

Value of Serum Tests in Combined Drug Therapy of Endocarditis

THOMAS A. DRAKE, CORINNE J. HACKBARTH, AND MERLE A. SANDE*

The Medical Service, San Francisco General Hospital, University of California, San Francisco, California 94110

Received 19 May 1983/Accepted 23 August 1983

Two *in vitro* tests, the serum killing level and the serum bactericidal rate assays, were evaluated for correlation with therapeutic efficacy in the rabbit model of *Staphylococcus aureus* endocarditis. Animals were treated with nafcillin alone and in combination with tobramycin or gentamicin. Both were effective therapies, but rapidity of vegetation sterilization by the single and combined regimens was shown by the serum bactericidal rate assay but not the serum killing level assay. As a direct measure of bactericidal activity in serum during therapy, the serum bactericidal rate assay may be a clinically useful supplemental test for providing information that the serum killing level assay cannot.

Since the development of antimicrobial therapy for endocarditis, the need has existed for an *in vitro* test which would predict therapeutic efficacy. A serum inhibitory assay was first described by Schlichter in 1947 (10, 11) and was later modified to a serum bactericidal assay (the serum killing level [SKL] assay) as the importance of bacterial killing became recognized. As currently performed, dilutions of serum receiving the same inoculum are subcultured after 18 to 24 h of incubation to determine the highest dilution in which $\geq 99.9\%$ of the inoculum has been killed. A peak SKL of 1:8 or greater is generally accepted as being predictive of adequate therapy, although this remains to be firmly established in clinical studies (2). Stratton et al. (13) have shown that the SKL is a reasonable measure of the magnitude of antibiotic concentration in serum relative to the minimum bactericidal concentration (MBC) of the infecting organism.

Studies of penicillin-aminoglycoside synergy in experimental endocarditis suggest that the rate of bacterial killing is an important determinant of antibiotic efficacy (4, 9). This parameter of antibiotic activity is not well demonstrated by the SKL assay. As organisms are quantitated only once during the assay, 18 to 24 h after inoculation, it is the cumulative bactericidal activity, rather than the kinetics of bacterial killing, that is measured.

In an adaptation of an assay suggested by Kunin (7), we performed 8-h time-kill studies with single 1:2 dilutions of serum samples to measure the rate of bactericidal activity (serum bactericidal rate [SBR] assay). Results of this measure of bacterial killing were then compared

with SKLs for correlations with the efficacy of single and combined drug therapy of *Staphylococcus aureus* endocarditis in rabbits. SBRs reflected the greater *in vivo* efficacy of combination drug therapy, whereas SKLs did not.

MATERIALS AND METHODS

Organism. A single *S. aureus* strain which had been isolated from a patient with endocarditis was used. At the beginning of the study, this strain of *S. aureus* was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and poured over glass beads, which were then kept at -70°C and removed singly as needed throughout the study.

Antibiotics. Sodium nafcillin was obtained from Wyeth Laboratories, Inc. (Philadelphia, Pa); tobramycin was supplied by Eli Lilly & Co. (Indianapolis, Ind.); gentamicin was provided by Schering Corp. (Bloomfield, N.J.).

Susceptibility tests. Minimum inhibitory concentrations and MBCs of nafcillin, tobramycin, and gentamicin were determined by standard macrobroth dilution techniques (1). Synergy studies were performed by the time-kill method in Mueller-Hinton broth (MHB). Portions (0.1 ml) of an overnight culture were added to 10 ml of MHB containing one of the following: nafcillin, 10 $\mu\text{g/ml}$; tobramycin, 1.0 $\mu\text{g/ml}$; gentamicin, 1.0 $\mu\text{g/ml}$; nafcillin, 10 $\mu\text{g/ml}$, plus tobramycin, 1.0 $\mu\text{g/ml}$; nafcillin, 10 $\mu\text{g/ml}$, plus gentamicin, 1.0 $\mu\text{g/ml}$; or no antibiotic. Cultures were incubated at 35°C for 24 h without shaking. Samples (0.1 ml) were removed from each flask at 6 and 24 h of incubation and quantitatively cultured.

Animal studies. Endocarditis was established in 64 2- to 3-kg white New Zealand rabbits by previously described methods (9). A polyethylene catheter was inserted through the carotid artery into the left ventricle of the heart and secured in place while the rabbits were under pentobarbital anesthesia. One hour after the insertion of the catheters, 10^6 CFU of *S. aureus* in

1 ml of saline from an overnight culture was injected via the catheters. Administration of nafcillin (200 mg/kg) was begun 24 h after infection, alone or in combination with either tobramycin or gentamicin (3.4 mg/kg) intramuscularly, and was repeated every 8 h for a total of seven doses. Control animals included in each experiment received no injections. Sixteen hours after the last drug dose, animals were sacrificed by intravenous injection of 125 mg of pentobarbital. The hearts were removed, and vegetations on the aortic valve were excised, weighed, homogenized in 2.5 ml of sterile saline, and quantitatively cultured. Bacterial titers in the vegetations were expressed as CFU per gram of wet vegetation. The lowest concentrations detectable ranged from \log_{10} 2.3 to 3.4, depending on the vegetation weight. Sixteen animals had negative blood cultures at the start of therapy and were excluded from the analysis of results.

Serum antibiotic concentrations, SKL assay, and SBR assay. Blood was obtained from ear arteries 30 to 60 min and 7 to 8 h after dosing, usually after the first or third dose. Serum was removed and stored at -70°C until use. Antibiotic concentration, SKL, and SBR were determined for each specimen, unless testing was limited by the volume of serum obtained. Serum antibiotic concentrations were determined by the agar well diffusion technique, with a *Bacillus globigii* strain as the indicator organism. When samples contained both an aminoglycoside and nafcillin, either the aminoglycoside was eliminated by adding cellulose phosphate (12) or the beta-lactam was hydrolyzed by adding beta-lactamase (8).

SKLs were determined by standard techniques (1) with inocula of ca. 5×10^5 CFU/ml from an overnight culture. Dilutions were made in pooled serum obtained from healthy rabbits. Tubes were vortexed immediately after inoculation and again just before sampling after 24 h of incubation without shaking. Duplicate assays with dilutions prepared in MHB were performed with some samples. The SKL was defined as the greatest dilution in which 99.9% of the inoculum was killed after 24 h of incubation.

SBRs were determined by adding 0.5 ml of inoculum (5×10^5 CFU/ml) from an overnight culture in MHB to 0.5 ml of rabbit serum (peak or trough sample) in clear plastic tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.), vortexing the mixture, and incubating it at 35°C without shaking. Samples (0.1 ml) were taken after vortexing at 2, 4, 6, and 8 h for quantitative culture. The SBR was determined as the mean log drop per hour in bacterial titer over the 8-h incubation period.

Statistics. Comparisons were made using Student's *t*-test; for multiple comparisons, Bonferroni's correction for significance was used.

RESULTS

Antimicrobial susceptibility tests. The test strain of *S. aureus* was inhibited and killed by clinically achievable concentrations of the antibiotics used. Minimal inhibitory concentrations and MBCs were, respectively, 0.25 and 1.0 μg of nafcillin per ml, and 0.125 and 0.25 μg of gentamicin or tobramycin per ml. Time-kill studies demonstrated acceleration in bactericidal activity with the beta-lactam plus either aminoglyco-

side, as defined by a $\geq 10^2$ reduction in bacterial titers at various times compared with the activity of each drug alone.

Efficacy of therapy in animals. Nafcillin alone reduced vegetation titers below the levels found in untreated control animals (Fig. 1), and the addition of an aminoglycoside improved the efficacy even further. Control rabbits survived an average of 2.4 days (range of 1 to 4 days) and had mean bacterial concentrations (± 1 standard deviation) in vegetations of $8.9 \pm 0.8 \log_{10}$ CFU/g at autopsy. In contrast, rabbits treated with nafcillin had mean concentrations of $5.4 \pm 1.6 \log_{10}$ CFU/g. The addition of tobramycin or gentamicin to nafcillin reduced the titers to $3.9 \pm 1.0 \log_{10}$ and $3.8 \pm 1.3 \log_{10}$ CFU/g, respectively ($P < 0.05$ for both). Survival was greater than 90% in all treatment groups. These in vivo findings correlated well with the equal potentiation of bacterial killing rates by each aminoglycoside in vitro. Peak antibiotic levels in serum were comparable to those expected to be achieved in man (5). The mean (\pm standard deviation) peak concentrations were: nafcillin, $32.2 \pm 18.2 \mu\text{g/ml}$; tobramycin, $6.3 \pm 2.3 \mu\text{g/ml}$; and gentamicin, $5.9 \pm 1.7 \mu\text{g/ml}$.

SKLs. The SKLs did not reflect the differences in in vivo efficacy between the antibiotic regimens (Fig. 2). No significant increase in SKLs was found when either aminoglycoside was combined with nafcillin, despite improved in vivo efficacy. SKLs of peak samples from treated animals ranged from 1:2 to 1:32 (median 1:8) with nafcillin, 1:4 to 1:32 (median 1:16) with

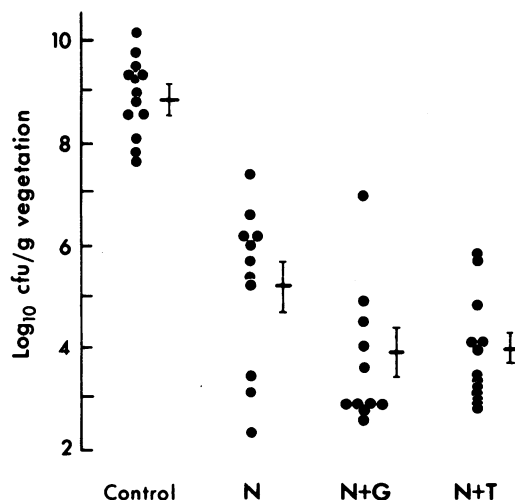


FIG. 1. Bacterial concentrations in aortic valve vegetations of controls and rabbits treated for 56 h. N, Nafcillin; N+T, nafcillin plus tobramycin; N+G, nafcillin plus gentamicin. Error bars indicate \pm standard error of the mean.

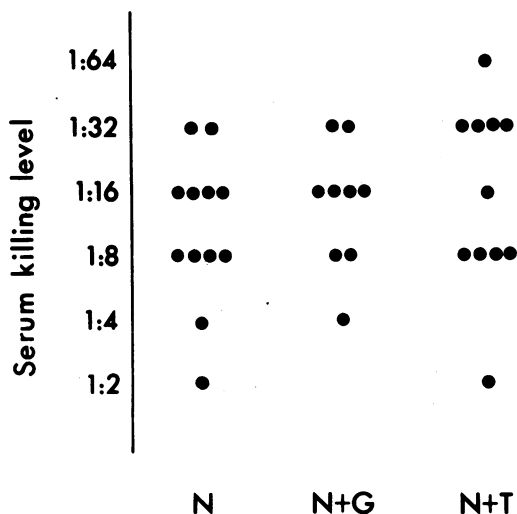


FIG. 2. Peak SKLs of treated rabbits. Samples were diluted with pooled normal rabbit serum. N, Nafcillin; N+T, nafcillin plus tobramycin; N+G, nafcillin plus gentamicin.

nafcillin plus gentamicin, and 1:2 to 1:64 (median 1:16) with nafcillin plus tobramycin. Extensive overlap was present among all three treatment groups. Determination of SKLs with broth dilutions rather than serum gave slightly higher SKLs for all three treatment groups but did not affect the degree of overlap. Median SKLs of trough samples were 1:2 for all three treatment groups.

SBRs. Eight-hour time-kill curves in peak serum samples diluted 1:2 with an inoculum in MHB reflected the enhanced efficacy of combination therapy (Fig. 3). Killing was more rapid and of greater magnitude in samples from rabbits receiving an aminoglycoside plus nafcillin, with little or no overlap in the cumulative drop of log CFU between samples from single and combined drug regimens at 4 to 8 h of incubation. Presence of an aminoglycoside produced marked killing in the initial 2 h ($-1.0 \log_{10}$ CFU/h) and less rapid killing in the subsequent 6 h (-0.6 to $-0.7 \log_{10}$ CFU/h). These rates were significantly higher than those with nafcillin alone, which were $-0.3 \log_{10}$ CFU/h during both the initial 2 and subsequent 6 h. Much greater intersample variability was observed among samples containing an aminoglycoside. Although the differences in SBR results were significant when single and combined drug regimens were compared, within a given treatment regimen, serum samples with the most rapid killing did not necessarily correlate with the lowest vegetations titers. Serum killing curves of trough samples showed no difference between treatment groups. All were comparable to those

curves observed in peak samples containing nafcillin alone. Use of a log-growth phase inoculum eliminated an early "lag" phase in killing seen with some samples containing nafcillin alone, but did not alter the rapid bactericidal activity of peak serum samples containing nafcillin and an aminoglycoside.

DISCUSSION

In this study, the validity of the SKL assay as a measure of adequate therapy is not questioned, but rather its ability to reflect optimal therapy of *S. aureus* endocarditis, which it fails to do. The SBR assay is proposed as a useful adjunctive test to aid in this determination. It is, simply, an abbreviated time-kill study performed with the bacterial isolate and serum of the individual being treated. The assay is a modification of one suggested by Kunin (7), which has the particular value of measuring the relative antibiotic killing rate. Though no prior studies have specifically examined SKLs in a comparison of single and combination therapies, Durack noted that SKLs were no different in animals receiving penicillin than in those receiving penicillin plus streptomycin during treatment of *Streptococcus sanguis* endocarditis, despite a marked difference in efficacy between the two treatment regimens (4). In a clinical study of *S. aureus* endocarditis, addition of gentamicin to nafcillin increased the rapidity of bloodstream sterilization, but peak SKLs were similar for both treatment groups (6).

This study documents the improved efficacy of combined nafcillin-aminoglycoside regimens over those with nafcillin alone in the therapy of experimental *S. aureus* endocarditis. Peak or

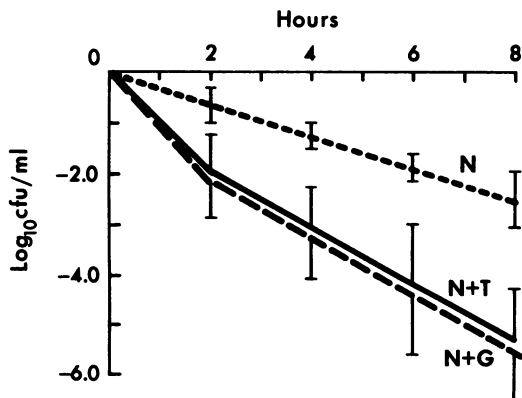


FIG. 3. SBRs of peak serum samples of treated rabbits, plotted as log drop in titer from inoculum over 8 h, averaged for each group at each sampling time during the assay. Error bars represent ± 1 standard deviation. N, Nafcillin; N+T, nafcillin plus tobramycin; N+G, nafcillin plus gentamicin.

trough SKLs do not reflect this enhanced activity, whereas peak SBRs do. The explanation lies in the fact that enhancement of nafcillin activity occurs rapidly and is dependent on aminoglycoside concentration. Sequential culturing of SKL dilutions at 2, 8, and 24 h demonstrated that the enhanced rate of killing by sera containing aminoglycosides occurred only at lower dilutions, usually 1:8 or less, and was evident only at the 2- and 8-h sampling times (data not shown). The killing effect of the aminoglycoside is concentration dependent, i.e., the rate at which the drug kills *S. aureus* increases with increasing concentrations over the therapeutic range. This is not, however, true of nafcillin, which reaches a maximum rate of bacterial killing at a relatively low concentration (the MBC, which usually is far below the levels generally obtained in serum). Therefore, the maximum SBR will occur with the highest aminoglycoside concentrations (a 1:2 dilution of serum). As the serum is progressively diluted, the killing rates will be correspondingly reduced until only the nafcillin activity is present. This concentration dependency of nafcillin-plus-aminoglycoside "synergy" against *S. aureus* has been demonstrated in a different manner by Watanakunakorn and Glotzbecker (15). Of 35 strains, 30 had a fourfold or greater decrease in the minimum inhibitory concentration of nafcillin in the presence of 5 µg of tobramycin per ml. Only 13 of the same 35 strains had such a decrease with 1.25 µg of tobramycin per ml. It is, therefore, unlikely that the SKLs for these antibiotics would reflect any potentiation of *in vivo* efficacy.

Although the SBR assay directly measures the rate of killing, it does not reflect the magnitude of antibiotic concentrations relative to the MBC as well as the SKL assay does (13). It may still be important, therefore, to obtain SKLs of 1:8 or greater for peak samples, as this assures measurable bactericidal activity in serum (SKL, 1:2 or greater) for at least three half-lives of the drug in question. The SBR assay complements the SKL assay by measuring a parameter of drug activity (rate of killing) that is not reflected by the latter. Klustersky and colleagues recognized this distinction and have assessed the relative potency of drug combinations by determining both SKLs and time-kill curves in dilutions of serum obtained after dosing volunteers (14). The assay used resembles that reported here, although the killing curves were obtained with a 1:8 rather than a 1:2 dilution of serum. A 1:2 dilution is preferable since alterations in concentration-dependent effects of the drugs are minimized. The other accepted laboratory tests that evaluate drug interactions (checkerboard and time-kill kinetic studies) are also limited. Although they may effectively predict drug-to-drug interac-

tions, neither reflects accurately the actual drug concentrations in the individual patient or the influence of serum on the results.

Our results, which support the value of the SBR assay, are specifically applicable for combined nafcillin-plus-aminoglycoside therapy of endocarditis caused by methicillin-susceptible *S. aureus*. Although only a single strain was tested in this study, it is typical of numerous *S. aureus* strains that we have examined *in vitro* for aminoglycoside enhancement of nafcillin activity. Whether or not the SBR assay will predict the relative effectiveness for a variety of single antibiotics or significantly different dosage schedules has yet to be studied. Also, the actual rate of eradication of bacteria from vegetations *in vivo* is far slower than that observed in the SBR assay; this is possibly related to the demonstrated metabolic inactivity of organisms in established vegetations (3). Evidence for the predictive utility of the SBR assay, as for any *in vitro* test, must be strictly empirical until the mechanisms of bacterial killing *in vivo* are elucidated.

The SBR assay could easily be adapted for the clinical microbiology laboratory. With limited sampling times (e.g., 0, 2 to 4, and 6 to 8 h only), the assay would be simple to perform in conjunction with the SKL assay, with final results available the following day. The potential therapeutic value of this test would be to assure the physician that (i) the choice of antimicrobial agents is, in fact, producing a rapid bactericidal effect *in vivo*, and (ii) the addition of another drug to the regimen increases, rather than decreases, the bactericidal rate. The SKL assay alone cannot reliably provide this guidance. The unique properties of the SBR assay warrant its further evaluation, both in experimental and clinical settings, for infections where rapid eradication of bacteria would be expected to favorably influence the outcome.

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