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Bactericidal Killing Activities of Cefepime, Ceftazidime, Cefotaxime, and Ceftriaxone against *Staphylococcus aureus* and β-Lactamase-Producing Strains of *Enterobacter aerogenes* and *Klebsiella pneumoniae* in an In Vitro Infection Model

SHIRLEY M. PALMER,[1]† S. LENA KANG,[1]‡ DIANE M. CAPPELLETTY,[1] AND MICHAEL J. RYBAK[1,2]*

The Antiinfective Research Laboratory, Department of Pharmacy Services, Detroit Receiving Hospital, and University Health Center, College of Pharmacy and Allied Health Professions,[1] and the Department of Internal Medicine, Division of Infectious Diseases, School of Medicine,[2] Wayne State University, Detroit, Michigan 48201

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Cefepime (CP) is a new injectable cephalosporin with a broad spectrum of activity and stability against common chromosomally and plasmid-mediated β-lactamases. The bactericidal activities of CP, ceftazidime (CZ), cefotaxime (CTX), and ceftriaxone (CAX) against reference and clinical strains of *Staphylococcus aureus*, an isogenic pair of *Enterobacter aerogenes* strains (wild type and a CZ-resistant derepressed mutant), and a *Klebsiella pneumoniae* isolate possessing a TEM-10 β-lactamase were investigated in a two-compartment pharmacodynamic in vitro infection model which simulates human pharmacokinetics. An inoculum of ~10^6 CFU/ml was used in all model experiments. Antibiotics were administered to simulate the following regimens: CP at 2 g every 12 h (q12h), CZ at 2 g q8h, CTX at 2 g q8h, and CAX at 2 g q24h. Human albumin was added during experiments with CAX and staphylococci to simulate protein binding. Samples were removed at multiple time points over a 48-h period to determine the inoculum size for time-kill curves. Development of resistance was detected by inoculating samples obtained at 0, 24, and 48 h onto antibiotic-containing agar plates. The time to 99.9% killing was used to compare drug regimens. Against staphylococci, the time to bacterial eradication was significantly delayed with CAX-albumin. All regimens had similar activities against the wild-type *Enterobacter* strain; however, regrowth was noted with CZ, CTX, and CAX against the CZ-resistant strain. There were no differences between the CP, CTX, and CAX regimens against *K. pneumoniae*. Of interest, no regrowth of any organism was noted with CP. These data indicate that CP has activity against *S. aureus* and CZ-resistant gram-negative bacilli.

Cefepime is a new injectable cephalosporin with a broad spectrum of activity against many gram-positive and gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It has poor affinity for inducible chromosomally mediated cephalosporinases (Bush group 1) and is resistant to hydrolysis by most common chromosomally and plasmid-mediated enzymes, including the recently described extended-broad-spectrum β-lactamases of the Bush 2b′ classification (4, 5). In vitro data indicate that cefepime retains activity against gram-negative organisms which have developed resistance to other broad-spectrum cephalosporins and thus may be useful for the treatment of infections involving these bacteria. Like other broad-spectrum cephalosporins, cefepime will probably be used empirically in patients for whom the infecting pathogen has not yet been identified. Therefore, activity against gram positive organisms that is comparable to those of other commonly used agents will be necessary. The purpose of this investigation was to compare the bactericidal activity of cefepime with those of ceftazidime, cefotaxime, and ceftriaxone against *S. aureus* and β-lactamase-producing strains of *Enterobacter aerogenes* and *Klebsiella pneumoniae.*

* Corresponding author. Mailing address: The Antiinfective Research Laboratory, Department of Pharmacy Services, Detroit Receiving Hospital, 4201 St. Antoine Blvd., Detroit, MI 48201. Phone: (313) 745-4554. Fax: (313) 993-2522.

† Present address: College of Pharmacy, University of Florida, Gainesville, FL 32610-0486.

‡ Present address: University of the Pacific, College of Pharmacy, Loma Linda University Medical Center, Loma Linda, CA 92354.

MATERIALS AND METHODS

Bacterial strains. Two strains of methicillin-sensitive *S. aureus* were studied: an American Type Culture Collection strain (25923) and a clinical isolate (380). The strains selected had susceptibilities to the targeted antibiotics similar to those reported in the literature. In order to assess activity against gram-negative organisms which produce Bush type 1 β-lactamase, an isogenic pair of *E. aerogenes* strains was studied (wild-type, strain 3893; derepressed mutant, strain 3978). To assess cefepime's activity against gram-negative bacilli which produce extended-broad-spectrum β-lactamases (Bush group 2b′), a strain of *K. pneumoniae* (2324) possessing a TEM-10 enzyme was selected. MICs and MBCs for each organism at inocula of 10^5, 10^6, and 10^7 CFU/ml were determined for each drug by utilizing broth microdilution techniques according to the procedures outlined by the National Committee for Clinical Laboratory Standards (20).

Antibiotics. Cefepime powder (lot 903) was supplied by Bristol-Myers Squibb, Princeton, N.J. Ceftazidime analytical powder (batch UCRZ 2125) and powder for injection (lot BE897A) were provided by Glaxo, Research Triangle Park, N.C. Ceftriaxone analytical powder (lot 5157) and powder for injection (lot 531900) were supplied by Hoffman-La Roche, Nutley, N.J. Cefotaxime analytical powder (lot RP10233) and powder for injection (lot L562/018008) were provided by Hoechst-Roussel, Sommerville, N.J.

Medium. Mueller-Hinton broth (Difco, Detroit, Michigan) supplemented with 25 mg of calcium per ml and 12.5 mg of magnesium per ml (SMHB) was utilized for susceptibility testing and model experiments.

Preliminary experiments conducted in our laboratory indicated that the addition of albumin (4 g/dl) to the medium (to simulate serum protein binding) significantly affected the bactericidal activity of ceftriaxone against *S. aureus* but had no effect on time-kill curves with ceftriaxone against the gram-negative bacillus with the highest MIC, *E. aerogenes* 3978 (tested at concentrations of 20, 100, and 250 μg/ml [data not shown]). Model experiments with ceftriaxone and staphylococci were conducted in duplicate with SMHB and SMHB containing physiologic concentrations of human albumin (4 g/dl) (Fig. 1).

In vitro model. A two-compartment in vitro model which has been previously

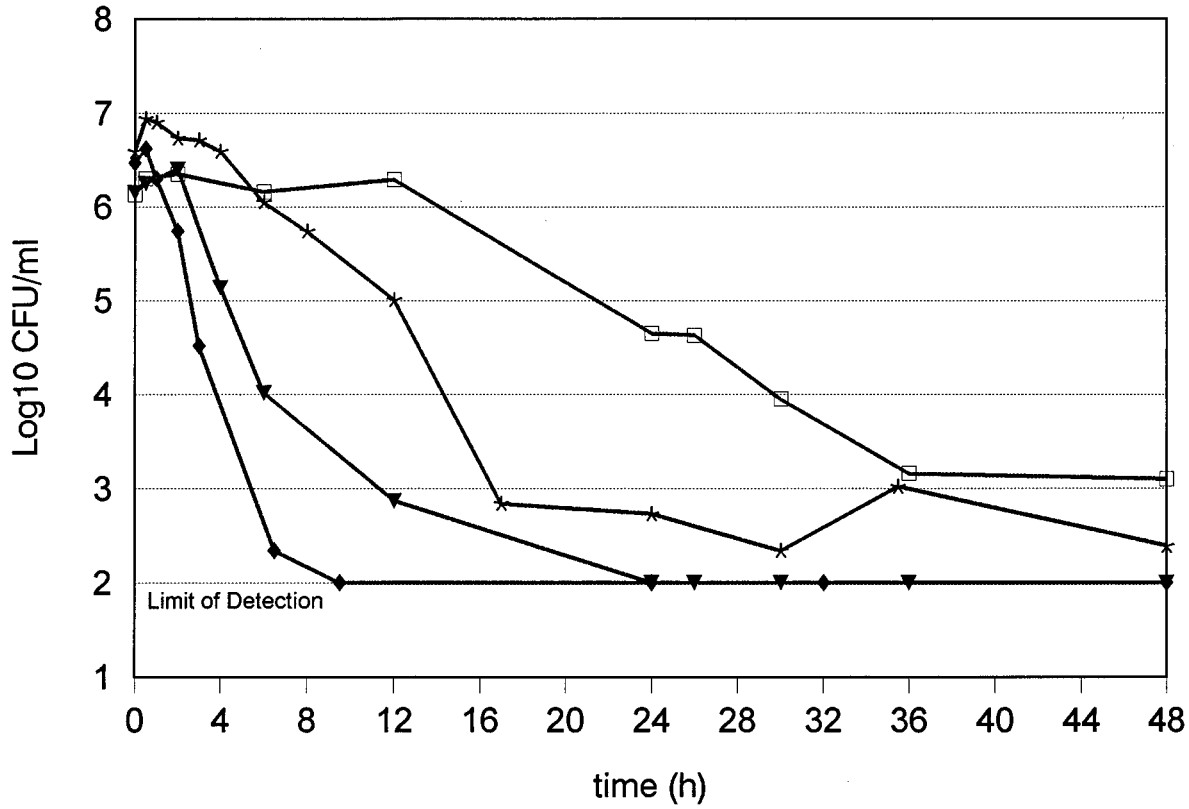

FIG. 1. Activity against *S. aureus* of ceftriaxone in SMHB and in SMHB with 4 g of albumin per dl. ✳, *S. aureus* 25923 in SMHB; □, *S. aureus* 25923 in SMHB plus 4% albumin; ◆, *S. aureus* 380 in SMHB; ▼, *S. aureus* 380 in SMHB plus 4% albumin.

described was utilized (17). Organisms were inoculated into the peripheral compartment, which consisted of an 8- to 12-ml hollow glass T-tube with inert polysulfone membranes (pore size, 0.2 or 0.45 μm) fixed to each end. The pore sizes were selected to prohibit passage of bacteria (0.45 μm for staphylococci, 0.2 μm for gram-negative organisms) yet permit bidirectional diffusion of antibiotics and nutrients between the peripheral and central compartments. Antibiotics were delivered as a bolus into the central compartment, which was represented by a glass reservoir ranging in size from 250 to 500 ml. Magnetic stirring devices placed into each compartment circulated medium to ensure an equal distribution of drug and to aid in temperature maintenance. Clearance of the antimicrobial agents was achieved by using a peristaltic pump to supply fresh, antibiotic-free medium to the central compartment, resulting in the simultaneous displacement of antibiotic-containing medium. The model apparatus was maintained at 37°C in a water bath. All experiments were performed in duplicate.

Stock suspensions of organisms were prepared from an overnight growth of three to five colonies suspended in 3 ml of SMHB and incubated at 37°C. Each sample was diluted appropriately in SMHB and reincubated at 37°C for 1.5 to 2 h to ensure exponential growth. One milliliter of the final culture was used to inoculate the peripheral compartment, achieving a starting inoculum of $\sim 10^6$ CFU/ml.

Antibiotics were administered as described above to simulate clinically achievable peak concentrations in serum following a 2-g intravenous infusion (cefepime, 130 μg/ml; ceftazidime, 160 μg/ml; cefotaxime, 130 μg/ml; and ceftriaxone, 250 μg/ml) (1, 16, 23, 27). The peristaltic pump was set to simulate elimination half-lives of 2 h for cefepime, ceftazidime, and cefotaxime and 8 h for ceftriaxone. Antibiotics were redosed at intervals which would be considered appropriate for adults with normal renal function (cefotaxime and ceftazidime, every 8 h [q8h]; cefepime, q12h; ceftriaxone, q24h).

Pharmacodynamic analysis. Samples (0.1 ml) were removed from the peripheral compartment at time zero (predose), 30 min postbolus, and throughout the dosing interval over a 48-h period (1 to 4, 6, 8 to 16, 24 to 26, 32 to 36, and 48 h) to determine CFU of viable bacteria per milliliter. Serial 10-fold dilutions with sterile, cold 0.9% saline were made, and 20-μl aliquots were plated in triplicate on Tripticase soy agar. The plates were incubated for 18 to 24 h at 37°C; the colonies were then counted, and the inoculum size was determined. At times when the bacterial counts were expected to be below the limits of detection or when antibiotic carryover was expected to be a problem, 100-μl samples were diluted in 10 ml of cold 0.9% saline and filtered by using a Millipore system (gridded 0.45-μm-pore-size filters). Filters were placed aseptically on Trypticase soy agar and incubated for 18 to 24 h, at which time the colonies were counted. Experiments conducted in our laboratory have demonstrated a reliable lower limit of detection of 100 CFU/ml with this method. Kill curves were constructed by plotting the $\log_{10}$ CFU per milliliter versus time over the 48-h period. The time to achieve a 99.9% reduction in the initial inoculum was determined by

TABLE 1. Antimicrobial susceptibilities

Antibiotic[a]	MIC/MBC (μg/ml) for the following strain at the indicated inoculum size (CFU/ml):														
	S. aureus 25923			*S. aureus* 380			*E. aerogenes* 3893			*E. aerogenes* 3978			*K. pneumoniae* 2324		
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
CP	1/16	2/32	8/32	4/8	4/8	4/64	≤0.06/≤0.06	≤0.06/0.25	4/32	0.5/1.0	0.5/2.0	8/16	2/16	2/16	16/32
CZ	8/32	8/32	32/128	8/8	8/16	16/128	<0.5/<0.5	≤0.5/4.0	8/32	64/128	64/128	>512/>512	128/>256	256/>256	>512/>512
CTX	1/8	1/8	2/8	2/4	4/4	2/32	≤0.06/0.06	0.125/0.5	16/32	16/32	16/32	64/128	1/8	2/32	8/64
CAX	1/32	2/16	4/32	2/4	2/4	4/32	0.125/0.125	0.25/2.0	8/32	16/32	16/32	128/256	2/8	4/16	64/128

[a] CP, cefepime; CZ, ceftazidime; CTX, cefotaxime; CAX, ceftriaxone.

TABLE 2. Pharmacokinetics in the in vitro model[a]

Antimicrobial regimen	Central compartment			Peripheral compartment, drug concn (μg/ml) at:	
	Drug concn (μg/ml) at:		Half-life (h)		
	0.5 h	48 h		2 h	48 h
Cefepime, 2 g q12h	99.23 ± 18.49	<2.0	1.70 ± 0.12	30.07 ± 7.58	11.25 ± 2.03
Ceftazidime, 2 g q8h	159.78 ± 24.86	14.43 ± 4.84	1.87 ± 0.11	79.58 ± 3.36	38.22 ± 13.6
Cefotaxime, 2 g q8h	184.29 ± 18.09	11.21 ± 5.38	1.97 ± 0.18	55.14 ± 0.23	35.41 ± 7.19
Ceftriaxone, 2 g q24h	190.49 ± 11.08	19.03 ± 3.27	6.95 ± 0.85	39.38 ± 6.2	52.33 ± 4.05

[a] Values are means ± standard deviations for two to six experiments.

visual inspection of the kill curves. Development of resistance during therapy was detected by inoculating samples obtained at 0, 24, and 48 h onto freshly prepared agar plates containing antibiotics at concentrations equivalent to twice the MIC and 8, 16, 32, or 64 μg/ml (National Committee for Clinical Laboratory Standards breakpoints for each drug) and incubating for 48 h. When resistance was found, MICs for the isolate were obtained prior to and after serial passage onto antibiotic-free agar to determine if the resistance was stable.

Pharmacokinetic analysis. Additional samples of 0.1 ml each were obtained from the central and peripheral compartments at 0, 0.5, 2, 6, 8 to 12, and 16 to 48 h during the experiment to determine drug concentrations. All samples were stored at −80°C prior to assay. Concentrations of cefepime, ceftazidime, cefotaxime, and ceftriaxone were determined by high-performance liquid chromatography (2, 14, 15). The assays were linear in the range of 0 to 70 μg/ml (r^2 = 0.997 to 0.999), with interday coefficients of variation ≤10.6%. All standards and samples were prepared and diluted as necessary in SMHB.

Statistical analysis. Analysis of variance and Tukey's test for multiple comparisons were used to assess differences in time to 99.9% killing. A P value of <0.05 was considered significant.

RESULTS

Susceptibility testing. MICs and MBCs for each of the organisms studied are reported in Table 1. In general, a minimal (ca. twofold) increase in MICs was observed at the higher inoculum of 10^6 CFU/ml, but a significant increase in MICs was seen at an inoculum of 10^7 CFU/ml.

Pharmacokinetics. The pharmacokinetic parameters measured in the in vitro model are reported in Table 2. The mean half-lives in the central compartment were 1.77 ± 0.16 h for cefepime, ceftazidime, and cefotaxime and 6.95 ± 0.85 h for ceftriaxone. The concentrations of antibiotics in the peripheral compartment remained above the MICs for all organisms at 48 h, except in the cases of ceftazidime against *E. aerogenes* 3978 and *K. pneumoniae* 2324.

Pharmacodynamics. Results from the pharmacodynamic studies are presented in Table 3. Against *S. aureus* 25923, cefepime, ceftazidime, and cefotaxime achieved 99.9% killing within 24 h (Fig. 2). In experiments conducted with and without albumin, the bactericidal end point was achieved with ceftriaxone in 15.25 and 35.5 h, respectively (ceftriaxone-albumin versus ceftriaxone, $P < 0.006$; ceftriaxone-albumin versus cefepime, ceftazidime, and cefotaxime, $P < 0.05$). Although organisms were still detectable at 48 h with all regimens except cefepime, the susceptibilities had not changed. Each antimicrobial agent had similar killing activity against *S. aureus* 380, with the exception of ceftriaxone, which required 10.5 h to reach 99.9% killing in the presence of albumin ($P < 0.05$ versus ceftazidime, cefotaxime, and ceftriaxone; $P = 0.059$ versus cefepime) (Fig. 3).

All antibiotics exhibited similar activities against *E. aerogenes* 3893, achieving a 99.9% reduction in the bacterial count within 5 h (Fig. 4). The development of resistance was not detected. With the ceftazidime-resistant *Enterobacter* strain (*E. aerogenes* 3978), all regimens appeared to be similar over the first 6 h; however, significant regrowth occurred with ceftazidime, cefotaxime, and ceftriaxone (Fig. 5). Posttreatment isolates (*E. aerogenes* 3978) from the ceftazidime experiment exhibited an eightfold increase in MICs of cefepime and ceftazidime only. This resistance pattern remained stable after six passes on antibiotic-free medium. The susceptibilities of posttreatment isolates from the remaining experiments with *E. aerogenes* 3978 were unchanged. Against *K. pneumoniae* (ceftazidime resistant), only ceftazidime failed to reduce the bacterial inoculum (Fig. 6). The time to reach the bactericidal end point was not significantly different with the other three regimens, and MICs for posttreatment isolates were unaltered. Of interest, no regrowth of any organism was noted with cefepime.

DISCUSSION

Broad-spectrum cephalosporins have become the "workhorses" of the hospital antibiotic formulary, as they cover a wide variety of potential pathogens. Cephalosporins are frequently used either alone or in combination with an aminoglycoside for the empiric therapy of nosocomial infections. In our investigation, we chose to evaluate monotherapy with cefepime versus that with other commonly prescribed cephalosporins to

TABLE 3. Time required to achieve 99.9% killing[a]

Antimicrobial agent	*S. aureus* 25923		*S. aureus* 380		*E. aerogenes* 3893		*E. aerogenes* 3978		*K. pneumoniae* 2324	
	t99	Inoculum[b] at 48 h	t99	Inoculum at 48 h	t99	Inoculum at 48 h	t99	Inoculum at 48 h	t99	Inoculum at 48 h
Cefepime	23.25	≤2.0	7.25	≤2.0	4.5	≤2.0	4.5	≤2.0	3.5	≤2.0
Ceftazidime	21.25	3.12	6.0	≤2.0	4.5	≤2.0	4.75	9.15	NA	9.14
Cefotaxime	23	3.48	5.5	2.58	4.25	≤2.0	5.25	5.58	5.25	3.61
Ceftriaxone	15.25	2.39	4.75	≤2.0	5.0	≤2.0	3.5	5.76	4.5	2.08
Ceftriaxone-albumin	35.5[c]	3.1	10.0[d]	≤2.0						

[a] t99, time (hours) required to achieve a 99% reduction in $\log_{10}$ CFU per milliliter; NA, not achieved.
[b] Expressed as $\log_{10}$ CFU per milliliter. The detection limit was 2.0 $\log_{10}$ CFU/ml.
[c] $P \leq 0.018$ versus cefepime; $P \leq 0.024$ versus ceftazidime; $P \leq 0.038$ versus cefotaxime; $P \leq 0.006$ versus ceftriaxone.
[d] $P = 0.059$ versus cefepime; $P \leq 0.032$ versus ceftazidime; $P \leq 0.018$ versus cefotaxime; $P \leq 0.012$ versus ceftriaxone.

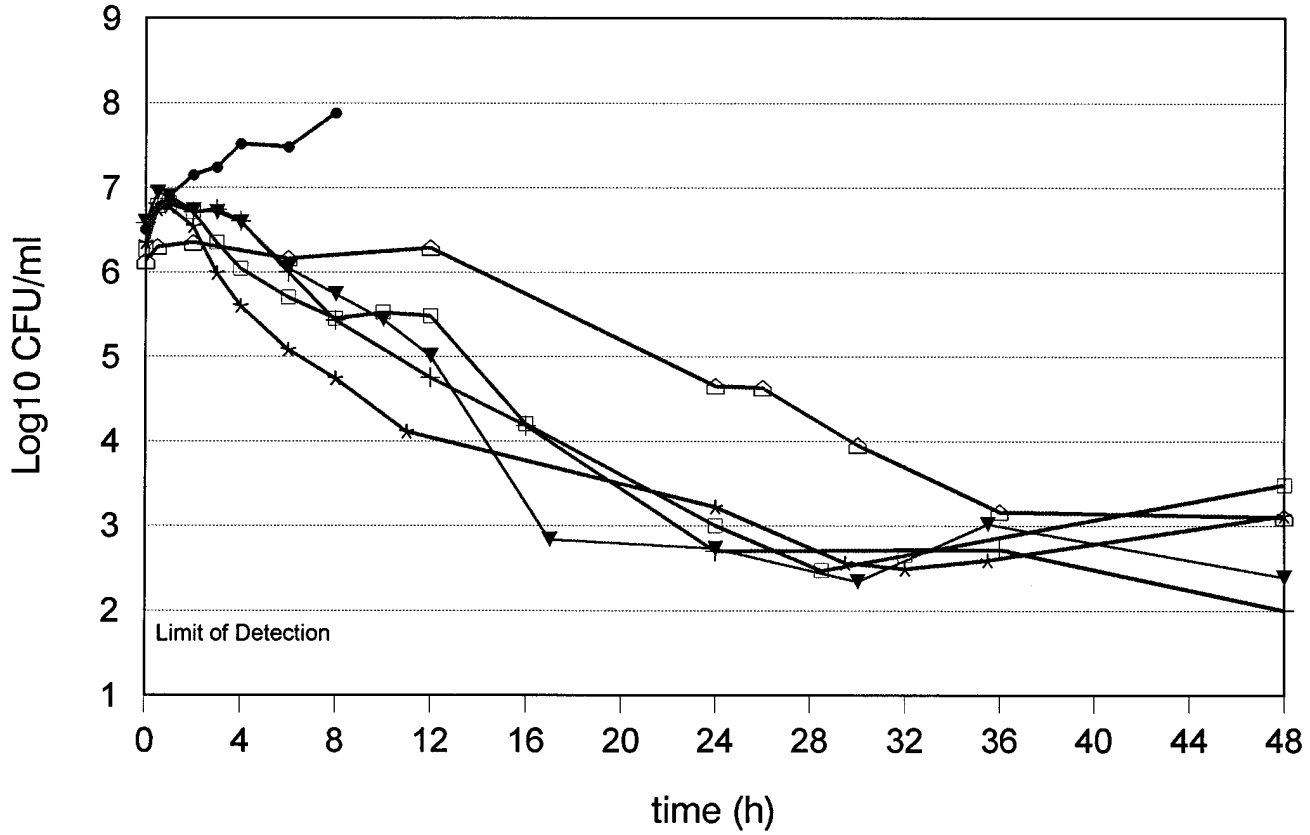

FIG. 2. Time-kill curves for *S. aureus* 25923. ●, growth control; +, cefepime; ✳, ceftazidime; □, cefotaxime; ▼, ceftriaxone (SMHB); ⌓, ceftriaxone (SMHB plus 4% albumin).

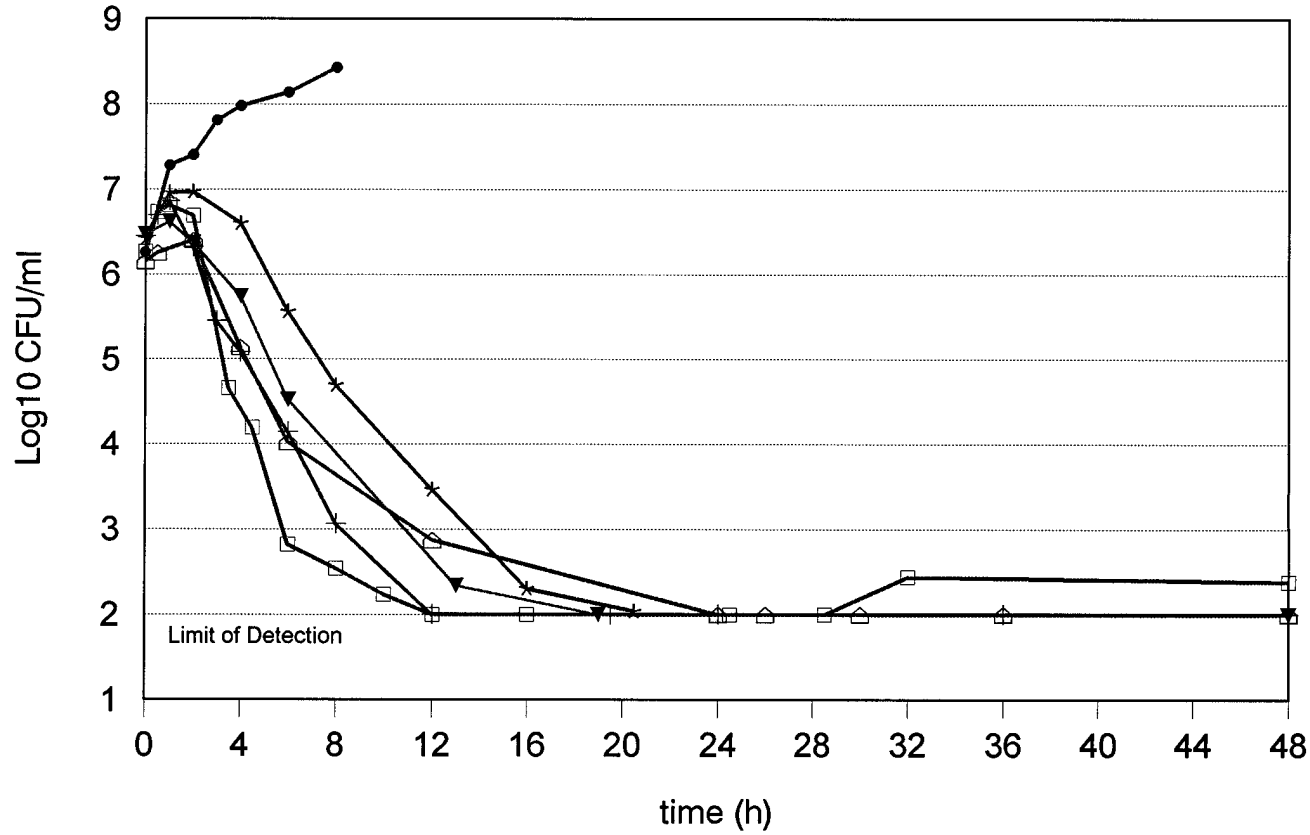

FIG. 3. Time-kill curves for *S. aureus* 380. ●, growth control; +, cefepime; ✳, ceftazidime; □, cefotaxime; ▼, ceftriaxone (SMHB); ⌓, ceftriaxone (SMHB plus 4% albumin).

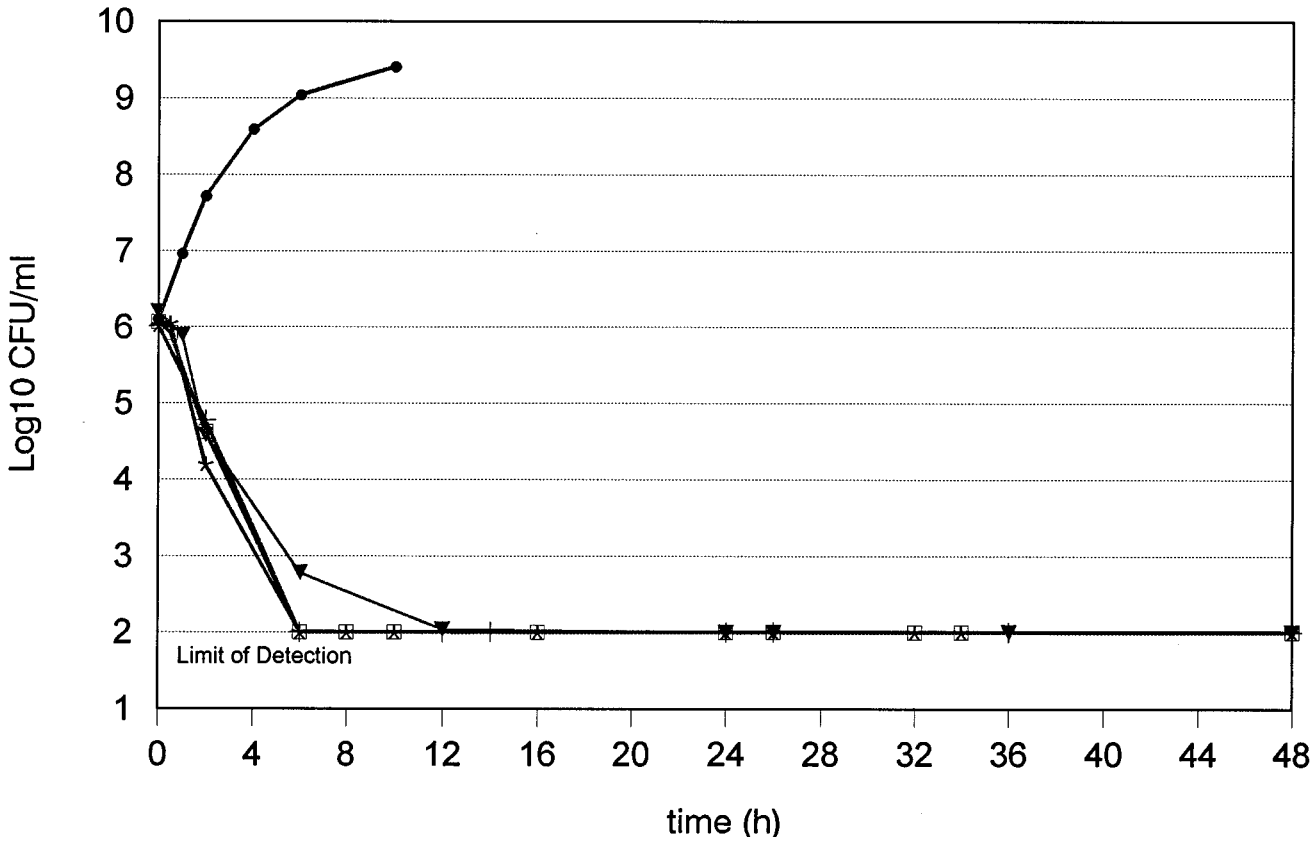

FIG. 4. Time-kill curves for *E. aerogenes* 3893. ●, growth control; +, cefepime; ✻, ceftazidime; □, cefotaxime; ▼, ceftriaxone (SMHB).

determine differences among the agents with respect to potency against these multidrug-resistant isolates.

The effect of protein binding on antimicrobial efficacy has been a subject of great debate. Nonetheless, there is considerable evidence from in vitro studies and in vivo animal models which indicates that the presence of serum proteins significantly impairs the activity of highly protein-bound β-lactams against *S. aureus* (9, 10, 13, 18, 32). Reports in the literature have also associated high levels of protein binding with treatment failures in patients with staphylococcal infections who are receiving teicoplanin and cefonicid (6, 7).

In general, the amount of free drug able to penetrate tissues is markedly influenced only when protein binding exceeds 90% (32). Cefepime, ceftazidime, and cefotaxime are minimally bound (19, 21, and 38%, respectively), while ceftriaxone exhibits concentration-dependent binding ranging from 84% at a concentration of 300 μg/ml in serum to 96% at concentrations of <70 μg/ml (30). In our investigation, the effect of protein binding upon the antistaphylococcal activity of ceftriaxone was found to be significant. In the presence of albumin, the onset of killing was delayed and the rate of bacterial eradication was markedly reduced so that the time to reach 99.9% killing was increased greater than twofold, resulting in significant differences between the ceftriaxone-albumin regimen and other drug regimens.

Selection of resistant isolates which constitutively overproduce chromosomal β-lactamases has remained a problem in the clinical setting. Although they were initially regarded as stable, most broad-spectrum cephalosporins are hydrolyzed to some extent by high levels of enzyme, rendering them useless against derepressed strains. It has been suggested that combination therapy with a cephalosporin or broad-spectrum penicillin and an aminoglycoside may be necessary in order to prevent the emergence of resistance during therapy; however, recent experience indicates that the addition of an aminoglycoside does not always prevent resistance from occurring (8). Cefepime appears to retain activity against these isolates, possibly because of a combination of factors, including faster penetration through the outer membranes of gram-negative bacteria, poor affinity for most β-lactamases, and increased stability to hydrolysis (3, 22, 24, 31). The *Enterobacter* strains utilized in our experiments were an isogenic pair obtained from a patient enrolled in the ceftazidime arm of a multicenter comparative trial of cefepime versus ceftazidime. The initial isolate (*E. aerogenes* 3893, ceftazidime sensitive) became resistant during therapy (*E. aerogenes* 3978), resulting in treatment failure. Analysis of the pretreatment and posttreatment isolates revealed that the strain had undergone spontaneous mutation to the stably derepressed state, producing copious amounts of β-lactamase of the Bush type 1 class (14a). In contrast, selection of resistance with *E. aerogenes* 3893 was not observed with any antimicrobial regimen in our model. It is possible that our inoculum was too low to detect resistant subpopulations, as these mutants are reported to occur in one of every 10^6 to 10^7 wild-type cells of strains possessing inducible type 1 cephalosporinases (28, 29). In addition, the duration of our experiments (48 h) may not have been sufficient for a resistant subpopulation to emerge. Cefepime had the greatest in vitro activity against the ceftazidime-resistant mutant; although the MICs were increased 10-fold (from 0.06 to 0.5

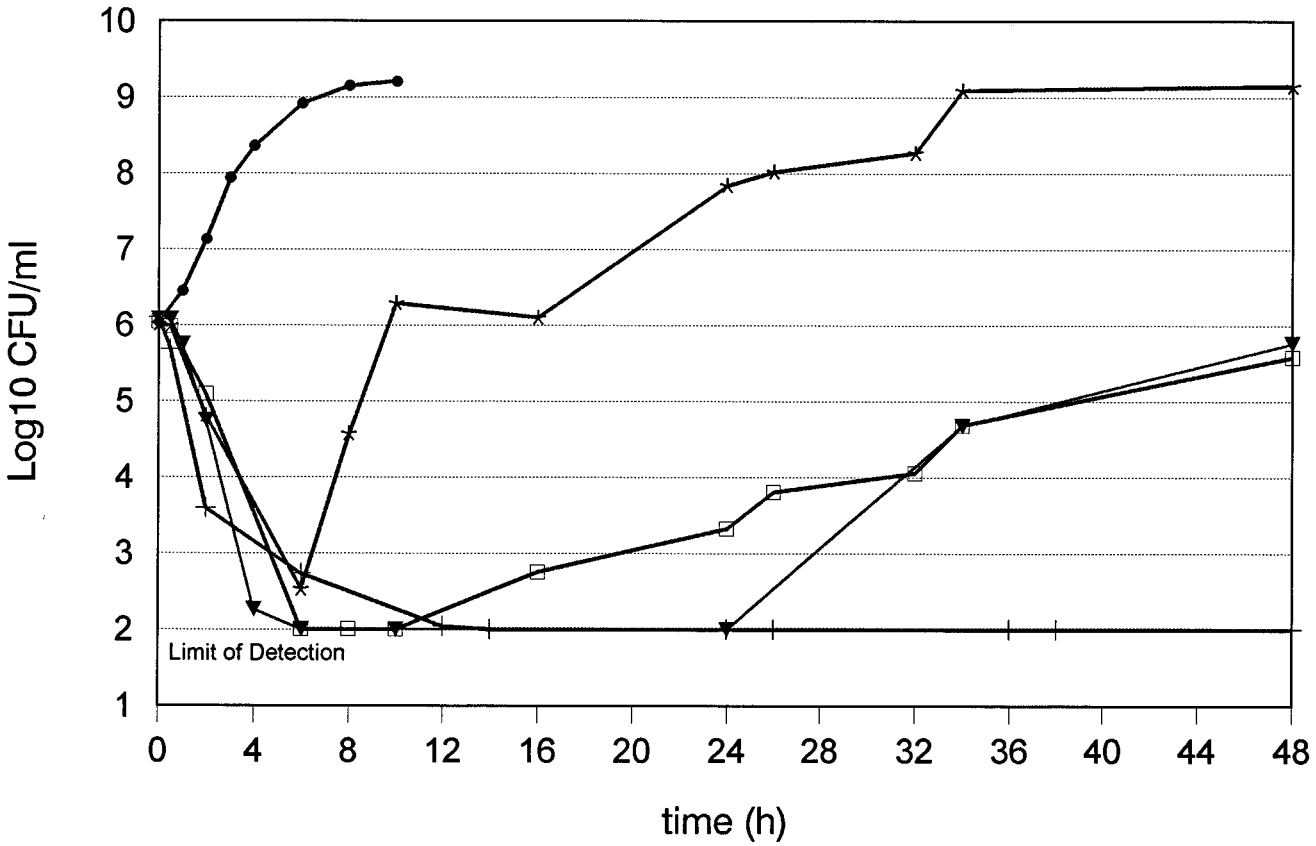

FIG. 5. Time-kill curves for *E. aerogenes* 3978. ●, growth control; +, cefepime; ✳, ceftazidime; □, cefotaxime; ▼, ceftriaxone (SMHB).

μg/ml), they still remained within the range for the organism to be considered susceptible, while MICs of the other agents increased more than 100-fold. These data are consistent with those reported by other investigators, as the MICs of cefepime are usually found to be proportionately lower than those of other broad-spectrum cephalosporins against ceftazidime-resistant gram-negative bacilli (11, 24). In our pharmacodynamic experiments, only cefepime was able to suppress regrowth of the mutant *Enterobacter* strain. Regrowth was noted with cefotaxime and ceftriaxone, despite adequate antibiotic concentrations in the peripheral compartment. It has been suggested that bacteria which adhere to a foreign body surface have decreased susceptibility to antimicrobial agents; once the bacteria have become dislodged, normal growth resumes and antimicrobial susceptibility is restored. It is possible that this "regrowth" was an artifact of the model, secondary to adherent bacteria on the interior surface of the infection compartment, as no change in the antimicrobial susceptibilities of post-treatment isolates was noted (12). Interestingly, the susceptibility pattern of *E. aerogenes* 3978 was altered following treatment with ceftazidime. We are not sure why this occurred. An alteration in porin channels would result in susceptibility differences; however, cefepime would be less likely to be affected because of its enhanced penetrability, and the other three agents would have been affected equally. Alterations in penicillin-binding proteins should also affect these agents in a similar manner. It is possible that a mutation in the enzyme may have occurred, similar to that seen with plasmid-mediated enzymes of the TEM family, which could affect substrate specificity and result in differences in MICs with the various cephalosporins. To our knowledge, this has not yet been described for chromosomally mediated, class 1 enzymes.

Plasmid-mediated enzymes which confer resistance to broad-spectrum cephalosporins were first described in Europe in the 1980s. Recently, several nosocomial outbreaks of members of the family *Enterobacteriaceae* (primarily *K. pneumoniae*) that produce extended-spectrum β-lactamases have been reported in hospitals in the United States (19, 21, 25, 26). Most isolates produced TEM-10 or TEM-26 β-lactamases, which hydrolyze ceftazidime and aztreonam but possess variable activities against other agents. Routine susceptibility testing suggests that these strains may remain susceptible to cephamycins, cefotaxime, and ceftriaxone, although Meyer et al. found that only imipenem had consistent bactericidal activity as measured by MBCs (19). Our in vitro model is advantageous in that it allows us to examine the effect of varying drug concentrations upon killing activity over time. In our experiments, cefepime, cefotaxime, and ceftriaxone all exhibited bactericidal activity against the TEM-10-producing *Klebsiella* strain, despite MICs and MBCs of the latter two agents being in the intermediate range.

The overuse of broad-spectrum cephalosporins, particularly ceftazidime, has been implicated in the emergence of multidrug-resistant gram-negative bacteria in institutions across the United States and in Europe (8, 19, 21, 26). Cefepime may differ from ceftazidime in that it does not appear to induce or select for resistance in bacteria which commonly produce chromosomally mediated enzymes. Cefepime may also retain activity against gram-negative bacilli that produce extended-spectrum β-lactamases, which are resistant to many broad-

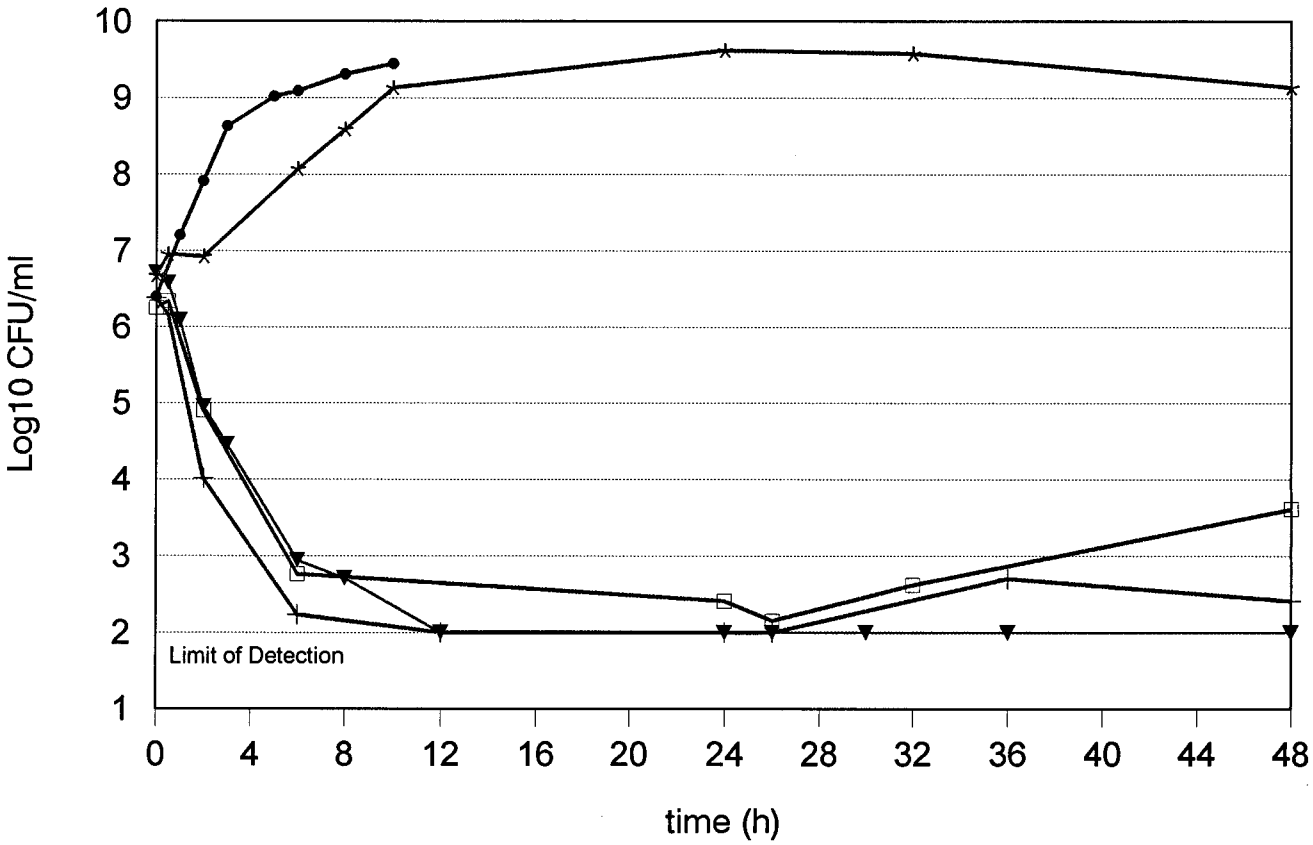

FIG. 6. Time-kill curves for *K. pneumoniae* 2324. ●, growth control; +, cefepime; ✳, ceftazidime; □, cefotaxime; ▼, ceftriaxone (SMHB).

spectrum agents. However, plasmid-mediated enzymes vary considerably in their affinity for cephalosporins, and some strains remain susceptible to agents such as cefotaxime, ceftriaxone, and the cephamycins. While cefepime's broad spectrum of activity may render it an attractive alternative to existing broad-spectrum cephalosporins, further clinical experience is necessary to determine if the potential for resistance will remain low. Since most β-lactams, including these cephalosporins, are subject to an inoculum effect, it is possible that greater differences between the various drug regimens would have been seen with a larger inoculum. Therefore, investigators should exercise caution in extrapolating our results to the treatment of specific infections in which a larger organism load may be present.

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