Combination of Bacteriostatic and Bactericidal Drugs: Lack of Significant In Vitro Antagonism Between Penicillin, Cephalothin, and Rolitetracycline

FRANZ D. DASCHNER
Children Hospital, Division of Antimicrobial Therapy, University of Munich, 8 Munich 2, Germany

Received for publication 16 April 1976

Although it is generally believed that bactericidal and bacteriostatic drugs should not be combined in vivo, in vitro experiments using the checkerboard dilution technique revealed no antagonism between penicillin/cephalothin and rolitetracycline but rather additive or synergistic activity of either drug combination in 40 to 50% of 20 Escherichia coli and 14 Staphylococcus aureus strains. Slight antagonism occurred only between 3 and 8 h after combining penicillin/cephalothin and rolitetracycline in either bacteriostatic or bactericidal concentrations, but not after 24 h of incubation, nor was antagonism found with combinations of these drugs in bacteriostatic concentrations. Neither bacteriostatic nor bactericidal activity of penicillin/cephalothin and rolitetracycline was inhibited by pretreatment of one E. coli strain with bacteriostatic rolitetracycline or bacteriostatic penicillin/cephalothin concentrations. Penicillin and cephalothin could exert a bactericidal effect after 2-h exposure of the E. coli strain to bacteriostatic rolitetracycline concentrations. Combined action of subinhibitory penicillin and rolitetracycline concentrations resulted in more pronounced inhibition of growth than either drug alone. The higher activity of penicillin/cephalothin in combination with rolitetracycline on some E. coli and S. aureus strains might be due to a better access of rolitetracycline into bacterial cells whose cell walls have been weakened by cell wall-active, bactericidal drugs. Thus, growth of penicillin-induced spheroplasts of E. coli and stable staphylococcal L-forms was inhibited by much lower concentrations of rolitetracycline than were the corresponding parent cells with intact cell walls.

According to Jawetz and Gunnison, the antibiotic and chemotherapeutic drugs can be divided into substances the effect of which is mainly bactericidal (e.g., penicillin, cephalosporins) and substances that generally have only bacteriostatic effects (e.g., tetracyclines) (11). They noted that whereas two bactericidal drugs were often synergistic, a combination of bactericidal and bacteriostatic antibiotics often resulted in antagonism in vitro. Several exceptions, however, to this chemotherapeutic rule have been found. The bacteriostatic sulfonamides may act synergistically with the bactericidal polymyxins against Proteus species and Pseudomonas aeruginosa (20, 22). Antagonism has been described between two penicillins, ampicillin and carbenicillin, when tested against Enterobacter cloacae (2). Although increased killing of penicillin-susceptible Streptococcus viridans by penicillin plus streptomycin can be demonstrated in vitro, the addition of streptomycin is not needed for effective treatment of patients with S. viridans endocarditis (2). Furthermore, it has long been known from in vitro studies that decreasing or increasing the concentration of primarily bactericidal and bacteriostatic drugs results in either bacteriostatic or bactericidal activity (19). None of these studies, however, considered the original finding of Jawetz that even within a given test system, the results of drug combinations may depend on drug concentrations, time of interaction, inoculum size, and other laboratory variables (10). I therefore investigated some of these variables, especially subinhibitory, bacteriostatic, and bactericidal drug concentrations and time of interaction, combining bactericidal (penicillin G, cephalothin) and bacteriostatic (rolitetracycline [RTC]) drugs in vitro. In further experiments with cell wall-defective bacteria (L-forms, spheroplasts), I examined why the combination of bactericidal and bacteriostatic drugs may lead to synergism instead of antagonism (16). RTC was used because with this drug very high serum levels can be obtained in pa-
tients after intravenous application, levels that exceed the concentrations found to be bactericidal in this study (7, 15).

MATERIAL AND METHODS

Test organisms. All Escherichia coli and Staphylococcus aureus strains used in this study were isolated from children with nosocomial diseases, mostly urinary tract infections and septicemia. One E. coli strain isolated from urine was used throughout the study for the growth and killing curves. The four strains of stable staphylococcal L-forms (L-36, L-65, L-68, L-101) and the corresponding bacterial forms were obtained from B. M. Kagan, Cedars Sinai Medical Center, Los Angeles, Calif., and subcultured weekly as described previously (5).

Antibiotics. Potassium penicillin G was supplied by Bayer (Leverkusen), cephalothin by Eli Lilly & Co. (Bad Homburg), and RTC by Hoechst AG (Frankfurt), each as standard powder. Laboratory standard solutions were prepared by dissolving the antibiotic in Mueller-Hinton broth; they were stored at -22°C and thawed immediately before use. Further dilution was done in Mueller-Hinton broth (Merck AG, Darmstadt).

Media. Mueller-Hinton broth adjusted to pH 7.4 or brain heart infusion broth (Difco) supplemented with 10% sucrose for osmotic stabilization was used for the growth and killing curves. All synergy studies using the checkerboard dilution technique were done in freshly prepared Mueller-Hinton broth, adjusted to pH 7.4 and supplemented with phenol red as indicator to facilitate reading the end points, as described previously (4). Salt serum agar was prepared according to Kagan (12) using gamma globulin-free horse serum (Grand Island Lab., N. D.) instead of human serum.

Synergy studies. The effect of RTC on the inhibitory activity of penicillin G or cephalothin was observed using a microtiter variation of the checkerboard dilution technique, as described previously (4). Briefly, twofold dilutions of RTC in 0.025 ml were made in one direction with a microtiter dilutor. Twofold dilutions of penicillin or cephalothin were added in the same volume manually in the perpendicular direction, using a disposable pipette delivering 0.025 ml/drop. Where each of the antibiotics was used alone, 0.025 ml of broth was added to achieve a volume of 0.05 ml. The inoculum of 0.05 ml from an overnight culture, adjusted to contain approximately 10⁹ organisms/ml with a Coleman Junior spectrophotometer, was added by delivering 1 drop from a 0.05-ml disposable drop pipette. The final concentrations of RTC for E. coli (S. aureus) ranged from 0.09 to 12.5 μg/ml (0.02 to 3.12 μg/ml), those for cephalothin ranged from 0.9 to 125 μg/ml (0.006 to 0.9 μg/ml), and those for penicillin ranged from 117 to 15,000 μg/ml (6 to 768 μg/ml). Inhibitory end points were determined after overnight incubation at 37°C by noting the lowest concentration of antibiotics, alone and in combination, that allowed no visible growth. This was greatly facilitated by change of the indicator from red to yellow. Isobolograms for each strain were constructed by plotting the inhibitory end points on an arithmetic scale in the usual manner. The isobole defining additive effects of the two antibiotics was obtained by drawing a straight line between the minimal inhibitory concentrations (MICs) of each drug acting alone. Synergism would be evident if the line representing combined action of both drugs at various concentrations fell below the additive isobole (21). Since one-half variations in MICs are commonly observed with serial dilution tests, which have a marked effect on the shape of the isobole, synergism was considered present only when there was, with the combination, at least a fourfold reduction in the MICs of both antibiotics (e.g., a twofold reduction with one antibiotic and a fourfold with the other one, causing only a slight concave bowing of the isobole was not accepted as evidence of synergistic activity). A twofold reduction of the MICs of both or either was considered additive, and no lowering of the end points was regarded as indifference.

Growth curves. Growth curves (Fig. 4) of one E. coli strain in Mueller-Hinton broth or osmotically stabilized brain heart infusion broth were obtained by measuring optical density, as described previously (5, 6). Subinhibitory penicillin (150 μg/ml) or RTC concentrations (0.9 μg/ml) were added alone, in combination, or following pretreatment with one of these antibiotics after 3 h of incubation.

Killing curves. Serial colony counts were performed with broth cultures of one E. coli strain to determine the time-related bactericidal or bacteriostatic effects of penicillin or cephalothin alone or in combination with RTC. Broth with one or both antibiotics was inoculated with bacteria from an overnight culture and adjusted photometrically to contain an average final inoculum of 0.4 × 10⁶ organisms/ml. At 0, 1, 2, 3, 4, 5, 6, and 24 h, 0.1 ml from each tube was transferred as aseptically to a broth dilution in petri dishes with Endo agar (Difco). Colony-forming units per milliliter were counted after overnight incubation. Typical experiments are shown in Fig. 1 through 3.

Antibiotic susceptibility testing of L-forms. Antibiotic susceptibility testing was done by using the agar dilution technique. From salt serum agar with equal growth of single L-form colonies, 0.5- by 0.5-cm agar blocks were cut out and inversely moved to one-half of the salt serum agar plates containing twofold dilutions of RTC (range, 0.45 to 1,000 μg/ml). The other halves of the plates were inoculated with 0.005 ml of a standardized inoculum (approximately 5 × 10⁴ organisms) of the corresponding parent forms, which resulted in growth of single colonies. One plate was used to test all four L-forms and parent forms; one plate without antibiotics served as growth control. Plates were read after 24 h and again after 48 and 72 h. Antibiotic concentrations that inhibited visible growth of L-forms and parent forms of staphylococci were tabulated in the MICs.

Incubation. All experiments were done at least in triplicate under aerobic conditions at 37°C.

RESULTS

Synergy studies. Table 1 summarizes the effects of penicillin and cephalothin in combination with RTC on 20 E. coli and 14 S. aureus.
TABLE 1. Effect of combinations of penicillin (Pen)/
cephalothin (Ceph) and RTC on 20 E. coli and 14 S.
aureus strains as determined by using the
checkerboard dilution technique

<table>
<thead>
<tr>
<th>Effect of antibiotic combination</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen/RTC Ceph/RTC Pen/RTC Ceph/RTC</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Strains. Three E. coli and one S. aureus strain were inhibited synergistically by the cephalothin/RTC combination; penicillin/RTC or cephalothin/RTC combinations were found to be additive on at least one-half of the strains tested. More important, however, was the lack of true antagonism of combinations of any of the so-called bactericidal and bacteriostatic antibiotics used in this study. The mean MIC of RTC for the E. coli (S. aureus) strains was 1.61 (0.69) μg/ml, and that of penicillin was 1,374 (161) μg/ml; the MIC of cephalothin was 15.6 (0.28) μg/ml.

Effect of bacteriostatic/bactericidal penicillin or RTC concentrations alone and in combination (killing curves). Figure 1 shows that significant growth over 24 h of the E. coli strain used in this study could be inhibited by certain penicillin (300 μg/ml) or RTC (7.8 μg/ml) concentrations (bacteriostatic concentrations). Increasing the concentrations resulted in immediate and marked bactericidal activity of both drugs. Only slight regrowth occurred beyond 8 h and up to 24 h. Figure 2 summarizes the findings if bacteriostatic/bactericidal penicillin/RTC concentrations or one drug in a bactericidal and the other in bacteriostatic concentration were combined at the beginning of the experiment. Control curves without antibiotics were similar to the one shown in Fig. 1. No antagonism could be found with penicillin/RTC combined in bacteriostatic concentrations (7.8 μg of RTC and 300 μg of penicillin per ml). Slight antagonism occurred between 3 and 8 h after combining RTC or penicillin in either bacteriostatic or bactericidal concentrations. No antagonism, however, could be found after 24 h of incubation.

It is well known that penicillin can destroy bacteria only when they are in the growing stage, so that drugs causing bacteriostasis might render penicillin ineffective if administered simultaneously. In experimental infections in mice, antagonism tended to occur especially if therapy with chlortetracycline was started before therapy with penicillin (14). I therefore studied the effect of penicillin and RTC on E. coli cells that had been pretreated with either drug in bactericidal or bacteriostatic concentrations. Neither bacteriostatic nor bactericidal activity of penicillin and RTC was inhibited by pretreatment with bacteriostatic RTC or penicillin concentrations (Fig. 3). In this type of experiment penicillin could exert a prompt bactericidal effect even after 2 h of exposure of the E. coli strain to bacteriostatic RTC concentrations (7.8 μg/ml). Killing curves almost identical to those shown in Fig. 1, 2, and 3 were obtained if cephalothin instead of penicillin was used in bactericidal (15.0 μg/ml) or bacteriostatic (5.0 μg/ml) concentrations. Control tubes without antibiotics were assayed in all experiments to be sure that bacteria studied were always in the logarithmic growth phase. All control curves were almost identical to the one shown in Fig. 1.

Growth curves of E. coli and antibiotic susceptibility testing of staphylococcal L-forms. The effect of subinhibitory RTC (0.9 μg/ml) and

![Fig. 1. Timed killing curves of one E. coli strain by bacteriostatic/bactericidal penicillin (300/600 μg/ml) or RTC (7.8/15.6 μg/ml) concentrations as compared to a control curve without antibiotic.](http://aac.asm.org/)
penicillin (150 µg/ml) concentrations on the growth of *E. coli* was studied by measuring optical density at hourly intervals. The curves obtained for growth in Mueller-Hinton broth are shown in Fig. 4, and those obtained for growth in osmotically stabilized brain heart infusion broth are shown in Fig. 5. Combined action of subinhibitory penicillin and RTC concentrations invariably resulted in more pronounced inhibition of growth than with either drug alone. Addition of penicillin or RTC 3 h after pretreatment with the other drug, each in a subinhibitory concentration, led to growth retardation; the growth curves were parallel, approaching the rate of growth inhibition of *E. coli* by penicillin and RTC when both drugs were added at the beginning of the experiment (Fig. 4).

The better activity of both drugs in combination, at least within 3 h of incubation, than either component alone was less obvious when hypertonic osmotically stabilized brain heart infusion broth instead of Mueller-Hinton broth was used for the growth curves (Fig. 5). Addition of RTC in a subinhibitory concentration 3 h after pretreatment with penicillin, however, significantly decreased optical density, suggesting bactericidal activity of RTC. At this time 99.9% of all bacteria were transformed to spheroplasts by penicillin, which has been demonstrated previously (6) as well as in this study by phase-contrast microscopy. This suggested that cell wall alterations induced by penicillin, especially in hypertonic medium, might permit greater access of RTC into the bacterial cell, which thus better reaches its site of action. This assumption could be supported by my finding that growth of four different strains of stable staphylococcal L-forms (cell wall-deficient bacterial forms) was inhibited by significantly lower concentrations of RTC than was that of the corresponding parent forms with intact cell walls (Table 2).

**DISCUSSION**

Despite the theoretical problems posed by the use of antibiotic combinations, there are five possible justifications for their use in clinical practice (2): (i) the concept of proven clinical synergy; (ii) to broaden the spectrum; (iii) to treat mixed or potentially mixed infections; (iv) to prevent the emergence of resistant organisms; and (v) the use of two potentially toxic drugs in lower dosage to possibly reduce the
danger of severe side effects. It is, however, generally believed that, using two drugs, bactericidal and bacteriostatic antibiotics should not be combined because of their in vitro and in vivo antagonism. Antibiotic antagonism, especially between the penicillins chloramphenicol and tetracycline (13, 17, 18, 23), could be demonstrated in various clinical studies. Similarly designed studies, however, failed to show any untoward effect of combinations of bacteriostatic and bactericidal drugs as compared with using only one drug. Finland et al. (9) observed no difference in the antibacterial activity of human plasma when either penicillin or penicillin combined with chloramphenicol was administered to human subjects. Ahern and Kirby (1) treated 25 patients with pneumococcal pneumonia with penicillin alone and 25 with penicillin and chlorotetracycline and found neither antagonism nor a beneficial effect from the drug combination. Walker (25) treated patients with streptococcal pharyngitis with penicillin alone or with penicillin and chloramphenicol; duration of fever, frequency of relapses, or other symptoms were not influenced by using penicillin alone or in combination with chloramphenicol. Some recent experimental studies also question the concept of general antagonism between bacteriostatic and bactericidal drugs. Luboshitzky et al. (16) found striking examples of synergism between tetracyclines/cephaloridine on Serratia and between tetracycline/kanamycin on Enterobacter. The combination of chloramphenicol and cephalothin resulted in an additive effect against four out of six multiple-antibiotic-resistant strains of Staphylococcus epidermidis (24). None of these studies, how-

![FIG. 4. Growth of E. coli at subinhibitory concentrations of penicillin or RTC alone or in combination in Mueller-Hinton broth. Addition of penicillin or RTC both in subinhibitory concentrations to cells pretreated with the other drug led to prompt bacteriostasis.](image)

![FIG. 5. Growth of E. coli at subinhibitory penicillin or RTC concentrations alone or in combination in osmotically stabilized brain heart infusion broth. Addition of RTC in subinhibitory concentrations to penicillin-induced spheroplasts resulted in bactericidal activity (arrow).](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent form</td>
</tr>
<tr>
<td>36</td>
<td>125</td>
</tr>
<tr>
<td>65</td>
<td>500</td>
</tr>
<tr>
<td>68</td>
<td>1.9</td>
</tr>
<tr>
<td>101</td>
<td>125</td>
</tr>
</tbody>
</table>
ever, considered the original finding of Jawetz (10) that results of drug combinations may depend on the bacterial strains, drug concentrations, time of interaction, and other laboratory variables. I therefore investigated some of these variables and found not antagonistic, but additive, indifferent, or synergistic activity between cephalothin/penicillin and tetracycline on various strains of *S. aureus* and *E. coli* when tested by using the checkerboard dilution technique. Slight antagonism between penicillin/cephalothin and RTC, each combined in bactericidal and bacteriostatic concentrations, occurred only between 3 and 8 h, but not at 24 h, of incubation. There was also no antagonism when the drugs were combined in bacteriostatic concentrations. This was also true if penicillin/cephalothin or RTC was added to *E. coli* cells pretreated with either bacteriostatic RTC or penicillin concentrations. Penicillin as well as cephalothin could exert a bactericidal effect after a 2-h exposure of *E. coli* to bacteriostatic RTC concentrations. The latter findings may account for some of the conflicting results of in vitro and in vivo studies concerning the effect of various drug combinations, especially those between so-called primarily bacteriostatic and bactericidal antibiotics (1, 9, 14). Even the combined action of subinhibitory penicillin and RTC concentrations invariably resulted in more pronounced inhibition of growth than did using either drug alone. Bactericidal activity of subinhibitory RTC concentrations on penicillin-induced *E. coli* spheroplasts in osmotically stabilized fluid medium suggested that cell wall-defective bacteria (spheroplasts) might permit greater access of RTC into the bacterial cell, which thus better reaches its site of action. This hypothesis could be supported by comparing the RTC MICs of staphylococcal L-forms and their corresponding parent forms with intact cell walls. L-forms were invariably more susceptible to RTC than the parent forms.

My data confirm those of Mouton and Koelman (19) and Jawetz (10), who found that drug interaction patterns between bactericidal and bacteriostatic drugs are highly dependent on bacterial species, strains, and drug concentrations used. Better activity of tetracyclines on cell wall-defective bacteria as compared with bacterial cells with intact cell walls has been demonstrated previously by Kagan (12). Whether this may account, at least in part, for the additive or synergistic action of penicillin in combination with RTC on certain strains remains to be studied. Combinations of bactericidal drugs with tetracyclines may be necessary to broaden the spectrum in the treatment of certain mixed infections, e.g., with anaerobes, where tetracyclines were shown to be effective in vitro (3). Further clinical studies are necessary to prove the effectiveness of these drug combinations. One of these studies is currently being done in our hospital. At present, however, I would not recommend the use of combinations of bactericidal and bacteriostatic drugs unless antagonism has been excluded, preferably by methods using bactericidal end points.

**ACKNOWLEDGMENTS**

The enthusiastic technical assistance of Ingrid Emricsson is appreciated.

This work was supported in part by a grant from Hoechst AG, Frankfurt, Germany.

**LITERATURE CITED**


Downloaded from http://aac.asm.org/ on January 12, 2018 by guest


