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Activities of Tobramycin and Azlocillin Alone and in Combination Against Experimental Osteomyelitis Caused by *Pseudomonas aeruginosa*

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Azlocillin and tobramycin were used alone and in combination in the treatment of chronic osteomyelitis due to *Pseudomonas aeruginosa* in rabbits. This combination showed in vitro synergy measured by both the checkerboard technique and time-kill curves. A marked inoculum effect was demonstrated in vitro with azlocillin and the infecting strain of *P. aeruginosa*. The minimal inhibitory concentration of azlocillin, with an inoculum of 10^5 organisms, was 12.5 μg/ml; when the inoculum size was increased to 10^7 organisms, the minimal inhibitory concentration rose to more than 500 μg/ml. In therapeutic trials, the combination of azlocillin and tobramycin, given for 28 days, was significantly better than either no therapy or azlocillin alone, but was not significantly better than tobramycin alone. Even after 4 weeks of combined therapy with azlocillin and tobramycin, *P. aeruginosa* was recovered from the bones of 60% of the treated rabbits.

A previous study in an experimental model of osteomyelitis caused by *Pseudomonas aeruginosa* showed that the combination of carbenicillin and sisomicin was significantly more effective (when administered for 4 weeks) than either agent alone (5). However, that combination failed to sterilize the bones of 30% of the rabbits treated, and it was apparent that a more effective regimen would be desirable. Azlocillin and tobramycin were selected for further testing in this experimental model. Tobramycin is as active in vitro as sisomicin against the infecting strain of *P. aeruginosa*; azlocillin showed considerably greater activity than carbenicillin in initial in vitro testing against the infecting strain of *P. aeruginosa*. It seemed plausible that azlocillin, either alone or in combination with tobramycin, might be an effective regimen for treatment of experimental osteomyelitis caused by *P. aeruginosa*. The present study measured the efficacy of azlocillin and tobramycin alone and in combination in this model. This combination, given for 28 days, was more effective than either no therapy or azlocillin alone, but failed to sterilize the bones of 60% of the treated rabbits.

MATERIALS AND METHODS

Production of osteomyelitis. The technique for production of osteomyelitis has been described previously (5). New Zealand white rabbits (weight 1.5 to 2 kg) received an intramedullary injection into the tibia of sodium morrhuate and 2×10^7 colony-forming units (CFU) of *P. aeruginosa* (5). The strain used was isolated from a patient with osteomyelitis. It was sensitive to polymyxin, carbenicillin, azlocillin, gentamicin, tobramycin, sisomicin, and amikacin. It was resistant to bacteriolysis when tested with fresh serum from five normal individuals.

Determination of MICs. An inoculum (0.5 ml containing either 10^5 or 10^7 CFU of *P. aeruginosa* per ml in Mueller-Hinton broth) was added to 0.5 ml of serial dilutions of antibiotic standards. The mixture was incubated at 37°C for 18 h and then observed for turbidity. The lowest concentration of antibiotic for which no growth was visible was the minimal inhibitory concentration (MIC).

Tests for synergism. Broth dilution sensitivity tests were performed according to the checkerboard pattern, using inocula of either 10^5 or 10^7 CFU of *P. aeruginosa*. Twofold dilutions of one drug were tested in combination with concentrations of twofold dilutions of the other. For each checkerboard determination, MICs for each agent alone were measured in parallel. The same criteria used for measuring inhibition of growth in the MIC determinations were used for the checkerboards. Combinations were considered to be synergistic when the concentration of each agent in combination was reduced to less than or equal to 25% of the amount required for each agent alone to inhibit growth.

Determination of rates of killing by antibiotics. An overnight culture of the infecting strain of *P. aeruginosa* was diluted in Mueller-Hinton broth to 10^7 CFU/ml. Either a single antibiotic or a combination of antibiotics was added to the bacteria. A sample was removed immediately for determination of the number of CFU by the standard pour-plate technique. The bacteria-antibiotic mixture was incubated at 37°C; 6 and 24 h later, samples were removed for determination of the number of CFU by the pour-plate tech-

nique. Synergism was defined as at least 100-fold (2 $\log_{10}$), increased killing at 24 h by the combination compared with the most efficacious of individual drugs.

Measurement of antibiotics in serum and bone. Rabbits which were not included in the therapeutic trials were infected with *P. aeruginosa* as described above. After 14 days, groups of six rabbits received subcutaneous injections of either tobramycin or azlocillin and were killed 1, 2, or 4 h later. Blood was collected just before death; the diseased bone was removed and prepared as described previously (4). No measurements of levels of antibiotics in bone or serum were made after administration of cumulative doses of antibiotics or in rabbits receiving combinations of antibiotics.

Serum assay. Tobramycin was assayed by using techniques previously described for sisomicin (4); the test organisms used were *Bacillus subtilis* spores (Difco Laboratories, Detroit, Mich.). The minimum concentration of tobramycin which could be measured was 0.04 μg/ml. For assay of azlocillin, standard curves were constructed from the sizes of zones of inhibition of reference standards tested with *B. subtilis* spore suspensions. Sera and standards were diluted in a mixture of 50% normal rabbit serum–50% 0.1 M phosphate buffer (pH 7.3). The minimum concentration of azlocillin which could be measured was 0.7 μg/ml.

Bone assay. Bones were prepared for assay as previously described (4). The concentration of antibiotic is expressed as micrograms of antibiotic per gram of bone. To determine whether azlocillin or tobramycin is bound to bone powder, standards of each antibiotic were agitated either with suspensions of bone powder from uninfected rabbits or with buffer for 4 h before assay. No differences in zone sizes were seen, indicating that the antibiotics did not bind to bone powder.

Therapeutic trials. Groups of 25 rabbits were infected on different days; within each group, equal numbers of rabbits were assigned to the following regimens: (i) no antibiotic therapy, (ii) twice-daily injection at 8 a.m. and 4 p.m. of 10 mg of tobramycin per kg of body weight, (iii) four-times-daily injection at 9 a.m., 1 p.m., 5 p.m., and 9 p.m. of 400 mg of azlocillin per kg of body weight, and (iv) four-times-daily injection of 400 mg of azlocillin per kg of body weight and twice-daily injection of 10 mg of tobramycin per kg of body weight at the same times as in the groups given either antibiotic alone.

All injections of antibiotics were subcutaneous. Therapy was instituted 14 days after infection and given for 14 or 28 days. All rabbits were killed 70 days after infection. Cultures of the bone were obtained by flushing the marrow cavity as previously described (3) and by inserting a swab into the exposed proximal end of the tibia. Recovery of any number of CFU of *P. aeruginosa* was considered to represent a positive bone culture. The assay was designed to detect as few as 1 or 2 CFU/ml of bone flush. MICs were determined for all *Pseudomonas* strains recovered from the bones of treated rabbits.

Roentgenograms of all infected bones were performed after the rabbits were killed. All roentgenograms were read by one observer (C.W.N.) without knowledge of the mode of treatment. Three radiographic parameters (presence of periosteal new bone, presence of bone destruction, and the extent of involvement) were determined for each bone. A numerical score was assigned, and the mean was calculated for each treatment group (see Table 2). The presence or absence of sequestrum formation was determined radiographically for each rabbit.

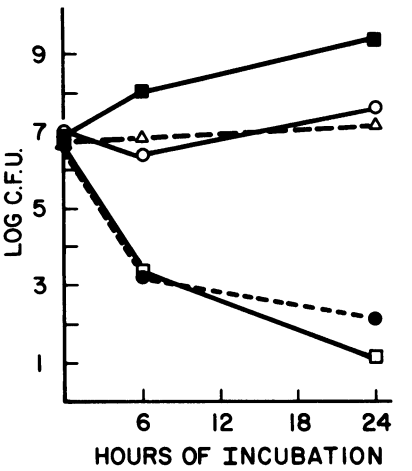

FIG. 1. A representative experiment of the rates of killing of *P. aeruginosa* by azlocillin, tobramycin, and azlocillin plus tobramycin in Mueller-Hinton broth. The control consisted of *P. aeruginosa* in Mueller-Hinton broth with no antibiotics. Symbols: ■, control; ○, tobramycin (0.39 μg/ml); △, azlocillin (100 μg/ml); ●, tobramycin (1.56 μg/ml); and □, tobramycin (0.39 μg/ml) + azlocillin (12.5 μg/ml).

RESULTS

MIC determinations and synergy testing. The MICs of tobramycin and azlocillin, for the infecting strain of *P. aeruginosa*, using an inoculum of 10^5 organisms, were 0.2 and 12.5 μg/ml, respectively. When the inoculum size was increased to 10^7 organisms, the MIC for tobramycin rose only to 0.39 μg/ml, but the MIC for azlocillin rose to more than 500 μg/ml. With the higher inoculum and azlocillin, there was visible growth in all tubes containing concentrations up to and including 500 μg of azlocillin per ml. However, tubes containing 125, 250, and 500 μg of azlocillin per ml were much less turbid than those with lower concentrations of azlocillin, suggesting partial, but incomplete, inhibition of growth by these higher concentrations of azlocillin.

The checkerboard technique showed evidence of synergy with inocula of either 10^5 or 10^7 CFU of *P. aeruginosa*. With the larger of these inocula, growth inhibition occurred with combinations of 0.05, 0.1, and 0.195 μg of tobramycin per ml with, respectively, 25, 12.5, and 6.25 μg of azlocillin per ml. Time-kill curves also demonstrated synergy when concentrations of both

TABLE 1. Antibiotic concentrations in serum and diseased bone after a single dose of antibiotic

Time after injection (h)	Drug concn after dose of[a]:			
	Tobramycin (10 mg/kg)		Azlocillin (400 mg/kg)	
	Serum (μg/ml)	Bone (μg/g)	Serum (μg/ml)	Bone (μg/g)
1	13.8 ± 1.6	2.6 ± 1.1	383 ± 49.0	29.0 ± 8.0
2	5.7 ± 1.3	2.5 ± 0.8	238 ± 33.7	16.9 ± 4.4
4	2.0 ± 1.5	1.7 ± 0.4	25.5 ± 14.5	1.2 ± 0.5

[a] Results are expressed as means ± standard deviation. For each time interval after injection, six rabbits with osteomyelitis were studied.

agents, which produced no killing when used alone, were combined (Fig. 1).

Measurement of antibiotic concentrations in serum and bone. Concentrations of azlocillin and tobramycin in serum and osteomyelitic bone (after a single dose of antibiotic) are shown in Table 1. Tobramycin was present in bone at levels eight times the MIC 4 h after injection. With azlocillin, levels above the MIC for the infecting strain of *P. aeruginosa* were present in bone at 2 h after injection, but not at 4 h.

Therapeutic trials. The results of therapy are shown in Table 2. Deaths in the treated rabbits usually occurred within the first 10 days of therapy and were associated with severe diarrhea, weakness, and the inability to eat or drink. There were no significant differences noted in the radiological severity of disease or in the percentage of rabbits with sequestrum formation regardless of type of treatment.

Of the untreated animals, 92% had positive bone cultures when killed. The combination of azlocillin and tobramycin when given for 4 weeks was significantly more effective than either no therapy or azlocillin alone for 28 days (chi-square test, $P < 0.05$), but was not significantly more effective than tobramycin alone. Even after 4 weeks of combination therapy with azlocillin and tobramycin, *P. aeruginosa* was isolated from the bones of 60% of the treated rabbits.

MICs of azlocillin and tobramycin for all *P. aeruginosa* isolates recovered from either treated or control rabbits were equivalent to those of the parent strain. No tobramycin-resistant microcolonies were recovered.

DISCUSSION

This model of chronic osteomyelitis, caused by *P. aeruginosa*, was previously used to test therapy with carbenicillin and sisomicin; each agent alone was ineffective in sterilizing bone, but the combination of both drugs was significantly better. However, 4 weeks of combined therapy with carbenicillin and sisomicin still left 30% of the treated rabbits with bacteriologically positive bones. The present experiments, which used the same methodology as the earlier study, gave poorer results; 60% of the rabbits treated with the combination of azlocillin and tobramycin were treatment failures.

Before beginning the study, it seemed reasonable that the combination of tobramycin and azlocillin might be an effective regimen. Compared with sisomicin, tobramycin is as active against the infecting strain of *P. aeruginosa* and achieved comparable serum and bone levels. Azlocillin, when tested in the usual manner

TABLE 2. Results of treatment with azlocillin and tobramycin alone and in combination for experimental osteomyelitis due to *P. aeruginosa* in rabbits

Antibiotic	Duration of therapy (days)[a]	No. of animals[b]	Severity of disease[c]	Rabbits with sequestra on day 70 (%)	Rabbits with positive bone culture on day 70 (%)
None	—	24	2.2 ± 1.0	33	92
Azlocillin	28	21	2.0 ± 0.9	33	95
Tobramycin	14	22	1.7 ± 0.9	32	77
Tobramycin	28	21	1.6 ± 0.8	29	76
Azlocillin + tobramycin	14	21	1.7 ± 0.8	30	65
Azlocillin + tobramycin	28	20	1.7 ± 0.9	30	60

[a] Azlocillin was not tested in a 14-day regimen. See the text for the dose of each antibiotic.

[b] Number of rabbits surviving to day 70; each group initially contained 25 rabbits.

[c] Radiological evaluation on day 70 based on a scale of 0 to 3 (means ± standard deviation): 0, no disease; 1, minimal periosteal reaction and bone destruction limited to area proximal to site of injection; 2, severe periosteal reaction, bone destruction limited to proximal half of tibia, and new bone formation; and 3, severe periosteal reaction, bone destruction involving entire length of tibia, and extensive new bone formation.

against an inoculum of 10^5 *P. aeruginosa* per ml, demonstrated greater activity than did carbenicillin (respective MICs were 12.5 and 62.5 μg/ml). Levels of azlocillin in the diseased bone were still above the MIC for *P. aeruginosa* at 2 h but not at 4 h, whereas levels of carbenicillin in the diseased bone were lower than the MIC even at 1 h after injection. Finally, the combination of tobramycin and azlocillin was synergistic as measured both by the checkerboard technique against inocula of both 10^5 and 10^7 organisms and by time-kill curves against an inoculum of 10^7 organisms.

Despite theoretical reasons why the combination of tobramycin and azlocillin might be effective in this model, this study demonstrated that the combination was not. Although the combination was more effective than either no therapy or azlocillin alone, it was not significantly better than tobramycin alone. What explanations can be invoked to explain these poor results? First, the dosage schedule for azlocillin and tobramycin was less than optimal, and it left prolonged periods of time without effective antibiotic levels. However, similar problems existed in the prior study where carbenicillin plus sisomicin were administered at identical intervals. Second, some studies have reported significantly less bactericidal activity for azlocillin against *P. aeruginosa* compared with that of carbenicillin or ticarcillin (2, 8); others have not (1, 7). Our own studies demonstrated no differences in the rates of killing for *P. aeruginosa* by azlocillin or carbenicillin. We did, however, detect a marked difference in MIC determinations when the inoculum size was increased 100-fold. The MIC of azlocillin increased greater than 40-fold (from 12.5 to more than 500 μg/ml), whereas the MIC of carbenicillin rose only 4-fold (from 62.5 to 250 μg/ml). The number of *P. aeruginosa* recovered from the bones of untreated rabbits generally ranges from 10^5 to 10^6 CFU/ml, and it is possible that the inoculum effect (demonstrated in vitro) may be important in the relative failure of azlocillin in vivo. However, this is a speculation at the present time.

This experimental model of osteomyelitis caused by *P. aeruginosa*, which mimics the human disease both pathologically and radiographically (6), also appears to resemble the human counterpart in being difficult to treat successfully. Although the experimental studies with combinations of carbenicillin and sisomicin demonstrated partial efficacy, the present study with azlocillin and tobramycin was less encouraging. Whether other newer penicillins with greater bactericidal activity and more resistance to the beta-lactamase of *P. aeruginosa* or the newer cephalosporins with enhanced activities against *P. aeruginosa* will be more effective in combination with aminoglycosides than the presently tested drugs will require examination. An optimal program of therapy for the treatment of *P. aeruginosa* osteomyelitis remains elusive.

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LITERATURE CITED

1. **Coppens, L., and J. Klastersky.** 1979. Comparative study of anti-pseudomonas activity of azlocillin, mezlocillin, and ticarcillin. Antimicrob. Agents Chemother. 15:396–399.
2. **Fu, K. P., and H. C. Neu.** 1978. Azlocillin and mezlocillin: new ureido penicillins. Antimicrob. Agents Chemother. 13:930–938.
3. **Norden, C.** 1970. Experimental osteomyelitis. I. A description of the model. J. Infect. Dis. 122:410–418.
4. **Norden, C.** 1975. Experimental osteomyelitis. IV. Therapeutic trials with rifampin alone and in combination with gentamicin, sisomicin and cephalothin. J. Infect. Dis. 132:493–499.
5. **Norden, C., and E. Keleti.** 1980. Experimental osteomyelitis caused by *Pseudomonas aeruginosa*. J. Infect. Dis. 141:71–75.
6. **Norden, C., R. Myerowitz, and E. Keleti.** 1980. Experimental osteomyelitis due to *Staphylococcus aureus* or *Pseudomonas aeruginosa*: a radiographic-pathological correlative analysis. Br. J. Exp. Pathol. 61:451–460.
7. **Stewart, D., and G. P. Bodey.** 1977. Azlocillin: in vitro studies of a new semisynthetic penicillin. Antimicrob. Agents Chemother. 11:865–870.
8. **White, A. R., K. R. Comber, and R. Sutherland.** 1980. Comparative bactericidal effects of azlocillin and ticarcillin against *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 18:182–189.