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Bactericidal Interactions of a β-Lactam and β-Lactamase Inhibitors in Experimental *Pseudomonas aeruginosa* Endocarditis Caused by a Constitutive Overproducer of Type Id β-Lactamase

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We investigated the in vitro and in vivo effects of a combination of a β-lactam (ceftazidime) and a β-lactamase inhibitor (dicloxacillin) to synergistically kill a ceftazidime-resistant variant, *Pseudomonas aeruginosa* PA-48, which overproduces type Id cephalosporinase constitutively. In vitro, dicloxacillin plus ceftazidime exerted bactericidal synergy at ~10^5 CFU/ml of inoculum (but not at ~10^7-CFU inoculum), whereas other β-lactamase inhibitors (sulbactam, clavulanic acid) showed no enhanced killing of PA-48 when combined with ceftazidime at clinically achievable levels for each agent. Dicloxacillin was a potent competitive inhibitor of the extracted Id cephalosporinase from strain PA-48 in short-term comixture studies (<10 min [K_i = 2 nM]); in contrast, longer-term comixture studies (90 min) indicated that dicloxacillin functions as a competitive substrate for the enzyme. Growth of PA-48 cells in the presence of dicloxacillin (12.5 to 100 μg/ml) had no significant effect on the production rates or functional activity of the Id enzyme. In experimental aortic valve endocarditis due to the ceftazidime-resistant variant (PA-48), rabbits received either no therapy, ceftazidime (25 mg/kg intramuscularly, every 4 h), or ceftazidime plus dicloxacillin (200 mg/kg intramuscularly, every 4 h). The combination regimen reduced mean bacterial densities of PA-48 within cardiac vegetations significantly below those in the other groups at both days 3 and 6 of treatment ($P < 0.005$). However, mean vegetation bacterial densities remained >6 $\log_{10}$ CFU/g in the combined treatment group. This modest in vivo synergistic effect (as compared to striking in vitro synergy at ~10^5-CFU inoculum) most likely reflects the high densities of PA-48 achieved in vivo within cardiac vegetations (>8 $\log_{10}$ CFU/g).

The medical therapy in human cases of *Pseudomonas aeruginosa* endocarditis has been disappointing, particularly in aortic and mitral valve involvements (25, 26). One of the limiting factors in this regard, as exemplified in human as well as experimental *Pseudomonas* endocarditis, has been in vivo development of antibiotic resistance. Such resistances have been variably directed towards the aminoglycoside or β-lactam components of the combination therapy regimens generally used to treat *Pseudomonas* endocarditis (1, 19, 23, 26). The mechanism of β-lactam resistance in these situations has usually been associated with either inducible or constitutive β-lactamase overproduction (3, 19, 26).

One possible strategy to circumvent the above β-lactamase-related resistances is to interfere with the function of the enzyme by using β-lactamase inhibitors. The present study was designed to (i) evaluate several β-lactamase inhibitors in vitro for their ability, when combined with a β-lactam agent (ceftazidime), to synergistically kill a ceftazidime-resistant strain of *P. aeruginosa* which constitutively overproduces β-lactamase; (ii) define the ability of β-lactamase inhibitors to specifically abrogate enzyme activity in vitro; and (iii) determine the in vivo effect of combinations of β-lactam plus β-lactamase inhibitors in experimental endocarditis caused by the variant, *P. aeruginosa* PA-48, which constitutively overproduces type Id β-lactamase.

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MATERIALS AND METHODS

Organism. The *Pseudomonas* strain used in this study (PA-48) is a ceftazidime-resistant mutant strain isolated from cardiac vegetations during the therapy of experimental endocarditis with ceftazidime monotherapy (1). The identification, serotyping and rabbit serum resistance of the parental strain for PA-48 used to initially infect rabbits (PA-96) have been defined previously (5). The resistant variant (PA-48) has been characterized in detail elsewhere (3). Briefly, PA-48 is a mutant of PA-96 that constitutively overproduces the type Id β-lactamase which is inducible in the parent strain (PA-96). Periplasmic β-lactamase contents of PA-48 exceed those of the parental strain by ~300 times in the uninduced state (3). The enzyme exhibits an M_w of ~41,000, an alkaline pI (8.1), and a substrate profile which includes cephaloridine and cefamandole, typical of the Id category (cephalosporinases [30]). In addition, this enzyme demonstrates evidence of slow hydrolysis of ceftazidime, a β-lactamase-stable cephalosporin (3). The MICs and MBCs (μg/ml) for PA-48 of selected β-lactams at an inoculum of ~10^5 logarithmic-phase cells per ml in Mueller-Hinton broth (MHB) are as follows: ceftazidime, 128 and 128; cefoperazone, 64 and 128; moxalactam, 64 and 128; and azlocillin, 128 and 128 (1).

Time-kill curves. The ability of PA-48 to be synergistically

killed in vitro by combinations of ceftazidime (Glaxo Research, Durham, N.C.) and various β-lactamase inhibitors was defined by the kill-curve technique. The β-lactamase inhibitors tested were dicloxacillin (Bristol Pharmaceuticals, Syracuse, N.Y.), sulbactam (Pfizer Pharmaceuticals, Pearl River, N.Y.), and clavulanic acid (Beecham Pharmaceuticals, Bristol, Tenn.). PA-48 was grown overnight in MHB and diluted in fresh MHB to approximate a nephelometer standard of $\sim 2 \times 10^5$ CFU/ml. Nephelometer approximations were confirmed by dilution plate colony counts. Ceftazidime was added to achieve a final concentration of 25 μg/ml (~1/5 MIC). The β-lactamase inhibitors were added to achieve final concentrations as follows: dicloxacillin (25 μg/ml), sulbactam (12.5 μg/ml), and clavulanic acid (5 μg/ml). These concentrations were chosen to encompass levels achievable in human serum, as well as representing levels below which inactivation of β-lactamases susceptible to their respective actions occur (6, 10, 11, 29). One tube each contained PA-48 in ceftazidime alone and in antibiotic-free MHB as a growth control. In a parallel experiment, timed killing was measured by using an initial inoculum of $\sim 10^7$ CFU/ml, a *Pseudomonas* bacterial density regularly achieved within aortic valve vegetations of rabbits with experimental endocarditis (1). After a time-zero quantitative culture was obtained, all tubes were incubated at 37°C; samples for quantitative culture into antibiotic-free MH agar were serially taken at 4, 24, and 48 h of incubation, and time-kill curves were constructed comparing surviving $\log_{10}$ CFU per milliliter versus incubation time. Bactericidal synergy was considered present if β-lactam plus β-lactamase inhibitor combinations caused a >2 $\log_{10}$ decrease in surviving CFU per milliliter at 24 and 48 h as compared with the single agents.

Enzyme inactivation studies. To directly assess the ability of dicloxacillin to inhibit the production rates or activity of the β-lactamase of the mutant, cells were grown in the presence of various concentrations of dicloxacillin (0 to 100 μg/ml). Overnight cultures of PA-48 in MHB were inoculated at a 1:100 dilution into fresh medium supplemented with dicloxacillin and grown into log phase (optical density at 600 nm, 0.6 to 0.8). Cells were harvested by centrifugation, washed twice in buffer to remove excess dicloxacillin, and broken by three passes through a French press (15,000 psi). The unbroken cells were removed by centrifugation, and the membranes and soluble fraction were centrifuged for 1 h at 100,000 × *g* at 4°C. The supernatant was used as the enzyme preparation. This method proved more reliable at reproducibly releasing the periplasmic enzyme than the freeze-thaw technique (15). Protein concentrations were determined by the method of Sanderman and Strominger (27). The spectrophotometric nitrocefin assay previously described (3) was used to determine the kinetics of the β-lactamase activity via Lineweaver-Burk double reciprocal plotting (17). Results for the V_{max} were expressed in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein in the enzyme preparation. Inhibition of the hydrolytic function of the extracted β-lactamase by dicloxacillin was characterized by the method of Dixon and Webb (7). To further assess inhibition by dicloxacillin of the β-lactamases in the growing cells of PA-48, a modification of the method of Nicas and Hancock was used (21). Dicloxacillin at 10 nM increments from 10 to 100 nM was coincubated with 90 μM nitrocefin and whole log-phase PA-48 cells in phosphate buffer, pH 7.0 (final volume, 1 ml). The assay was run for 3 min or until stable reproducible rates were obtained. The inhibition of the nitrocefin hydrolytic rate by viable PA-48 cells was plotted versus the concentration of the inhibitor (dicloxacillin); the dicloxacillin concentration inhibiting nitrocefin hydrolysis by 50% was calculated. Potential leakage of β-lactamase from the periplasm was corrected for by subtracting the rate of hydrolysis of an equal portion of cell-free supernatant from the observed hydrolytic rate. Periplasmic enzyme leakage never accounted for more than 1% of the total hydrolytic activity in the whole-cell enzyme assay.

To determine whether dicloxacillin functioned as a pure competitive inhibitor or as a competitive substrate of the extracted Id enzyme, longer-term comixture experiments were done. Briefly, French press-extracted Id enzyme from PA-48 (42 μg) was incubated with dicloxacillin (50 μM final concentration) at 20°C for 90 min; a parallel tube contained the same amount of enzyme in phosphate buffer (pH 7.0) alone. At 10-min intervals, sample from each enzyme tube was removed and the hydrolytic rate of nitrocefin (50 μM) was determined as described above. The relative activity of the enzyme plus dicloxacillin mixture at each 10-min interval was determined ([hydrolytic rate of enzyme plus dicloxacillin/hydrolytic rate of enzyme alone] × 100%) and plotted versus time.

Induction and treatment of experimental *Pseudomonas* endocarditis. Fifty female New Zealand White rabbits weighing ~2.5 kg were anesthetized with 50 mg of ketamine hydrochloride given intramuscularly (i.m.) (Bristol). Transaortic valve catheterization was performed with a polyethylene catheter by the transcarotid approach as previously described (1). Each animal was then inoculated intravenously with $\sim 10^8$ CFU of PA-48 at 24 h postcatheterization. The catheter was left in place throughout the experiment, inducing a form of endocarditis that is a severe test of antimicrobial efficacy (1). After confirming induction of *Pseudomonas* endocarditis with positive blood cultures 24 h postinoculation, animals were randomized to one of three therapy groups: untreated controls; ceftazidime at 25 mg/kg i.m., given every 4 h; or ceftazidime at the above dosage regimen plus dicloxacillin at 200 mg/kg i.m., every 4 h. The ceftazidime dosages chosen regularly achieve levels in rabbit serum at ~1 h postdosage of >25 μg/ml (approximating the in vitro concentrations used in kill-curve assays). The dicloxacillin dosage was chosen as it consistently produces peak levels in serum of >20 μg/ml, a concentration similar to that used in the in vitro bactericidal synergy studies. The ceftazidime treatment group was included as an additional control in this study, in order to confirm in vivo stability of ceftazidime resistance during therapy with this agent. All therapy was begun 24 h after induction of endocarditis. Animals were randomly assigned to receive 3 or 6 days of therapy. A treatment group for dicloxacillin alone was not included in this study. The MIC and MBC of dicloxacillin for PA-48 were each >500 μg/ml; also, pilot studies in our laboratory had indicated that dicloxacillin administered at 200 mg/kg i.m. every 4 h yielded intravegetation densities of PA-48 no different from those of untreated controls.

Sacrifices. Animals were sacrificed by sodium pentobarbital overdosage after 3 or 6 days of treatment. At sacrifice, aortic valve vegetations were excised, weighed, and quantitatively cultured as previously described (1). No attempt was made to inactivate ceftazidime or dicloxacillin in vegetation homogenates, as all sacrifices were performed at least 12 h after the last drug dose; also, serial dilutions of the vegetation homogenate during quantitative culturing minimized antibiotic carry-over. Portions of each vegetation homogenate were also quantitatively cultured into MH agar

containing ceftazidime (50 μg/ml) to confirm persistence of ceftazidime resistance during the therapy period. To delineate the relative proportions of ceftazidime-resistant and ceftazidime-susceptible isolates within each vegetation, a resistance ratio was calculated ($\log_{10}$[number of resistant isolates/total number of isolates] [4]).

Antibiotic levels in serum. Antibiotic levels were determined in serum samples before and ~1 h after drug administrations on therapy day 3. Ceftazidime levels were determined in animals receiving monotherapy or combination therapy by bioassay, with *Escherichia coli* ATCC 10536 as the indicator organism. Dicloxacillin levels were determined by high-performance liquid chromatography. The sensitivity level of the bioassay for ceftazidime was 0.5 μg/ml, and that for the dicloxacillin assay by high-performance liquid chromatography was 0.05 μg/ml.

Vegetation antibiotic levels. Because animal sacrifices were performed >12 h after the last antibiotic doses, we determined achievable antibiotic levels in vegetations in a separate experiment. Three additional animals with aortic valve endocarditis due to PA-48 (induced as before) were each given a single dose of dicloxacillin (200 mg/kg, i.m.) and then sacrificed 1 h later. At 10 min before sacrifice, each animal received 1,000 U of heparin intravenously to facilitate the removal of surface blood. At sacrifice, individual aortic valve vegetations were removed, carefully blotted dry on filter paper, and then dried in sterile vials at 70°C for 48 h to remove antibiotic-containing extracellular tissue fluid (11). These procedures reduce blood contamination of vegetations to <3% (19). The vegetations were then weighed and homogenized in sterile saline solution (1 ml) and assayed for dicloxacillin levels by high-performance liquid chromatography. Because individual vegetation weights varied from ~0.001 to 0.25 g in this study (mean, ~0.01 g), results were expressed in micrograms of dicloxacillin per gram of vegetation. Ceftazidime levels achieved within cardiac vegetations were not determined, as these have been recently delineated in detail (20) and have been shown to closely approximate levels in serum.

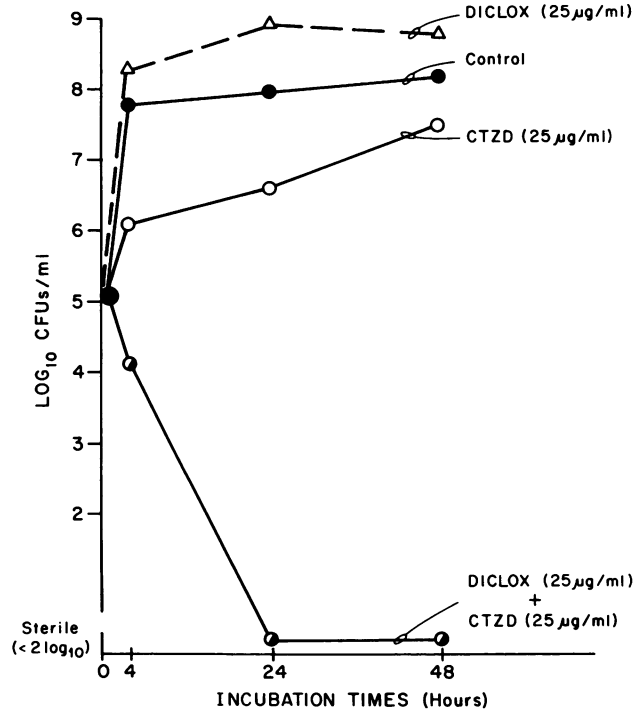

FIG. 1. Effect of dicloxacillin (DICLOX) on ceftazidime (CTZD) killing of a stably derepressed *P. aeruginosa* mutant (PA-48) which constitutively overproduces type Id β-lactamase. The initial inoculum was 10^5 CFU/ml.

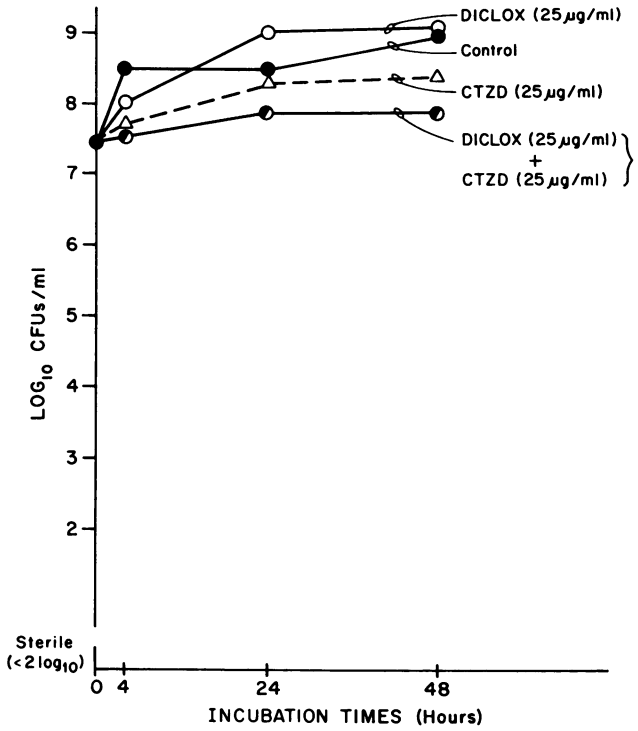

FIG. 2. Effect of dicloxacillin (DICLOX) on ceftazidime (CTZD) killing of PA-48. The initial inoculum was 10^7 CFU/ml.

Statistical evaluation. The Chi-square test with Yates correction factor was used for comparing proportional data, and the analysis of variance test was used for comparing the $\log_{10}$ CFU per gram of vegetation in the therapy groups. A P value of <0.05 was considered significant.

RESULTS

Time-kill curves. At ~10^5-CFU inoculum, the combination of dicloxacillin plus ceftazidime exerted a synergistic bactericidal effect in vitro (Fig. 1); by 24 h of incubation, bacterial counts (CFU per milliliter) in the antibiotic combination group were >6 $\log_{10}$ below those in the other groups. In contrast at ~10^7-CFU inoculum, no enhanced killing was observed with dicloxacillin plus ceftazidime (Fig. 2).

TABLE 1. Kinetics of nitrocefin hydrolysis by type Id β-lactamase after growth of source strain PA-48 in presence or absence of dicloxacillin

Dicloxacillin (μg/ml)	K_m (μM ± SD)	V_{max} (nmol/min per mg ± SD)
100	88 ± 8	1,458 ± 100
50	88 ± 9	882 ± 54
25	82 ± 2	960 ± 51
12.5	85 ± 5	1,304 ± 268
0	81 ± 5	813 ± 66

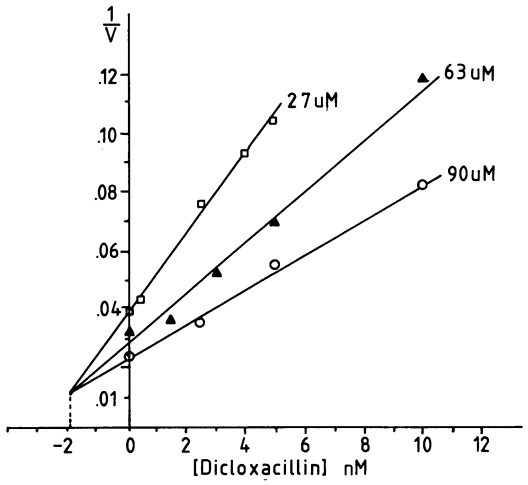

FIG. 3. Dixon plot of the inhibition, by different concentrations of dicloxacillin, of nitrocefin hydrolysis. The reciprocal of the rate of hydrolysis (1/*V* [velocity] in micromoles of nitrocefin hydrolyzed per milligram of protein per minute) was plotted against the inhibitor concentration for three different concentrations of nitrocefin: 27, 63, and 90 μM.

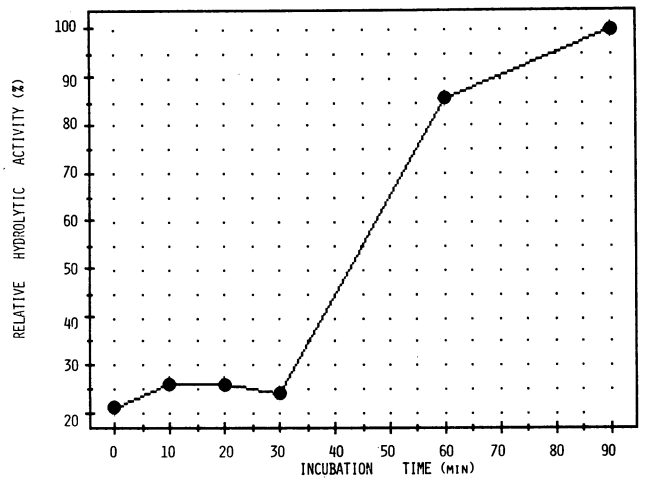

FIG. 5. Relative hydrolytic capacity of nitrocefin by enzyme plus dicloxacillin comixture as compared to enzyme alone. Relative activity (*y* axis) = (hydrolytic activity of enzyme plus dicloxacillin/hydrolytic activity of enzyme) × 100%.

For the other β-lactamase inhibitors tested (sulbactam and clavulanic acid), no bactericidal synergy was observed at either the ~10^5- or ~10^7-CFU inoculum (comparative kill curves were indifferent [data not shown]).

Enzyme inactivation studies. Table 1 depicts the characteristics of the β-lactamase of PA-48 extracted after growth of the organism in vitro in the presence of various concentrations of dicloxacillin. Periplasmic protein concentrations were virtually identical in cells grown in the presence or absence of dicloxacillin (~4 mg/ml, data not shown). Since the periplasmic β-lactamase of this strain consistently accounts for ~14% of the periplasmic protein content (3), it is unlikely that dicloxacillin caused a reduction in enzyme production rates. Also, the kinetics of hydrolysis of nitrocefin by the extracted periplasmic β-lactamase from PA-48 cells grown in the presence or absence of dicloxacillin were not significantly different (Table 1), suggesting that dicloxacillin does not irreversibly abrogate the function of this enzyme.

To determine the affinity of dicloxacillin as a competitive inhibitor of nitrocefin hydrolysis by the extracted Id β-lactamase from PA-48, Dixon inhibition plots were constructed (Fig. 3). Dicloxacillin proved to be a potent competitive inhibitor of nitrocefin hydrolysis by this enzyme, with a K_i of dicloxacillin for the β-lactamase of 2 nM; this K_i is similar to that previously reported by others (31). This avid short-term binding of dicloxacillin to the Id β-lactamase was ~5,000 times that recently observed for ceftazidime to the same enzyme (3). By using viable whole cells of PA-48 grown in MHB, the inhibition of β-lactamase-mediated nitrocefin hydrolysis by dicloxacillin was determined. Dicloxacillin at 34.9 nM (± 8.7 nM [standard deviation]) inhibited the rate of nitrocefin hydrolysis by 50% (Fig. 4), in agreement with data from Then and Angehrn (31).

Of interest, in longer-term coincubation studies, dicloxacillin formed an unstable substrate-enzyme complex with a half-life of ~44 min (Fig. 5). The activity of the enzyme (when coincubated with dicloxacillin) returned to that of control enzyme incubated in the absence of dicloxacillin within 90 min, suggesting either slow dicloxacillin hydrolysis or dicloxacillin release from the substrate-enzyme complex in a form incapable of rebinding to enzyme.

In vivo studies. (i) Mortality. Of 15 untreated control animals, 12 (80%) died before their assigned sacrifice time; in contrast, there was no significant difference in mortality

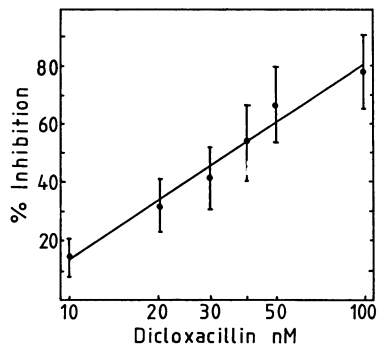

FIG. 4. Inhibition of nitrocefin hydrolysis by dicloxacillin in a whole-cell assay. The percent inhibition of nitrocefin (90 μM) hydrolysis is plotted against various dicloxacillin concentrations (10 to 100 nM). The data points represent the mean ± standard deviation of six independent experiments using strain PA-48 cells harvested in logarithmic phase. The line was drawn by regression analysis. The 50% inhibitory dose for dicloxacillin was determined to be 34.9 ± 8.7 nM.

TABLE 2. Mean vegetation bacterial densities

Group	Mean vegetation bacterial densities (log_{10} CFU/g ± SEM) on therapy day:	
	3	6
Control	8.13 ± 0.25 (3/8)[a]	–[b]
CTZD[c]	7.84 ± 0.21 (6/18)[d]	8.04 ± 0.22 (7/19)[e]
CTZD + DICLOX[f]	6.34 ± 0.39 (8/23)[d]	6.52 ± 0.4 (6/18)[e]

[a] Number of rabbits sacrificed/number of vegetations sampled.
[b] –, All controls had died or been sacrificed.
[c] CTZD, Ceftazidime.
[d,e] $P < 0.005$ for data followed by the same superscript.
[f] DICLOX, Dicloxacillin.

rates between animals receiving ceftazidime monotherapy (2 of 16; 13%) or ceftazidime plus dicloxacillin (5 of 19; 26%). The mortality rate for animals given ceftazidime monotherapy is similar to prior experience with this model (1).

(ii) Bacterial densities within vegetations. After 3 days of therapy, mean bacterial densities of *P. aeruginosa* were ~1.5 $\log_{10}$ CFU/g less in vegetations from recipients of ceftazidime-dicloxacillin combination therapy as compared with animals given ceftazidime monotherapy ($P < 0.005$; Table 2). Similarly, intravegetation bacterial densities in recipients of combination therapy remained significantly below those of ceftazidime monotherapy recipients after 6 days of treatment ($P < 0.005$).

As in previous studies with this model (1), the ceftazidime resistance phenotype persisted throughout the experimental period. Resistance ratios were low at each sacrifice time (mean ± standard error of the mean = −0.64 ± 0.08; range, 0 to −3.0), confirming that most of the organisms within vegetations remained ceftazidime resistant (4).

Intravegetation antibiotic levels and levels in serum. Mean dicloxacillin levels found in serum at 1 h postdosing were 107 ± 16 μg/ml (range, 63 to 178 μg/ml); <0.05 μg of dicloxacillin per ml was detectable just before dosing (trough). For ceftazidime, mean levels in serum 1 h postdosing were 78 ± 8.80 μg/ml; no significant differences were seen between animals receiving ceftazidime as monotherapy or combined with dicloxacillin (range, 50 to 110 μg/ml). Less than or equal to 0.5 μg of ceftazidime per ml was detected in trough samples.

Mean dicloxacillin levels achieved within aortic vegetations were 83 ± 19 μg/g (range, 33 to 167 μg/g).

DISCUSSION

Recent experiences in our laboratory and others have demonstrated that in vivo development of β-lactam resistance is an important determinant of therapeutic efficacy in treating invasive pseudomonal infections (1, 16, 19, 22, 23). This problem has remained prevalent despite the development of cephalosporins with potent antipseudomonal activity in vitro and which are relatively resistant to β-lactamase hydrolysis (e.g., ceftazidime, cefoperazone). Among clinically derived *P. aeruginosa* strains from human or experimental animal infections, the major mechanisms of resistance to such new cephalosporins have been (i) excessive (usually constitutive) β-lactamase production (16, 19, 22, 23) or perhaps (ii) transmembrane permeability defects (23). The role of β-lactamase in causing the resistance phenotype has been variously ascribed to either a nonhydrolytic (14, 15, 18, 28, 31) or hydrolytic (32) barrier concept.

Irrespective of the exact mechanism(s) involved in β-lactamase-mediated resistance to the antipseudomonal cephalosporins, one possible therapeutic strategy in circumventing such resistance is use of combinations of β-lactams plus β-lactamase inhibitors. Such inhibitors would theoretically inactivate the periplasmic β-lactamases and allow β-lactam access to lethal penicillin-binding proteins. Such approaches have been used against a variety of plasmid-mediated β-lactamases of *Staphylococcus aureus*, *Haemophilus influenzae*, and the *Enterobacteriaceae*; fixed combinations of various β-lactams (e.g., amoxicillin) plus either clavulanic acid or sulbactam have been successfully evaluated in this regard (8, 13). Unfortunately, the chromosomally mediated, constitutively overproduced cephalosporinases of *P. aeruginosa* variants have poor affinity for sulbactam and clavulanic acid and are not inhibited by these compounds at clinically attainable levels (11). The semisynthetic penicillins such as dicloxacillin and oxacillin, in contrast, reliably interfere with the activity of these cephalosporinases, a property used in the classification schema of these enzymes (type Id [30]).

The current study investigated the ability of dicloxacillin to act synergistically in vivo with ceftazidime in experimental endocarditis due to a ceftazidime-resistant variant which constitutively overproduces the type Id cephalosporinase. Several interesting findings emanated from this study. The dicloxacillin-ceftazidime combination exerted a synergistic bactericidal effect in vitro at an initial inoculum of $\sim 10^5$ CFU/ml; however, no synergy was observed at initial bacterial densities of $\sim 10^7$ CFU/ml, presumably related to overwhelming the enzyme-inhibitory capacity of dicloxacillin by additional periplasmic enzyme molecules. The bactericidal synergy seen in vitro for dicloxacillin-ceftazidime combinations was partially mirrored in vivo. At each animal sacrifice, vegetation bacterial densities were significantly lower in animals receiving the drug combination compared with those given ceftazidime monotherapy. However, it should be emphasized that this in vivo effect was relatively modest; mean *P. aeruginosa* densities within vegetations of animals given combination therapy were still >6 $\log_{10}$ despite 6 days of treatment. It is not likely that impaired antibiotic penetrability into cardiac vegetations is the principal reason for the disparity between the dramatic in vitro bactericidal synergy and the modest in vivo effect. Both ceftazidime and semisynthetic penicillins (e.g., methicillin) have been shown to penetrate well overall into experimental cardiac vegetations (~80 to 100% of levels in serum [12, 20]). Moreover, we confirmed that dicloxacillin did achieve vegetation concentrations of ~80% of that in serum at 1 h postdosing. In addition, it appears that dicloxacillin is capable of adequately penetrating into the pseudomonal periplasmic space. At a concentration of only 34.9 nM (16.4 ng/ml), dicloxacillin inhibited the β-lactamase of viable PA-48 cells by 50%; achievable serum and vegetation levels of dicloxacillin approached 100 μg/ml in this study. It seems more likely that the in vitro-in vivo disparity we observed is mainly related to the high bacterial densities of *P. aeruginosa* achieved in vivo. Our in vitro enzyme inactivation studies also indicated that dicloxacillin functions as a competitive substrate for the type Id cephalosporinase from the infecting pseudomonal strain, with a high enzyme affinity and substantial half-life; thus, it is probable that at high intravegetation bacterial densities, dicloxacillin may be slowly hydrolyzed or rendered incapable of rebinding to the enzyme. An additional problem in vivo may relate to the concentration of dicloxacillin or ceftazidime (or both) achieved in the more central parts of the vegetations. We have previously shown morphologic evidence by electron microscopy studies that substantially subinhibitory levels of β-lactams and aminoglycosides reach *P. aeruginosa* colonies within the depths of vegetations in experimental endocarditis (2).

The mechanism by which dicloxacillin exerts its inhibitory effect on the β-lactamase of the infecting *P. aeruginosa* strain (PA-48) appears different from that of other available enzyme inhibitors (e.g., clavulanic acid, sulbactam). The latter agents act by irreversibly binding to the enzyme molecule, totally abrogating functional activity (i.e., "suicide inhibitors" [9, 24]). In contrast, we demonstrated that dicloxacillin is an effective competitive inhibitor or competitive substrate of the Id β-lactamase activity (depending on coincubation times) without exerting significant effect on

the intrinsic functional properties of the enzyme. Because of the avid binding of dicloxacillin to this enzyme, with a K_i of 2 nM and a binding affinity ~5,000 times that of ceftazidime, this competitive inhibition likely allows the ceftazidime to reach its target penicillin-binding proteins and exert its lethal effect.

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