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Early Effects of β-Lactams on Aminoglycoside Uptake, Bactericidal Rates, and Turbidimetrically Measured Growth Inhibition in *Pseudomonas aeruginosa*

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In vitro studies of tircarcillin or cefsulodin combined with [^{3}H]tobramycin were performed with *Pseudomonas aeruginosa*. The rate of bacterial killing, the uptake of tobramycin, and the effects on optical density were measured. Both β-lactams increased the uptake of subinhibitory concentrations of tobramycin. This result was quantitatively associated with a 2- to 4-h time-kill potentiation and confirmed earlier studies on the mechanism of β-lactam–aminoglycoside synergy in *Escherichia coli* (P. H. Plotz and B. D. Davis, Science 135:1067–1068, 1962).

Studies by Plotz and Davis (20) on the mechanism of synergy between β-lactams and aminoglycosides demonstrated that penicillin enhances the intracellular entry of otherwise subinhibitory concentrations of streptomycin in *Escherichia coli*. The commonly held belief that this mechanism occurs in other *E. coli* strains or in different species of gram-negative bacilli has never been examined. Recent studies have demonstrated that β-lactam-enhanced aminoglycoside uptake and bacterial killing are absent in viridans group streptococci (17) but present in both enterococci and *Staphylococcus aureus* (17, 23). This finding suggests that this mechanism of drug potentiation is strain or species specific.

The purpose of this study was to examine the effects of β-lactam antibiotics on the stimulation of increased aminoglycoside uptake in *Pseudomonas aeruginosa*. We also wanted to confirm in gram-negative bacilli our findings with gram-positive cocci that increased uptake correlates temporally with enhanced killing by β-lactam–aminoglycoside combinations and that these effects are apparent after 2 to 4 h of incubation (17, 23). These results suggest that standard 24-h studies are unnecessary and possibly misleading.

P. aeruginosa ATCC 27853 was used (5). By using standard techniques (2, 22), MICs of the following drugs were determined for *P. aeruginosa*: ticarcillin (Beecham Laboratories, Bristol, Tenn.), cefsulodin (Abbott Laboratories, North Chicago, Ill.), and tobramycin (Eli Lilly & Co., Indianapolis, Ind.) in tryptic soy broth (Difco Laboratories, Detroit, Mich.). The MICs (in micrograms per milliliter) were as follows: tobramycin, 1; cefsulodin, 2; and ticarcillin, 32. Time-kill studies were performed by a standard technique (10) with log-phase cells in tryptic soy broth at 37°C. To determine cell viability, samples were diluted in normal saline, added to tryptic soy broth agar (1%), and counted at 24 h (4, 16). To prevent antibiotic carry-over, the cells were immediately diluted 1,000-fold in iced saline. Ticarcillin or cefsulodin was added either 30 min before the addition of tobramycin or with tobramycin. Optical density (OD) was determined turbidimetrically (16). [^{3}H]tobramycin uptake was measured by membrane filtration (4) with GF/C filters (Whatman, Inc., Clifton, N.J.). Unlabeled tobramycin was combined with [^{3}H]tobramycin (Amersham Corp., Arlington Heights, Ill.). The final specific activities were 5 to 20 μCi/mg. Samples were counted in a liquid scintillation counter by using a toluene-based scintillant (16). All studies were done at least in duplicate.

Figure 1A shows the effects of ticarcillin at one-quarter of the MIC on the uptake of tobramycin at one-half of the MIC. This figure shows the results of a representative experiment in which ticarcillin was added 30 min before tobramycin was added. The results were similar when the antibiotics were added simultaneously. Exposure of the cells to ticarcillin was associated with a marked stimulation of tobramycin uptake. Neither ticarcillin nor tobramycin alone inhibited cell growth, whereas the combination was associated with a bactericidal effect at 2 to 4 h (Fig. 1B). Figure 1C shows the effects of antibiotics on growth (as measured by OD), which increased without antibiotics and with either ticarcillin or tobramycin alone. The ticarcillin-tobramycin combination showed no effect from 0 to 2 h, after which an inhibition of turbidimetrically measured growth was observed. Experiments with ticarcillin at one-quarter of the MIC and tobramycin at one or two times the MIC also showed an excellent correlation among β-lactam-induced tobramycin uptake, bactericidal effect, and growth inhibition (data not shown). In these experiments, however, β-lactam-enhanced uptake and bacterial killing were apparent only before 1 h. Thereafter, the rates of tobramycin uptake and bacterial killing were similar to those with and without ticarcillin.

Experiments with cefsulodin and tobramycin each at one-quarter to one-half of the MIC showed no increased uptake of tobramycin, increased bacterial killing, or growth inhibition (as measured by OD). However, when cells were exposed to cefsulodin at the MIC in combination with tobramycin at one-half of the MIC (Fig. 2A), there was stimulation of tobramycin uptake. Cell viability was similar in cells not exposed to antibiotics and in cells exposed to cefsulodin (Fig. 2B). In contrast, the combination of cefsulodin and tobramycin was associated with an enhanced bactericidal effect. Consistently, cell growth was inhibited by the cefsulodin-tobramycin combination (Fig. 2C) as determined turbidimetrically. Studies with cefsulodin at the MIC and tobramycin at two times the MIC showed similar

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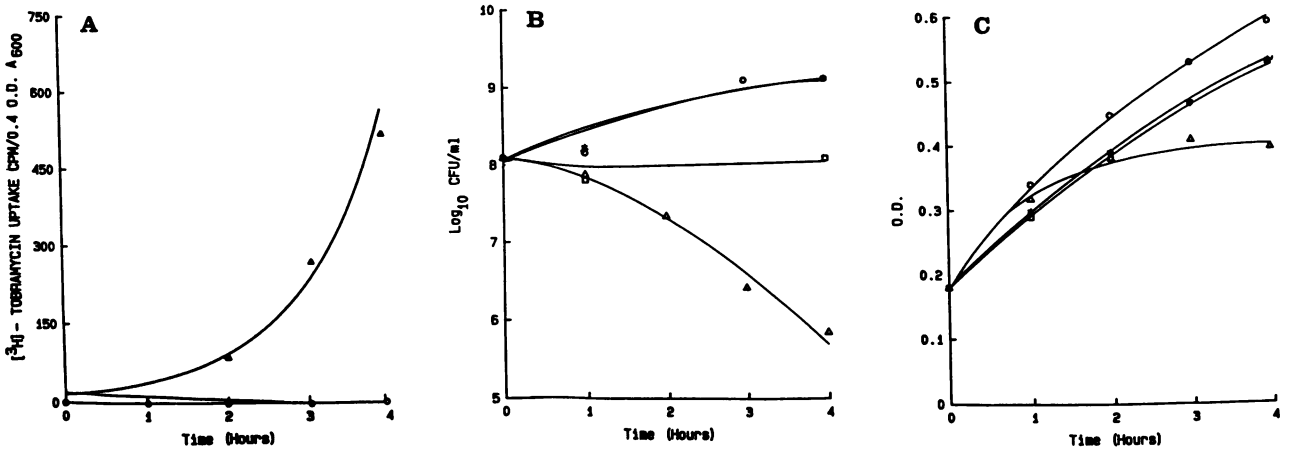

FIG. 1. Effects on *P. aeruginosa* ATCC 27853 of ticarcillin at one-quarter of the MIC and [^{3}H]tobramycin at one-half of the MIC alone or in combination. Ticarcillin was added 30 min before tobramycin was added. (A) Uptake of [^{3}H]tobramycin with (△) and without (○) ticarcillin. (B) Corresponding bactericidal effects with ticarcillin (○), ticarcillin and tobramycin (△), tobramycin alone (□), and control (*). (C) Corresponding effects on OD at 600 nm. Symbols are the same as for panel B.

results except that after 1 h, uptake and bacterial killing occurred with tobramycin alone.

It is believed that one mechanism of synergy between β-lactams and aminoglycosides in gram-negative bacilli is the β-lactam-enhanced increase of aminoglycoside uptake (1, 3). This assumption is based on studies examining the effects of these antibiotics in a single strain of *E. coli* (20). Our studies in a different gram-negative species demonstrated that β-lactams (i.e., ticarcillin and cefsulodin) stimulated the uptake of radiolabeled tobramycin. This finding was associated with an increased bactericidal rate and decreased cell replication. The onset of killing and growth inhibition (as measured by OD) were temporally associated with the uptake of tobramycin. While synergy studies with gram-positive cocci suggest that β-lactam–aminoglycoside potentiation is species specific (17), studies with both *P. aeruginosa* (present data) and *E. coli* (20; M. Miller, unpublished data) demonstrate that synergy in β-lactam–aminoglycoside combinations is associated with increased uptake of the aminoglycoside.

While we examined a single *P. aeruginosa* strain at a relatively high inoculum, abbreviated time-kill studies by others with *P. aeruginosa* (11) and enteric gram-negative bacilli (6, 7) have also shown early bactericidal synergism of β-lactam–aminoglycoside combinations at lower inocula. It is important to note that the β-lactams used in this study (ticarcillin and cefsulodin) preferentially bind to PBP 3 in *P. aeruginosa* (19, 21). Since preferential binding to different penicillin-binding proteins affects the degree and specificity of cell envelope perturbation (9), it is possible that other antipseudomonal β-lactams with different targets (i.e., imipenem, which binds to PBP 1 and 2) would affect the antibiotic barrier differently. Furthermore, Gerber et al. (5) have shown that an alternate mechanism of potentiation is present in vivo in *P. aeruginosa* ATCC 27853. β-Lactams prevented the regrowth of gentamicin-resistant mutants which were isolated from neutropenic rats treated with gentamicin alone. It is not possible at this time to determine the relative importance of antibiotic synergism or drug potentiation because of the prevention of mutational resist-

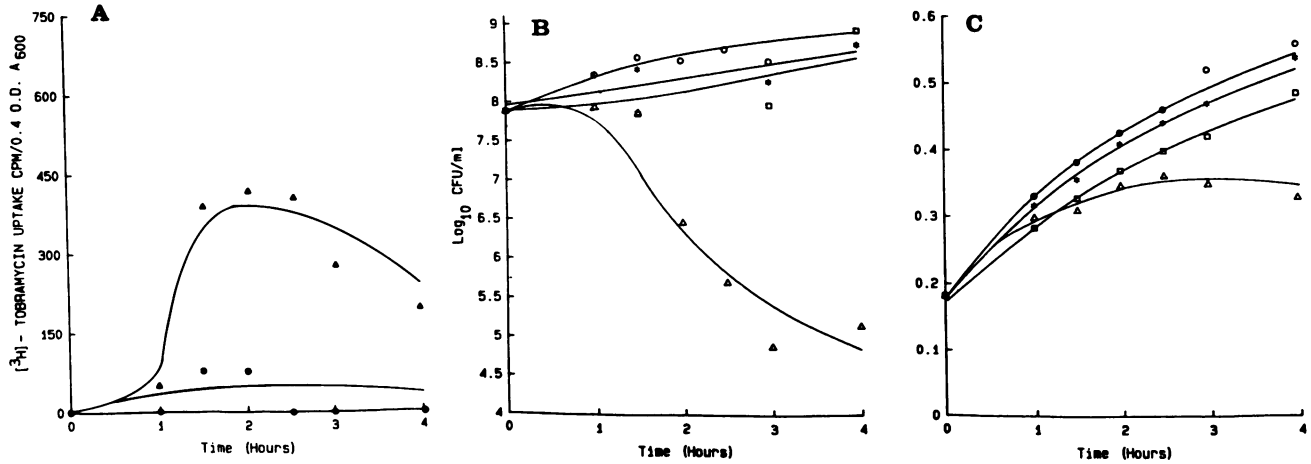

FIG. 2. Effects on *P. aeruginosa* ATCC 27853 of cefsulodin at the MIC and tobramycin at one-half of the MIC alone or in combination. (A) Uptake of [^{3}H]tobramycin with (△) and without (○) cefsulodin. (B) Corresponding bactericidal effects with cefsulodin and tobramycin (△), tobramycin alone (□), cefsulodin alone (○), and control (*). (C) Corresponding effects on OD at 600 nm. Symbols are the same as for panel B.

ance. Finally, these data support our belief, based on studies with gram-positive cocci (17), that standard 24-h time-kill studies may not reliably predict β-lactam–aminoglycoside synergism. Prolonged incubation is associated with mutant regrowth (15, 18), antibiotic degradation (6), and medium acidification (8). Abbreviated time-kill studies circumvent these problems. Moreover, an examination of the kinetics of increased aminoglycoside uptake and the bactericidal effect on replicating cells of β-lactams or chemicals which increase the $\Delta\mu$ H^+ demonstrates that aminoglycoside-associated killing is clearly apparent within minutes to hours (4, 12, 13, 15, 17, 18, 20, 23; present data). These kinetic studies support the suggestion of others (6, 7, 11, 14) that abbreviated time-kill studies may be preferable to conventional 24-h data in demonstrating synergy secondary to β-lactam-enhanced increased uptake of aminoglycosides.

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LITERATURE CITED

1. **Allan, J. D., and R. C. Moellering, Jr.** Management of infections caused by gram-negative bacilli: the role of antimicrobial combinations. Rev. Infect. Dis. **7**:5559–5569.
2. **Barry, A. L.** 1976. The antimicrobic susceptibility test: principles and practices. Lea & Febiger, Philadelphia.
3. **Davis, B. D.** Bactericidal synergism between β-lactams and aminoglycosides: mechanism and possible therapeutic implications. Rev. Infect. Dis. **4**:237–245.
4. **Eisenberg, E. S., L. J. Mandel, H. R. Kaback, and M. H. Miller.** 1984. Quantitative association between electrical potential across the cytoplasmic membrane and early gentamicin uptake and killing in *Staphylococcus aureus*. J. Bacteriol. **157**:863–867.
5. **Gerber, A. U., A. P. Vastola, J. Brandel, and W. A. Craig.** 1982. Selection of aminoglycoside-resistant variants of *Pseudomonas aeruginosa* an *in vivo* model. J. Infect. Dis. **146**:691–697.
6. **Glew, R. H., and R. A. Pavuk.** 1983. Early synergistic interaction between semisynthetic penicillins and aminoglycosidic aminocyclitols against *Enterobacteriaceae*. Antimicrob. Agents Chemother. **23**:902–906.
7. **Glew, R. H., and R. A. Pavuk.** 1984. Early synergistic interactions between amikacin and six β-lactam antibiotics against multiply resistant members of the family *Enterobacteriaceae*. Antimicrob. Agents Chemother. **26**:378–381.
8. **Houang, E. T., C. Hince, and A. J. Howard.** 1983. The effect of composition of culture media on MIC values of antibiotics. Antibiotics (Berlin) **12**:31–48.
9. **Kitano, K., and A. Tomasz.** 1979. Triggering of autolytic cell wall degradation in *Escherichia coli* by beta-lactam antibiotics. Antimicrob. Agents Chemother. **16**:838–848.
10. **Krogstad, D. J., and R. C. Moellering, Jr.** 1980. Combinations of antibiotics, mechanisms of interaction against bacteria, p. 298–341. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
11. **Lerner, S., E. J. Dudek, W. E. Boisvert, and K. D. Berndt.** Effect of highly potent antipseudomonal β-lactam agents alone and in combination with aminoglycosides against *Pseudomonas aeruginosa*. Rev. Infect. Dis. **6**:5678–5688.
12. **Mandel, L. J., E. S. Eisenberg, N. J. Simkin, and M. H. Miller.** 1983. Effect of *N*, *N'*-dicyclohexylcarbodiimide and nigericin on *Staphylococcus aureus* susceptibility to gentamicin. Antimicrob. Agents Chemother. **24**:440–442.
13. **Mates, S. M., E. S. Eisenberg, L. J. Mandel, L. Patel, H. R. Kaback, and M. H. Miller.** 1982. Membrane potential and gentamicin uptake in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **79**:6693–6697.
14. **Mates, S. M., L. Patel, H. R. Kaback, and M. H. Miller.** 1983. Membrane potential in anaerobically growing *Staphylococcus aureus* and its relationship to gentamicin uptake. Antimicrob. Agents Chemother. **23**:526–530.
15. **Mawer, S. L., and D. Greenwood.** 1978. Specific and nonspecific resistance to aminoglycosides in *Escherichia coli*. J. Clin. Pathol. (London) **31**:12–15.
16. **Miller, M. H., S. C. Edberg, L. J. Mandel, C. F. Behar, and N. H. Steigbigel.** 1980. Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **18**:722–729.
17. **Miller, M. H., M. A. El-Sokkary, S. A. Feinstein, and F. D. Lowy.** 1986. Penicillin-induced effects on streptomycin uptake and early bactericidal activity differ in viridans group and enterococcal streptococci. Antimicrob. Agents Chemother. **30**:763–768.
18. **Miller, M. H., M. A. Wexler, and N. H. Steigbigel.** 1978. Single and combination antibiotic therapy of *Staphylococcus aureus* experimental endocarditis: emergence of gentamicin-resistant mutants. Antimicrob. Agents Chemother. **14**:336–343.
19. **Noguchi, H., M. Matsuhashi, and S. Mitsuhashi.** 1979. Comparative studies of penicillin-binding proteins in *Pseudomonas aeruginosa* and *Escherichia coli*. Eur. J. Biochem. **106**:41–49.
20. **Plotz, P. H., and B. D. Davis.** 1962. Synergism between streptomycin and penicillin: a proposed mechanism. Science **135**:1067–1068.
21. **Rodriquez-Tebar, A., F. Rojo, J. C. Montilla, and D. Vasquez.** 1982. Interaction of β-lactam antibiotics with penicillin-binding proteins from *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **14**:295–298.
22. **Thrupp, L. D.** 1980. Susceptibility testing of antibiotics in liquid media, p. 73–113. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
23. **Zenilman, J. M., M. H. Miller, and L. J. Mandel.** 1986. In vitro studies simultaneously examining effect of oxacillin on uptake of radiolabeled streptomycin and on associated bacterial lethality in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **30**:877–882.