growth in the L-broth before testing. This procedure should eliminate any possible induction effect of the antibiotic-containing gradient plates.

Control mice. Fifty-five infected but untreated mice were used as controls. Twenty-four hours after challenge, control animals were killed, and autopsy showed a peritonitis in all cases with ~1 ml of peritoneal exudate. Bacterial counts from this fluid averaged 3.23×10^{10} cfu/ml (range, 5.7×10^{9}–1.03×10^{11}), with no significant differences between the four strains tested. Because we injected 0.5 to 3×10^{8} cfu into the peritoneal cavity, this meant that an actual infection occurred regularly. Both MICs and growth patterns on antibiotic-gradient agars were similar to those of the initial strains.

Treated mice. A total of 128 mice were infected and treated. In all cases but one, the number of colony-forming units per milliliter of peritoneal fluid was smaller by 0.50 to 6.90 logarithm units in treated animals than in control, untreated animals, a result indicating that the treatment had antibacterial activity (table 2). Results were analyzed by using Student's *t* test, and the following trends were observed. BMY 28142 (25 mg/kg, two doses) was the most-effective therapy, followed by carumonam (25 mg/kg, two doses; $P < .01$), and then by ceftriaxone (500 mg/kg, one dose, $P < .05$ against carumonam), Sch 34343 (50 mg/kg, two doses, $P < .05$ against ceftriaxone, 500 mg/kg, one dose), and by ceftriaxone (50 mg/kg, one dose; an insignificant difference compared with Sch 34343, $P < .001$ against ceftriaxone, 500 mg/kg, one dose).

No resistance occurred after BMY 28142 and Sch 34343 therapies (table 2). In contrast, resistance emerged after ceftriaxone and carumonam therapies. The percentages of animals in which resistance was observed were as follows: 100% after ceftriaxone (50 mg/kg, two doses); 87% after ceftriaxone (50 mg/kg, one dose); 35% after ceftriaxone (500 mg/kg, one dose); and 21% after carumonam. These results were analyzed by using the χ^2 test with the Yates' correction test, and the following trends were observed. Ceftriaxone (500 mg/kg, one dose) reduced the risk of resistance occurring after ceftriaxone (50 mg/kg, one dose; $P < .001$); carumonam yielded less resistance than did ceftriaxone (50 mg/ kg, one dose; $P < .001$) and a resistance rate similar to that observed with ceftriaxone (500 mg/kg, one dose; an insignificant difference). The absence of resistance after therapy with BMY 28142 and Sch 34343 was a result different from that obtained with ceftriaxone ($P < .001$ or $P < .05$ depending on the dose given to

Table 3. Pharmacokinetics of four β-lactam compounds in the peritoneal fluid of mice.

Antibiotic	Dose (mg/kg)	Mean (SD) of antibiotic concentration 60 min after dosing (μg/ml)	Apparent half-life in the peritoneal fluid (min)
Ceftriaxone	50	170 (1.1)	138
	500	268 (27.5)	ND*
Carumonam	25	30 (11.0)	42
BMY 28142	25	19 (3.7)	33.5
Sch 34343	50	16† (4.7)	28

* ND = not determined.
† Concentration determined after 45 min.

the mice), but a result similar to that obtained with carumonam (an insignificant difference).

The shift towards resistance was always important, but generally, greater increases in MICs were observed with strains 219 and 895 than with strains 908 and 218 (table 2).

Antibiotic assays. Results from 60 mice (table 3) showed that 60 min after dosing, concentrations of ceftriaxone, carumonam, BMY 28142, or Sch 34343 in the peritoneal fluid exceeded the MIC for the four strains tested (table 1) but not necessarily the MICs for the resistant clones (table 1). Apparent half-lives of the four β-lactam compounds in the peritoneal fluid suggested that 22 hr and 16 hr after dosing, when the mice were killed, the residual antibiotic concentrations in the peritoneal fluid were too low for interfering with the bacterial population analysis.

Discussion

In our animal model, bacterial resistance emerged rapidly after therapy with carumonam and ceftriaxone. For instance, with ceftriaxone, resistance rates in the mouse were 87% after the first injection of 50 mg/kg and 100% after the second injection. A major finding of this study was that the rate of resistance emerging in vivo varied according to the compound administered. Heterogeneous susceptibility to β-lactam compounds among the cells within an *Enterobacter* population, a phenomenon already described a decade ago [10], probably accounted for these differences. Thus, the gradients prepared with carumonam or ceftriaxone and plated with *Enterobacter* before therapeutic exposure showed clones growing far over the MIC level. These preexisting resistant clones were probably selected by the treatment and preferentially developed. In contrast, no resistant colonies grew on gradients prepared with Sch 34343, which, in turn, selected no resistance in the animals. BMY 28142, which allowed resistant clones to grow on the gradients but yielded no emergence of resistance in our model, seemed to be an exception. In fact, the level of resistance of the preexisting clones together with the pharmacokinetic data must be considered. In the case of BMY 28142, the resistant colonies were inhibited by 0.5–2 μg/ml (table 1) when the mean concentration of the drug in the peritoneal fluid, 60 min after dosing, was ~10 times higher than these inhibitory concentrations (table 3). This "therapeutic index" (i.e., antibiotic concentrations in the peritoneal fluid over the level of resistance of the clones) was regularly lower with ceftriaxone and carumonam than with BMY 28142. Also, the lower dose of ceftriaxone (50 mg/kg) produced lower concentrations in the peritoneal fluid, i.e., smaller therapeutic index and accordingly selected resistance more frequently than did the higher dose (500 mg/kg). Moreover, in the latter case, as after carumonam therapy, resistance emerged almost exclusively in strains 219 and 895, which contained the most-resistant clones.

Thus, for a given β-lactam compound, the resistant clones, together with the pharmacokinetic data, could be associated with the further emergence of resistance in the mouse treated with this antibiotic. On the contrary, the number of colonies growing over the boundary on a gradient was a poor predictor because ceftriaxone, carumonam, and BMY 28142 yielded a similar number of resistant clones on the gradient before therapy, but different resistance rates after therapy. The frequency of these resistant clones was $\sim 10^{-6}$–10^{-7}; this frequency was similar to that observed in other studies [10, 11]. It has been stressed [12] that the resistant clones are often overlooked by the conventional susceptibility tests that use an inoculum of only 10^4–10^5 cfu, a procedure causing "very major" errors. A comparison of the two pan-

els of table 1 gives an illustration of such discrepancies. Indeed, population analysis on antibiotic-gradient agar, using an inoculum of 10^8 cfu, provided a better prediction of further emergence of resistance than did the usual MIC determination by agar-dilution.

The nitrocefin test applied to the resistant clones was almost always positive (we found only two negative colonies from the BMY 28142 gradient) after subculture in antibiotic-free medium. This finding indicated that most of the resistant clones produced constitutively great amounts of β-lactamase.

The most-convincing evidence that β-lactamase is associated with the resistance is the transfer of the chromosomal gene from a stably derepressed mutant of *E. cloacae* to *Escherichia coli;* this transfer leads to acquisition of β-lactam resistance by the recipient *E. coli* [13]. In our study, however, the nitrofecin test showed that our initial strains were inducible by cefoxitin. In vivo, induction of β-lactamase may have an additional advantage over the constitutive production of the enzyme – the higher induced levels might allow neutralization of more β-lactam molecules.

Extrapolating the results from our experimental studies directly to the clinical situation would be speculative. In this study, we put the antibiotics to a very severe test by using a high bacterial inoculum and talcum, which acted as a foreign body. Such unfavorable conditions are not commonly encountered in the clinical setting. Nevertheless, some of our observations might be helpful for the clinician. Emergence of resistance after therapy with the newer β-lactam antibiotics was dose related, and the ability to produce resistance varied according to the β-lactam compound considered. This supports the idea of avoiding underdosing patients, especially at initiation of therapy, when the bacterial populations are high. Also, one may hope that drugs such as Sch 34343 or BMY 28142 will limit the risk of selecting resistance during therapy of *E. cloacae* infections.

References

1. Sanders CC, Sanders WE Jr. Microbial resistance to newer generation β-lactam antibiotics: clinical and laboratory implications. J Infect Dis 1985;**151**:399–406
2. Michea-Hamzehpour M, Pechère J-C, Marchou B, Auckenthaler R. Combination therapy: a way to limit emergence of resistance? Am J Med 1986;**80**:138–42
3. Imada A, Kondo M, Okonogi K, Yukishige K, Kuno M. In vitro and in vivo antibacterial activities of Carumonam (AMA-1080), a new N-Sulfonated moncyclic β-lactam antibiotic. Antimicrob Agents Chemother 1985;**27**:821–7
4. Vuye A, Pijck J. In vitro antibacterial activity of BMY-28142, a new extended-spectrum cephalosporin. Antimicrob Agents Chemother 1985;**27**:574-7
5. Phelps DJ, Carlton DD, Farrel CA, Kessler RE. Affinity of cephalosporins for β-lactamases as a factor in antibacterial efficacy. Antimicrob Agents Chemother 1986;**29**:845–8
6. Gutmann L, Kitzis MD, Acar JF. Sch 34343 activity against streptococci and β-lactam-resistant Enterobacteriaceae. J Antimicrob Chemother 1985;**15**:147–54
7. Then RL, Angehrn P. Trapping of nonhydrolyzable cephalosporins by cephalosporinases in *Enterobacter cloacae* and *Pseudomonas aeruginosa* as a possible resistance mechanism. Antimicrob Agents Chemother 1982;**21**:711–7
8. Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Acta Pathol Microbiol Scand [Suppl] 1971;**217**:1–90
9. Bryson V, Szybalski W. Microbial selection. Science 1952; **116**:45–51
10. Findell CM, Sherris JC. Susceptibility of *Enterobacter* to cefamandole: evidence for a high mutation rate to resistance. Antimicrob Agents Chemother 1976;**9**:970–4
11. Gootz TD, Sanders CC, Goering RV. Resistance to cefamandole: derepression of β-Lactamases by cefoxitin and mutation in *Enterobacter cloacae*. J Infect Dis 1982;**146**:34–42
12. Sanders CC. Failure to detect resistance in antimicrobial susceptibility tests. A "very major" error of increasing concern. Antimicrobic Newsletter 1984;**1**:27–31
13. Seeberg AH, Wiedemann B. Transfer of the chromosomal *bla* gene from *Enterobacter cloacae* to *Escherichia coli* by RP4::Mini-Mu. J Bacteriol 1984;**157**:89–94