Efficacy of Amikacin and Ceftazidime in Experimental Aortic Valve Endocarditis Due to Pseudomonas aeruginosa

ARNOLD S. BAYER,1* DEAN NORMAN,2,4 AND KWANG SIK KIM3,4

Departments of Medicine1 and Pediatrics,2 Harbor-University of California at Los Angeles Medical Center, Torrance, California 90509; Research and Medical Services, Wadsworth VA Hospital Center, Los Angeles, California 900732; and University of California at Los Angeles School of Medicine, Los Angeles, California 900244

Received 10 June 1985/Accepted 26 September 1985

The in vivo efficacies of amikacin, ceftazidime, and their combination were evaluated in experimental aortic valve endocarditis due to Pseudomonas aeruginosa. Eighty catheterized rabbits were infected with a P. aeruginosa strain susceptible to both amikacin and ceftazidime and then received no therapy (controls), amikacin (15 mg/kg per day), ceftazidime (100 mg/kg per day), or amikacin-ceftazidime. Amikacin-ceftazidime significantly lowered vegetation titers of P. aeruginosa at day 7 of therapy versus other regimens (P < 0.0005). However, by day 14 of therapy, vegetation titers in animals receiving amikacin or ceftazidime regimens or both were not different from those of untreated controls; this was associated with in vivo development of amikacin resistance in most infected vegetations (79%), a phenomenon not seen at day 7 of therapy. Amikacin resistance was unstable in vivo, being undetectable in vegetations examined 5 days after treatment with amikacin had been completed. In contrast, ceftazidime resistance (first noted at day 7 of therapy in 12% of vegetations) persisted after termination of treatment with this agent. These in vivo observations on loss of amikacin resistance and persistence of ceftazidime resistance were mirrored during in vitro passage studies of amikacin- or ceftazidime-resistant P. aeruginosa strains isolated from cardiac vegetations. Amikacin resistance was no longer detectable by passage 5 in antibiotic-free media; however, ceftazidime resistance was stable despite 15 such passages. In vivo development of aminoglycoside-β-lactam resistances was associated with poor bacteriologic efficacy in this model.

Studies of human infective endocarditis caused by Pseudomonas aeruginosa have emphasized the difficulties in achieving cures with antibiotic therapy alone, with treatment failures of 35 to 75% (15–17). A major problem in this regard has been the development of resistance in vivo to the antibiotics commonly used for this infection (e.g., β-lactams and aminoglycosides), resulting in refractory or recrudescent bacteremia and endocarditis (15–17). This phenomenon has been most frequently observed when there was involvement of left-sided cardiac valves (16).

Bayer et al. have recently reported the excellent in vivo responses of experimental tricuspid valve endocarditis due to P. aeruginosa to combination chemotherapy with amikacin-ceftazidime (A. S. Bayer, K. Lam, D. C. Norman, K. S. Kim, and J. O. Morrison, Chemotherapy [Basel], in press). In that study, the amikacin-ceftazidime regimen was significantly more effective than the single-drug regimens in reducing the mean vegetation titers of P. aeruginosa and reducing the prevalence of bacteriologic relapses after therapy was terminated. In the current study, we examined this synergistic combination in experimental left-sided P. aeruginosa endocarditis, the most refractory syndrome in human cases (16).

(Received in revised form 4 October 1985. Accepted for publication 27 October 1985.)

MATERIALS AND METHODS

Organism. The P. aeruginosa strain used in this study (P. aeruginosa PA-96) was a clinical isolate that had been used in a prior study of experimental endocarditis (5). Its identification, serotyping, and resistance to rabbit serum have been previously described (5).

Antibiotic susceptibility testing. MICs and MBCs of amikacin and ceftazidime were determined by the broth macrodilution technique (13) with Mueller-Hinton broth (MHB; BBL Microbiology Systems, Cockeysville, Md.). MHB has a concentration of 0.5 mg/100 ml of magnesium and 3.2 mg/100 ml of calcium. Logarithmic-growth-phase cultures were used to prepare inocula of PA-96 at ~5 × 107 or ~5 × 108 CFU/ml. The ~105 inoculum was utilized to determine the antibiotic susceptibility at bacterial densities likely to be encountered within cardiac vegetations in vivo (1). The MIC was defined as the lowest antibiotic concentration causing no visible turbidity. The MBCs were determined by subculturing 10 µl from each clear tube onto antibiotic-free MH agar plates. The MBC was the lowest concentration of antibiotic that effected 99.9% reduction of the original inoculum. The MICs and MBCs of selected amikacin- or ceftazidime-resistant organisms isolated from endocardial vegetations were also determined for gentamicin, kanamycin, moxalactam, cefoperazone, azlocillin, mezlocillin, and ciprofloxacin (inoculum size, ~5 × 106 CFU/ml).

In vitro bactericidal interaction testing. Bactericidal interactions of ceftazidime and amikacin against PA-96 were examined with the time-kill assay system. As before, a logarithmic-phase inoculum of either ~5 × 106 or ~5 × 107 CFU/ml was used. The final antibiotic concentrations tested were amikacin alone at one-half the MBC, ceftazidime alone at one-half the MBC, or amikacin-ceftazidime, each drug at one-quarter its MBC. A synergistic interaction was considered present when the amikacin-ceftazidime combination caused a ≥2 log10 decrease in CFU per milliliter at 24 h in comparison with the more active single agent (10).
Induction and treatment of *P. aeruginosa* endocarditis. Eighty female New Zealand White rabbits, weighing 2 to 2.5 kg each, were anesthetized with 50 mg of ketamine hydrochloride given intramuscularly (Bristol Laboratories, Syracuse, N.Y.). The left ventricle was catheterized with a polyethylene catheter by passing the catheter tip across the aortic valve as previously described (1). The catheter remained in place for the duration of the study. Each animal was inoculated intravenously with $10^8$ CFU of PA-96 24 h after catheterization. Positive blood cultures for *P. aeruginosa* at 24 h postinoculation were taken as presumptive evidence of endocarditis (1). Macroscopic and bacteriologic data acquired at the time of sacrifice provided ultimate confirmation of vegetative endocarditis.

The 80 rabbits were randomized (by computer-generated code) to one of four therapy groups of 20 rabbits each: untreated controls, amikacin at 15 mg/kg per day (intramuscularly, in two divided doses—8 a.m. and 5 p.m.), ceftazidime at 100 mg/kg per day (intramuscularly, in two divided doses—8 a.m. and 5 p.m.), and amikacin-ceftazidime at the above dosages and schedules. Therapy was begun 48 h after inoculation. The above regimens were identical to those that produced an excellent in vivo response in a previous study on experimental tricuspid valve endocarditis produced by the same *P. aeruginosa* strain (4). Pilot studies in our laboratory indicated that these dosage regimens consistently produced supra-MBC serum levels at $\pm 1$ h postinjection.

**Sacrifice of animals.** Animals were sacrificed by rapid intravenous injection of 150 mg of sodium pentobarbital on either day 7 or 14 of therapy or on day 5 after stopping therapy (to determine bacteriologic relapse). At sacrifice, aortic valve vegetations were individually excised and weighed. Each vegetation was homogenized in MHB and quantitatively subcultured as previously described (5). No attempt was made to inactivate amikacin or ceftazidime in vegetation homogenates, as sacrifices were performed at least 18 h after the last drug dose (18). Animals that had been dead longer than 6 h before postmortem examination were excluded from bacteriologic evaluation (18). Portions of each homogenate were also subcultured onto MH agar containing 50 µg of either ceftazidime or amikacin per ml (depending on the therapeutic regimen) to detect in vivo development of resistance. Concentrations of 50 µg/ml were chosen because they were at or above the breakpoints for resistance of either agent ($\geq 32$ µg/ml [11]). In calculating mean titers, culture-negative vegetations were considered to contain $\pm 2 \log_{10}$ CFU/g based on average vegetation weight (0.01 g [3]). To delineate the relative proportions of antibiotic-resistant and antibiotic-susceptible isolates contained within individual vegetations, a resistance ratio was calculated (4). This is defined as the $\log_{10}$ of the ratio of the number of resistant organisms to the total number of organisms within a vegetation [$\log_{10}$ (number of resistant isolates per total number of isolates)].

**In vitro passage studies.** Stabilities of antimicrobial resistances induced in vivo were determined by in vitro passage experiments. Two ceftazidime-resistant and two amikacin-resistant *P. aeruginosa* variants, isolated from vegetations of animals receiving the respective single agents, were studied. Each strain was grown in antibiotic-free MHB overnight and then parallel plated onto both antibiotic-free and antibiotic-containing MH agar (50 µg of amikacin or ceftazidime per ml, depending on the therapy group). The serial passage of each strain in antibiotic-free MHB, with parallel plating as above, was performed an additional 14 times.

Concentrations of antibiotics in serum. On day 3 of antibiotic treatment, blood was drawn 1 h after drug administration and just before the next dose of antibiotic for measurement of near peak and trough concentrations in serum, respectively. Amikacin levels in serum were determined by a radioimmunoassay technique (American Diagnostics Corp., Newport Beach, Calif. [9]); ceftazidime levels in serum were determined by high-pressure liquid chromatography (8). The sensitivities of these assays are 0.5 µg/ml for amikacin and 5 µg/ml for ceftazidime.

**Serum bactericidal titers (SBTs).** All SBTs were determined by the microtiter method (14) in sera obtained at the 1-h posttreatment period referred to above. The diluent was heat-inactivated (at 56°C for 30 min), freshly pooled, normal rabbit serum. The serum bactericidal titer was defined as the highest serum dilution causing $\geq 99.9\%$ killing of the original inoculum (14).

**Cross-resistance.** To determine whether there was cross-resistance to ceftazidime and amikacin. *P. aeruginosa* variants cultured on antibiotic-containing plates were replica plated (6) onto agar containing the respective single drug (e.g., ceftazidime at 50 µg/ml).

**Statistical evaluation.** The chi-square ($x^2$) test with the Yates correction factor and Student's $t$ test were used for comparing differences in proportions between groups of animals and for comparing differences between $\log_{10}$ CFU per gram of vegetations, respectively; *P* values less than or equal to 0.05 were considered significant.

**RESULTS**

**Antimicrobial susceptibility tests.** At an inoculum of $10^5$ CFU/ml, the MIC/MBC for amikacin and ceftazidime were 2/2 and 8/8 µg/ml, respectively. MICs and MBCs of both ceftazidime and amikacin were increased fourfold at an inoculum of 10$^5$ CFU/ml. The amikacin and ceftazidime combination exhibited synergy only at an inoculum of 10$^5$ CFU/ml.

**Animal experiments.** (i) Mortality. Ten animals, three each assigned to control and amikacin groups and two each assigned to the ceftazidime and amikacin-ceftazidime groups, died within 12 h of inoculation and were not included in the data analysis. Mortality rates of remaining animals were: controls (11 of 17 [65%]), amikacin (5 of 17 [29%]), ceftazidime (5 of 18 [27%]), amikacin-ceftazidime (4 of 18 [22%]). All deaths in animals receiving ceftazidime or amikacin-ceftazidime occurred during week 2 of therapy or in the posttherapy period. In animals receiving amikacin alone, two died during week 1 of therapy, while three died during week 2 of therapy. In contrast, all deaths in the control group occurred within week 1 postinoculation.

(ii) Blood cultures. Blood cultures taken 24 h after inoculation were positive for *P. aeruginosa* in all 80 catheterized animals, presumptively confirming induction of endocarditis (1). Blood cultures taken at time of sacrifice were uniformly positive in control animals and remained positive in 89% of the recipients of amikacin monotherapy. In contrast, terminal blood cultures were positive in only 19 and 20% of the recipients of ceftazidime either alone or in combination with amikacin, respectively. The frequencies of positive blood cultures in the latter two therapy groups were significantly lower than those observed in controls or animals receiving amikacin alone (*P* < 0.0005).

(iii) Titers of *P. aeruginosa* in endocardial vegetations. All vegetations from untreated controls (*n* = 15) yielded *P. aeruginosa* on culture (no spontaneous cures). The range of
titers for these controls was similar to that previously cited in this model (1).

On day 7 of therapy, titers for the rabbits treated with amikacin-ceftazidime were significantly lower than those for amikacin- or ceftazidime-treated groups (P < 0.0005, Table 1). On day 14, mean vegetation titers of recipients of the combination regimen were significantly higher than those on day 7 (P < 0.0005).

On day 5 after termination of treatment, the mean vegetation titers of all three treated groups were higher (>log10 9) and not significantly different from those of untreated controls sacrificed earlier.

(iv) Development of resistance in vivo. No vegetations obtained from recipients of amikacin on day 7 of treatment contained resistant isolates (Table 2). In contrast, 93% of the vegetations obtained on day 14 yielded resistant isolates. Resistance ratios in these vegetations were high, ranging from −3.85 to −5.46 (mean ± standard error of the mean, −4.78 ± 0.15). Amikacin resistance appeared to be unstable, for no resistant isolates were recovered from vegetation sampled on day 5 after the last dose of amikacin.

No vegetations obtained on day 7 of therapy from animals treated with ceftazidime yielded resistant isolates, and only 1 of 20 vegetations obtained on day 14 of therapy yielded a resistant isolate. As opposed to amikacin resistance, ceftazidime resistance appeared to be stable, with three of seven vegetations sampled at day 5 posttherapy yielding resistant isolates. Resistance ratios ranged from −3.39 to −8.63 (mean ± standard error of the mean, −5.15 ± 1.41).

The above findings in the single-dose groups were mirrored in animals receiving combination therapy. Neither amikacin- nor ceftazidime-resistant isolates were detected at day 7 of therapy. By day 14 of treatment, however, 9 of 14 vegetations yielded amikacin-resistant isolates, with resistance ratios ranging from −1.64 to −7.14 (mean, −3.46 ± 0.55); 3 of 14 vegetations yielded ceftazidime-resistant isolates, with resistance ratios ranging from −1.5 to −7.14 (mean, −4.05 ± 1.44). At day 5 after stopping combination therapy, none of 17 vegetations obtained yielded amikacin-resistant isolates, while 1 of the 17 vegetations contained ceftazidime-resistant organisms.

(v) In vitro features of antibiotic resistances—passage and cross-resistance studies. Fifteen serial passages of each of two ceftazidime-resistant isolates through antibiotic-free media showed that this phenotypic trait was stable. In contrast, two isolates resistant to amikacin reverted to amikacin-susceptible status within five passages in such media. The stability of ceftazidime resistance and lability of amikacin resistance in vitro thus mirrored the in vivo observations above among endocardial isolates after stopping antibiotic treatment.

Resistance to ceftazidime induced in vivo was not limited to this agent but carried over to other β-lactams, including cefoperazone, moxalactam, azlocillin, and mezlocillin (Table 3). Similarly, amikacin resistance induced in vivo was not limited to this agent but carried over to other aminoglycosides, including gentamicin and kanamycin. Both ceftazidime- and amikacin-resistant isolates remained highly susceptible to ciprofloxacin, a quinolone derivative with anti-DNA gyrase activity (22).

### Table 1. Mean vegetation titers (log10 CFU/g) and vegetation sterilization rates for various antibiotic regimens in experimental endocarditis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of rabbits sacrificed</th>
<th>No. pos. veg./total*</th>
<th>Mean veg. titers*</th>
<th>No. of rabbits sacrificed</th>
<th>No. pos. veg./total</th>
<th>Mean veg. titers*</th>
<th>No. of rabbits sacrificed</th>
<th>No. pos. veg./total</th>
<th>Mean veg. titers*</th>
<th>No. of rabbits sacrificed</th>
<th>No. pos. veg./total</th>
<th>Mean veg. titers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>15/15</td>
<td>9.54 ± 0.20*</td>
<td>3</td>
<td>14/14</td>
<td>9.6 ± 0.27*</td>
<td>4</td>
<td>15/15</td>
<td></td>
<td>9.91 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>5</td>
<td>17/17</td>
<td>8.79 ± 0.41*</td>
<td>5</td>
<td>18/20</td>
<td>6.21 ± 0.74*</td>
<td>2</td>
<td>7/7</td>
<td>10.51 ± 0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>6</td>
<td>21/22</td>
<td>7.29 ± 0.39*</td>
<td>4</td>
<td>14/14</td>
<td>8.59 ± 0.52*</td>
<td>4</td>
<td>17/17</td>
<td>9.47 ± 0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin-ceftazidime</td>
<td>6</td>
<td>11/18</td>
<td>3.8 ± 0.79*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of culture-positive vegetations per total sample.

### Table 2. Amikacin- and ceftazidime-resistant isolates from cardiac vegetations

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of resistant vegetations/no. of vegetations sampled (%)</th>
<th>No. of resistant vegetations/no. of vegetations sampled (%)</th>
<th>No. of resistant vegetations/no. of vegetations sampled (%)</th>
<th>No. of resistant vegetations/no. of vegetations sampled (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>0/17 (0)</td>
<td>3/14 (93)</td>
<td>0/14 (0)</td>
<td>3/14 (93)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0/22 (0)</td>
<td>5/12 (42)</td>
<td>2/17 (12)</td>
<td>2/17 (12)</td>
</tr>
<tr>
<td>Amikacin-ceftazidime</td>
<td>0/18 (0)*</td>
<td>4/9 (44)</td>
<td>0/17 (0)*</td>
<td>4/9 (44)</td>
</tr>
</tbody>
</table>

* Vegetations containing amikacin-resistant P. aeruginosa isolates.

* Vegetations containing ceftazidime-resistant P. aeruginosa isolates.
Replica-plating studies provided no evidence for cross-resistance, indicating that development of resistance to ceftazidime or amikacin was an independent event requiring exposure to the drug.

**Antibiotic levels.** Amikacin and ceftazidime levels in serum at 1 h postdosage equalled or exceeded the MBC of PA-96 determined in vitro for both $10^8$ and $10^7$ inocula (Table 4). No antibiotics were detectable at trough samplings. There were no significant differences in mean amikacin or ceftazidime levels in the single drug versus combination therapy groups.

**Serum bactericidal titer.** No serum bactericidal activity was detectable in 3 of 12 serum samples from recipients of amikacin alone; moreover, the serum bactericidal activity in the remaining nine samples was detectable at relatively low titers (1:2 to 1:4 [Table 5]). In contrast, all samples from animals receiving ceftazidime regimens had bactericidal activity at titers ≥1:4. The geometric means of the reciprocal bactericidal titers for these latter groups were significantly higher than that for amikacin alone ($P < 0.0005$).

**DISCUSSION**

Ceftazidime is a potentially important candidate for therapy of invasive infections with *P. aeruginosa*, such as infective endocarditis. This antibiotic is resistant to hydrolysis by β-lactamases of the Richmond-Sykes class I which are commonly found in *P. aeruginosa* (12); it also possesses potent antipseudomonal activity in vitro and frequently demonstrates bactericidal synergy with amikacin against aerobic gram-negative bacilli (2).

Our current study showed the following. (i) The amikacin-ceftazidime combination effected a rapid clearance of *P. aeruginosa* from vegetations during the first 7 days of therapy, similar to what we have previously observed in experimental right-sided *P. aeruginosa* endocarditis due to the homologous strain (2a). (ii) Bacteriologic relapses occurred during week 2 of treatment in the combination therapy group, coincident with the appearance of aminoglycoside- or β-lactam-resistant isolates within vegetations. (iii) Amikacin resistance was unstable, disappearing within 5 days of the end of therapy. (iv) Resistance to ceftazidime was stable, persisting throughout the 5-day posttreatment period. (v) These observations on resistance in vivo were mirrored by the results of in vitro studies which showed that amikacin resistance was lost and ceftazidime resistance was retained during serial transfer through drug-free media. The in vitro and in vivo stability of ceftazidime resistance suggested that these variant strains of *P. aeruginosa* might represent stably derepressed mutants as described by Sanders and Sanders (19). (vi) Neither amikacin nor ceftazidime resistances were limited to these inducing antibiotics but were common to other structurally related compounds. (vii) The in vivo development of resistance to amikacin or ceftazidime did not cross antibiotic class lines; thus, resistant mutants induced by ceftazidime monotherapy did not manifest amikacin resistance on crossover replica-plating studies nor did aminoglycoside-resistant mutants induced by amikacin monotherapy manifest ceftazidime resistance.

It is not entirely clear why amikacin-ceftazidime therapy resulted in both treatment failures and development of antibiotic resistances in vivo in left-sided endocarditis, when the identical treatment regimens exerted such a potent in vivo effect in right-sided endocarditis due to the same strain without development of resistances in vivo (2a). The only readily identifiable difference in these two model systems is that *P. aeruginosa* titers in aortic valve vegetations are ~2 log$_{10}$ higher than those seen in tricuspid valve vegetations (1, 2a, 5); this may relate to enhanced pseudomonal growth under conditions of higher oxygen tensions as seen on the left side versus the right side of the heart (20). These disparate results in the therapy of right- versus left-sided *P. aeruginosa* endocarditis models vis-à-vis efficacy and development of resistance may represent an in vivo inoculum effect (7, 21).

The precise mechanisms leading to development of multi-modal antibiotic resistance in our studies are unclear and...
require further study. Impaired penetration of amikacin or ceftazidime or both into left-sided cardiac vegetations is one possible factor. This concept is supported indirectly by the ability of ceftazidime and amikacin-ceftazidime to sterilize most blood cultures in the setting of supra-MIC and supramBC antibiotic levels and high serum bactericidal activities at a time when cardiac vegetations contained high P. aeruginosa titers.

We are currently in the process of a detailed in vitro examination of the mechanism(s) involved in the in vivo development of β-lactam and aminoglycoside resistances among our endocardial isolates of P. aeruginosa. Our preliminary data have indicated that membrane permeability defects are involved in the aminoglycoside resistance observed, while derepression of β-lactamase production and transmembrane permeability defects are involved in the β-lactam resistance (A. S. Bayer, D. C. Norman, and K. S. Kim, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, A 42, p. 8).

LITERATURE CITED