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# Failure of Time-Kill Synergy Studies Using Subinhibitory Antimicrobial Concentrations To Predict In Vivo Antagonism of Cephalosporin-Rifampin Combinations against *Staphylococcus aureus*

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**Results of in vitro time-kill synergy studies using subinhibitory, inhibitory, or suprainhibitory concentrations of bactericidal agents were compared with treatment outcomes of experimental infective endocarditis due to a methicillin-susceptible strain of *Staphylococcus aureus*. For rifampin-cephalosporin combinations, in vitro synergy testing using recommended fractions of the MIC failed to predict antagonism in vivo while concentrations above the MIC corresponded with antagonism in vivo.**

Standardized in vitro tests of susceptibility, such as determination of the MIC, correlate well with the outcomes of methicillin-susceptible *Staphylococcus aureus* experimental endocarditis treated with beta-lactam monotherapy (16). Fewer data about the correspondence of in vitro and in vivo results with combinations of antimicrobial agents are available, despite the fact that antimicrobial combinations are recommended to increase the bactericidal activity, to reduce the risk for emergence of resistance, or to shorten the duration of treatment of *S. aureus* endocarditis.

In vitro studies have indicated that rifampin is very active against most strains of *S. aureus* (2, 11). The use of rifampin alone is not recommended because of the possibility of a one-step mutation to ribosomal resistance (1 in $10^6$ to $10^7$ bacteria) (12, 14, 20). The adjunctive role of rifampin in combination therapy with a beta-lactam for *S. aureus* endocarditis, however, remains controversial (14, 19, 21).

Among laboratory methods to assess antimicrobial interaction in vitro, the time-kill method corresponds best with outcomes in animal models (3). Published recommendations for testing two bactericidal agents propose using either subinhibitory (subbactericidal) fractions of at least one (1) or both agents (4) or "average" concentrations in serum obtainable during therapy (7). Whether or not antimicrobial concentrations independent of the antimicrobial concentration ratio are a determining factor for outcomes of time-kill studies remains unclear (18, 22).

The purposes of this study were (i) to determine the effect of antimicrobial concentrations on the outcomes of time-kill studies using rifampin or gentamicin and nafcillin, cefazolin, or cefpirome in a constant concentration ratio and (ii) to compare these in vitro results with treatment outcomes of experimental infective endocarditis in order to determine the in vitro concentrations that best corresponded with in vivo outcomes.

**In vitro studies.** A macrodilution method involving an inoculum of $5 \times 10^5$ CFU/ml for 25 strains of methicillin-susceptible *S. aureus* recovered from patients with infective endocarditis treated at the Mayo Clinic was used to test the MIC (8). A representative isolate was selected for in vivo studies. Time-kill synergy studies were performed at recommended subinhibitory concentrations (one-fourth and one-half the MIC) (1, 4) and also at inhibitory (MIC) and suprainhibitory (twice and four times the MIC) concentrations of all antimicrobial agents. Tests were performed in triplicate, and results were expressed as mean $\log_{10}$ CFU per milliliter. Synergy or antagonism was defined as an $\geq$100-fold increase or decrease and indifference was defined as a <10-fold increase or decrease in killing after incubation with the combination compared with that of the most active single agent (4).

**In vivo studies.** Catheter-associated aortic valve experimental infective endocarditis was established in New Zealand White rabbits by a modification of the method of Garrison and Freedman (5) as described previously (16). Treatment was initiated 24 h after infection with $5 \times 10^5$ CFU of staphylococci per ml and continued for 3 days. Antimicrobial dosages (nafcillin at 200 mg/kg of body weight intramuscularly three times daily [t.i.d.], cefazolin at 50 mg/kg intramuscularly t.i.d., cefpirome at 40 mg/kg subcutaneously t.i.d., gentamicin at 1.5 mg/kg intramuscularly t.i.d., and rifampin at 5 mg/kg subcutaneously twice daily) were chosen to result in peak concentrations in serum in rabbits similar to those in humans receiving recommended doses (9). Mean concentrations (in micrograms per milliliter) were achieved 30 min after a single dose in uninfected rabbits as follows: nafcillin, 60; cefazolin, 154; cefpirome, 59; gentamicin, 4.4; and rifampin, 1.9. Treated animals were sacrificed at least 12 h after the last antimicrobial dose; control animals were sacrificed 24 h after infection because they rarely survived 3 days. Aortic valve vegetations were removed aseptically, and vegetations were weighed, homogenized, and cultured quantitatively by a pour plate method. Results were expressed as $\log_{10}$ CFU of staphylococci per gram of valve vegetation. For quantitative nonparametric analysis, samples with no growth on the lowest dilution ("sterile" vegetations) were assigned half of the value of the lowest detectable quantity of microorganisms. The overall null hypothesis that no differences in mean $\log_{10}$ CFU of staphylococci per gram of valve vegetation existed among any of the treatment groups was analyzed statistically by the Kruskal-

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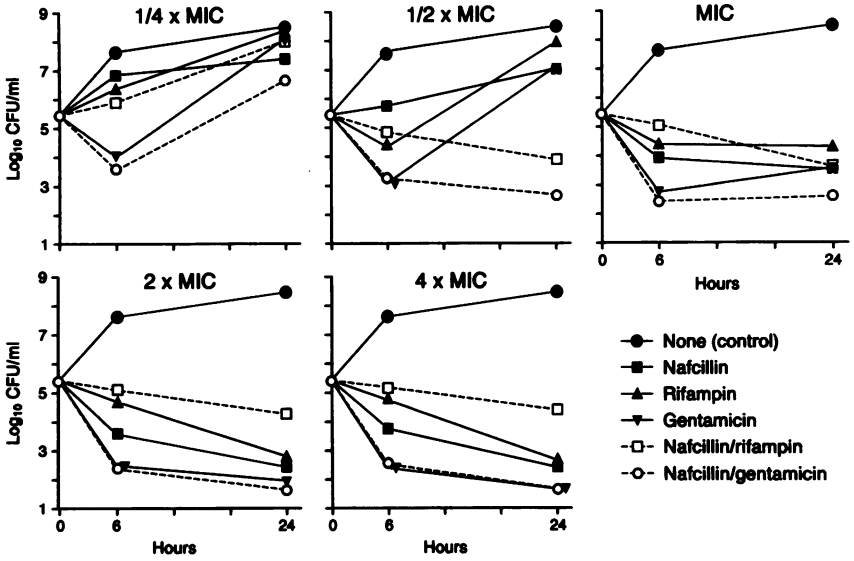


FIG. 1. In vitro activities of subinhibitory, inhibitory, and suprainhibitory concentrations of nafcillin and rifampin or gentamicin at fixed concentration ratios compared with the activities of the single agents against the *S. aureus* study strain as assessed by the time-kill technique.

Wallis test (10). Pairwise comparisons of treatment groups were performed by the Wilcoxon rank sum test (10).

The MICs (in micrograms per milliliter) for the strain used in vivo were 0.5 for nafcillin, 0.5 for cefazolin, 1 for cefpirome, 0.015 for rifampin, and 1 for gentamicin. Results of time-kill studies of the study strain are shown in Fig. 1 for rifampin-nafcillin and gentamicin-nafcillin combinations. Similar results were obtained with the respective cephalosporin-containing combinations. Occasional regrowth of cultures containing rifampin alone, but not in the presence of any beta-lactam, at concentrations at or above the MIC was attributed to rifampin-resistant mutants (6, 12). These overgrown cultures were excluded from the evaluation. Table 1 shows the results of treatment of experimental endocarditis in rabbits. Preliminary overall analysis revealed a significant difference ($P < 0.01$) in mean $\log_{10}$ CFU per gram of valve vegetation among treatment groups.

TABLE 1. Results of treatment of experimental endocarditis in rabbits

| Treatment | No. dead/no. treated | No. with sterile vegetations/no. surviving | $\log_{10}$ CFU/g of valve vegetation | |
|---|---|---|---|---|
| | | | Median | Range from 25th to 75th percentile |
| None | 0/9 | 0/9 | 11.29 | 8.73–11.51 |
| Cefazolin + rifampin | 5/14 | 0/9 | 6.45 | 6.16–6.56 |
| Cefpirome + rifampin | 3/15 | 0/12 | 6.43 | 6.02–7.06 |
| Nafcillin + rifampin | 2/15 | 4/13 | 5.93 | 0.56–6.52 |
| Cefpirome | 1/17 | 4/16 | 2.69 | 1.39–5.56[a] |
| Cefazolin | 1/19 | 0/18 | 2.63 | 2.16–5.82[b] |
| Cefpirome + gentamicin | 2/14 | 0/12 | 2.15 | 2.04–2.87 |
| Nafcillin | 1/15 | 2/14 | 1.97 | 1.52–2.89[c] |
| Cefazolin + gentamicin | 3/16 | 6/13 | 1.68 | 0.56–2.29 |
| Nafcillin + gentamicin | 2/18 | 9/16 | 0.56 | 0.56–4.77 |

[a] $P < 0.01$ for value for cefpirome compared with value for cefpirome plus rifampin.

[b] $P < 0.01$ for value for cefazolin compared with value for cefazolin plus rifampin or cefazolin plus gentamicin.

[c] $P < 0.05$ for value for nafcillin compared with value for nafcillin plus gentamicin.

Because time-kill studies demonstrated indifference after 6 h of incubation for all regimens at any of the different concentrations tested, evaluation of time-kill studies after 6 h did not distinguish between regimens. After 24 h of incubation with rifampin in combination with all three beta-lactams, time-kill studies using recommended concentrations below the MIC failed to predict in vivo antagonism observed with the combination of rifampin and cefazolin ($P < 0.10$) or cefpirome ($P < 0.01$) but not with nafcillin ($P = 0.58$). Time-kill studies using the same antimicrobial combinations at concentrations equal to or greater than twice the MIC corresponded with treatment outcomes with rifampin-cephalosporin combinations. In contrast, the in vitro synergy of gentamicin-containing regimens with subinhibitory concentrations of one-half the MIC did correspond with the in vivo finding of significantly more effective combination treatment with gentamicin and nafcillin ($P < 0.05$) or cefazolin ($P < 0.01$) but not with cefpirome ($P = 0.41$) compared with the respective beta-lactam monotherapy.

The concentration-dependent variability in the results of our time-kill studies using rifampin in combination with nafcillin, cefazolin, or cefpirome against *S. aureus* is consistent with the findings of Zinner et al. (22), who used various concentrations of rifampin and methicillin against a different strain of *S. aureus*. Our study, in contrast to that of Zinner et al. (22), used a fixed concentration ratio of rifampin to beta-lactams for in vitro testing, and it supports the hypothesis that antimicrobial concentrations, independent of the ratio of the agents, are important determinants for outcomes of time-kill synergy studies. Our results with rifampin in combination with three different beta-lactams demonstrate that the concentration-dependent outcome of time-kill studies using rifampin is not specific for a single beta-lactam, and the agreement between our findings and those of Zinner et al. (22) suggests that the variability in the results of time-kill studies is not unique to the strain of *S. aureus* used in our study. The finding of our study that the same beta-lactams combined with gentamicin demonstrated less concentration-dependent variability extends the results of previous studies in which it was found that outcomes

of time-kill synergy studies varied to a lesser degree with the antimicrobial concentrations when rifampin in combination with vancomycin (22) or coumermycin or ciprofloxacin (17) was used against *S. aureus*. These results suggest that outcomes of time-kill studies are affected by antimicrobial concentrations primarily when rifampin is tested in combination with beta-lactams against *S. aureus*.

In our study, the addition of rifampin to nafcillin did not decrease the efficacy of nafcillin monotherapy; however, the antagonism we observed with rifampin-cephalosporin combinations is in agreement with results of other studies (14) using a different strain of *S. aureus*. In our study, combination therapy with gentamicin and beta-lactams except for cefpirome was more effective than was monotherapy with the respective beta-lactam, supporting previous studies (13, 14) which demonstrated synergy in vivo against a different strain of *S. aureus* with gentamicin–beta-lactam combinations.

Because in vitro synergy tests for bactericidal agents are not standardized and methods to determine the recommended "average" concentrations in serum obtainable during therapy (7) remain unclear, suprainhibitory concentrations of twice and four times the MIC were chosen arbitrarily in our study. In contrast to results with gentamicin–beta-lactam combinations, results of time-kill studies using recommended fractions of the MIC (1, 4) failed to predict antagonism in vivo of rifampin in combination with cefazolin or cefpirome. Our results were obtained only with a single strain, and the degree to which these findings can be generalized is unknown. These results, however, are in agreement with similar findings with a different strain of *S. aureus* as described above. Further, although our results were obtained with a single strain, the failure to predict frank antagonism in vivo necessitates caution in using the time-kill technique with subinhibitory concentrations of both agents in rifampin–beta-lactam combinations against *S. aureus*. Results of time-kill studies using concentrations above the MIC of rifampin and cephalosporins corresponded with antagonism in vivo in this study, but further studies are needed to confirm this correspondence.

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