ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1986, p. 418–423
0066-4804/86/030418-06$02.00/0

Vol. 29, No. 3

Discrepancies between MBC and Actual Killing of Viridans Group Streptococci by Cell-Wall-Active Antibiotics

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Received 25 July 1985/Accepted 18 December 1985

We determined the MBC of amoxicillin and vancomycin, two antibiotics advocated for treatment and prophylaxis of bacterial endocarditis, for 24 strains of viridans group streptococci isolated from patients with endocarditis. We found that the MIC of amoxicillin for all strains was ≤0.25 μg/ml and the MBC was either low (<0.5 μg/ml) in 6 nontolerant strains or high (>128 μg/ml) in 18 tolerant strains. The MIC of vancomycin for the 24 strains was ≤1 μg/ml, and the MBC was either low (<1 μg/ml) for 3 nontolerant strains or high (>128 μg/ml) for 21 tolerant strains. In addition to the MBC, we determined the actual reduction of the viable bacterial counts in each tube dilution after 24 h of incubation. This determination was made by subtracting the number of colonies observed on the subculture plate from the number of bacteria contained in the initial inoculum. For both antibiotics we found that the maximal reduction in viable counts was achieved at or very close to the MIC and did not increase with increasing antibiotic concentrations (up to 128 μg/ml). As expected, the six strains for which the amoxicillin MBC was <0.5 μg/ml and the three strains for which the vancomycin MBC was <1 μg/ml had a reduction of viable counts of more than 3 $\log_{10}$ (>99.9% killing). In contrast, among the strains defined as tolerant to amoxicillin and vancomycin, there were wide variations in the actual reduction of bacterial counts, ranging from 3 $\log_{10}$ to <1 $\log_{10}$. Therefore our observations suggest that the reduction of viable streptococcal counts reflects more accurately the bactericidal effect of amoxicillin and vancomycin than does the MBC, which artificially divides the strains into sensitive or tolerant strains.

In several types of bacterial infection, a bactericidal antibiotic regimen appears to be required to achieve cure. This has been suggested for meningitis (27), septicemia in leukopenic patients (18), and bacterial endocarditis (5, 6, 31). In the latter infection, the ability of antibiotics to kill bacteria also affects the efficacy of prophylaxis (8, 13).

Many studies have used the MBC and, more recently, the MBC/MIC ratio (25, 26) to describe the bactericidal activity of antibiotics in an attempt to correlate in vitro data with in vivo treatment results (3, 11, 20, 23). However, both the MBC and the MBC/MIC ratio have been subject to much criticism for two main reasons. First, the definition of the MBC (>99.9% killing of bacteria during 24 h of incubation in broth) is arbitrary and separates the bacteria into two populations, a segregation which might not have biological relevance (16). Second, the tube dilution method used to assess the MBC has been shown to suffer from several technical problems which render the MBC poorly reproducible (12, 22, 28).

The purpose of this study was to investigate whether broth dilution susceptibility tests, performed routinely to determine the MIC, could be used in a way that would reflect the bactericidal effect of antibiotics more precisely and reproducibly than the MBC or the MBC/MIC ratio. Because both treatment and prophylaxis of endocarditis may require bactericidal antibiotics, we tested the killing effect of amoxicillin and vancomycin (both recommended for prophylaxis and treatment of endocarditis) on 24 strains of viridans group streptococci isolated from patients with endocarditis.

(The results of this study were presented at the 43rd Congress of the Swiss Society for Microbiology in Lugano, Switzerland, 1984.)

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MATERIALS AND METHODS

Bacterial strains. Twenty-four strains of viridans group streptococci cultured from the blood of patients with endocarditis were studied. Of these, 21 were isolated from 1978 to 1981 at the Institut de Microbiologie of the Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, and 3 were kindly provided by R. Auckenthaler (Hôpital Cantonal, Geneva, Switzerland).

Strains were identified by standard methods (24) using the API 20S Strep Identification System (Analytab Products International SA, Meyrin, Switzerland). There were 10 *Streptococcus mitis*, 7 *S. sanguis*, and 2 *S. salivarius* strains. Five streptococcus strains (viridans group) could not be speciated. Strains were stored at −80°C in horse blood and subcultured on 5% human blood agar plates (BA plates [15 ml, 9 cm diameter]; Difco Laboratories, Detroit, Mich.) 1 to 5 days before each experiment.

Antibiotic susceptibility testing. Macrobroth dilution susceptibility tests were performed according to standard methods (1, 30). Serial twofold dilutions of amoxicillin trihydrate (Beecham Research Laboratories, Bern, Switzerland) and vancomycin hydrochloride (Eli Lilly, Bern, Switzerland) were prepared on the day of the study in Muller-Hinton Broth (Difco) supplemented with 1% IsoVitalex (MHB-IVX; BBL Microbiology Systems, Cockeysville, Md.) to ensure optimal growth of some fastidious strains. Exponential-phase bacterial suspensions were obtained according to standard procedures (19). These cultures were harvested after 2 h of exponential growth and, by using a nephelometer (Corning Evans Electroselenium Limited, Halstead, Essex, England), diluted in MHB-IVX to obtain inocula containing approximately 2×10^6 CFU/ml.

Alternatively, stationary-phase inocula were prepared by diluting overnight cultures in MHB-IVX to obtain approximately 2×10^6 CFU/ml. By using an automatic pipette, 1 ml

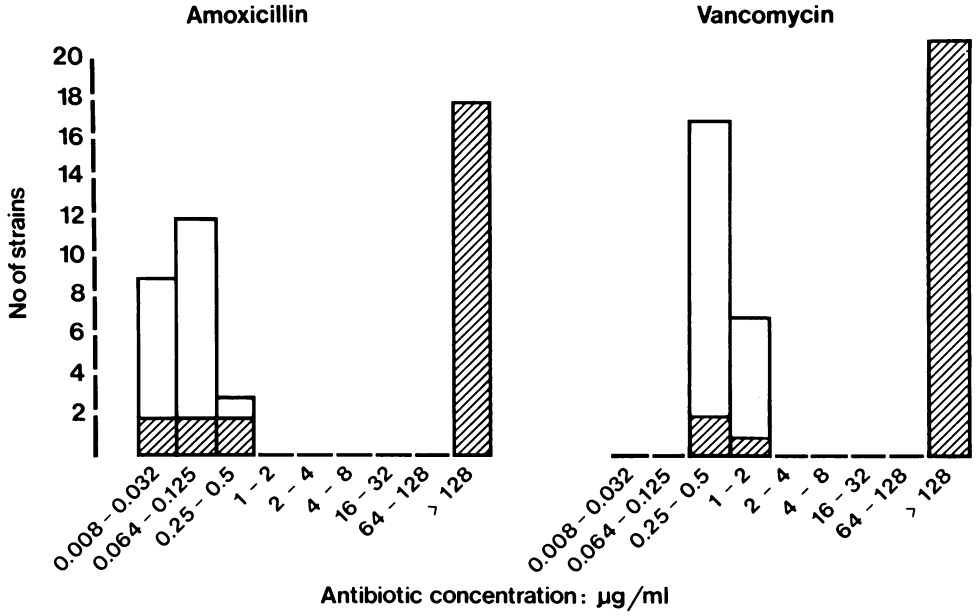

FIG. 1. Distribution of the 24 strains according to the MICs (□) and MBCs (▨) of amoxicillin and vancomycin.

of the bacterial suspension was added to 1 ml of each antibiotic dilution so that the final bacterial inoculum concentration in each tube was approximately 10^6 CFU/ml. Gentle mixing was carried out by repeated aspirations and flushing in order to avoid splashing the bacteria against the tube walls, which could in turn allow them to escape exposure to the antibiotic (15, 28). The colony count of the final inoculum was determined by plating an appropriate dilution of the control tube containing no antibiotic. To test the influence of inoculum density on antibiotic sensitivity testing, inocula of 10^5 and 10^4 CFU/ml were also prepared.

The tubes inoculated with bacteria were incubated for 24 h at 35°C, and the MIC was determined as the lowest antibiotic concentration preventing visible growth. In addition, the exact number of surviving organisms at 24 h in each dilution tube was determined by sampling 10 µl from all tubes without visible growth and from the first tube with growth, as well as from the control tube containing no antibiotic. Each 10-µl sample was spread with a glass rod over the entire surface of a BA plate (supplemented with 2,000 U of penicillinase per ml [Bacto-Penase concentrate; Difco] when amoxicillin was tested) to minimize or abolish the carry-over of antibiotics which might prevent growth (2, 4, 21). Colony counts on BA plates were read after 48 h of incubation at 35°C in 5% CO_2. Alternatively, when 10^5 and 10^4 CFU/ml inocula were tested, 100- and 200-µl samples were subcultured from each dilution tube to ensure the detection of very low numbers of surviving bacteria.

Expression of the antibiotic killing effect. The following parameters were used to express the bactericidal effect of amoxicillin and vancomycin. (i) The MBC was defined as the lowest antibiotic concentration that killed more than 99.9% of the initial inoculum after 24 h of incubation. A strain was defined as tolerant when the MBC/MIC ratio was greater than 32 (25, 26). (ii) In addition to the MBC, the actual reduction of the viable counts in each dilution tube (delta log CFU) was calculated by subtracting the $\log_{10}$ CFU/ml recovered in each tube after 24 h of incubation from the $\log_{10}$ CFU/ml of the inoculum. The results are the mean of three determinations performed in separate experiments.

RESULTS

MIC and MBC determinations. As shown in Fig. 1, all 24 viridans group streptococcal strains were uniformly susceptible to the growth inhibitory effect of amoxicillin (MIC, ≤0.25 µg/ml) and vancomycin (MIC, ≤1 µg/ml). In contrast, the MBC divided the strains into two groups. One group (six strains for amoxicillin and three strains for vancomycin) had very low MBCs equal to or twice their MICs. The MBC/MIC ratio was low, and the strains could therefore be defined as susceptible to the bactericidal effect of antibiotics. The other group (18 strains for amoxicillin and 21 strains for vancomycin) had very high MBCs (>128 µg/ml) and high MBC/MIC ratios ranging from 128 to 4,000 and were therefore defined as tolerant to amoxicillin and vancomycin.

Reduction of the viable bacterial counts after exposure to antibiotic (delta log CFU). Several observations were made when the actual reduction of bacterial counts was determined after 24 h of incubation in each tube containing increasing concentrations of amoxicillin and vancomycin.

(i) For each of the 24 strains tested, the maximal reduction in bacterial counts (expressed as the delta log CFU, i.e., the difference between the bacterial counts before and after exposure to antibiotic) was achieved by antibiotic concentrations very near the MIC and was not enhanced by increasing concentrations of antibiotic. This phenomenon is shown for three representative strains in Fig. 2 (amoxicillin) and Fig. 3 (vancomycin).

(ii) The bactericidal effect of the two antibiotics as measured by the delta log CFU varied greatly between strains. Figure 2 shows the results for three representative strains exposed to amoxicillin. *S. sanguis* II displayed only a 1-$\log_{10}$ reduction of the initial inoculum (i.e., ≈90% killing), whereas *S. mitis* strains 1 and 2 were much more susceptible to the bactericidal effect of amoxicillin. However, only *S. mitis* 2 had a >3-$\log_{10}$ reduction of viable counts at all antibiotic concentrations above the MIC and thus had a low MBC. In contrast, *S. mitis* 1, although only slightly less susceptible than *S. mitis* 2 to the bactericidal effect of amoxicillin, never displayed a reduction of viable counts

greater than 3 $\log_{10}$ (>99.9% killing) and therefore had a high MBC. Moreover, since 99.9% killing (delta log CFU, <3) was not achieved with either *S. sanguis* II or *S. mitis* 1, these two strains, which exhibited such different susceptibilities to amoxicillin, could both be considered tolerant when defined by the conventional MBC/MIC ratio. Similar results were obtained with vancomycin for the three representative strains (Fig. 3).

(iii) We found that the results were reproducible within a 0.5-$\log_{10}$ variation of the delta log CFU for each strain upon repeated experiments. Thus, since the bactericidal effect was both reproducible and constant with increasing antibiotic concentrations, it was possible to calculate for each strain a mean delta log CFU, which was defined as the mean of the various delta log CFU values observed at each antibiotic concentration above the MIC. For instance, the mean delta log CFU values after exposure to amoxicillin (Fig. 2) were 1.05 for *S. sanguis* II, 2.7 for *S. mitis* 1, and 3.05 for *S. mitis* 2. Figure 4 shows the bactericidal effect of amoxicillin and vancomycin expressed by the mean delta log CFU for the 24 viridans streptococcal strains. When the killing was expressed in this way, there was a regular distribution of the strains from the most susceptible to the least susceptible. There were, however, variations between the various species. The mean delta log CFU of the *S. sanguis* strains indicated that the strains of this species were significantly less susceptible to amoxicillin (P = 0.013) and vancomycin than were the *S. mitis* strains (P = 0.037) (Fisher exact test).

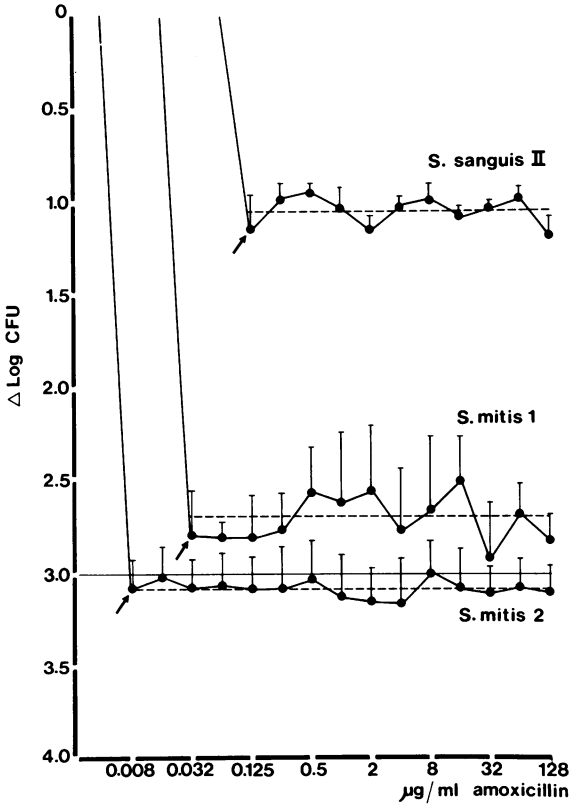

FIG. 2. Actual reduction of the viable counts for three representative strains after standard broth dilution testing with amoxicillin. Arrows indicate the MIC. The solid line at 3 $\log_{10}$ represents the cutoff value of 99.9% killing defining the MBC. The dashed lines for each strain represent the mean delta log CFU. The mean delta log CFU values for *S. sanguis* II, *S. mitis* 1, and *S. mitis* 2 were 1.05 (MBC, >128 μg/ml), 2.70 (MBC, >128 μg/ml), and 3.05 (MBC, 0.008 μg/ml), respectively.

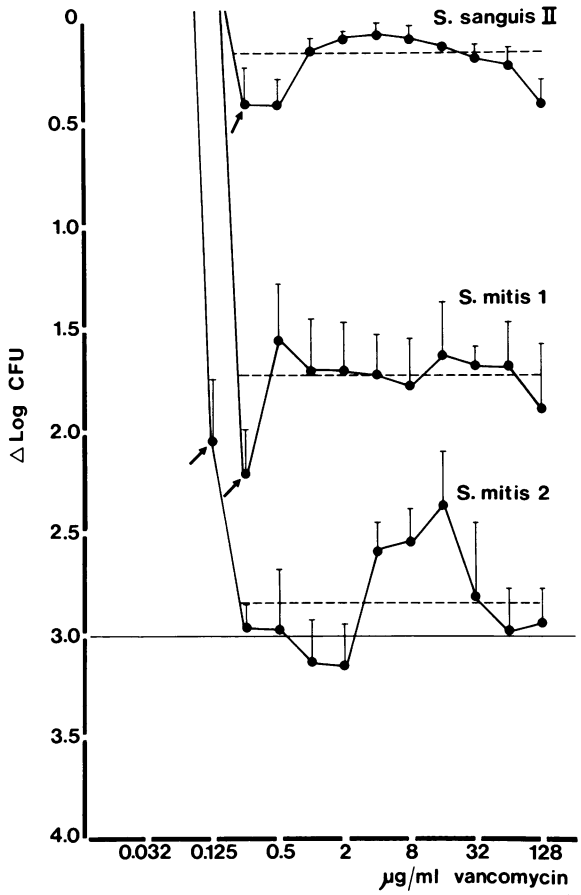

FIG. 3. Actual reduction of the viable counts for three representative strains after standard broth dilution testing with vancomycin. Arrows indicate the MIC. The solid line at 3 $\log_{10}$ represents the cutoff value of 99.9% killing defining the MBC. The dashed lines for each strain represent the mean delta log CFU. The mean delta log CFU values for *S. sanguis* II, *S. mitis* 1, and *S. mitis* 2 were 0.14 (MBC, >128 μg/ml), 1.72 (MBC, >128 μg/ml), and 2.84, respectively. The MBC for *S. mitis* 2 was difficult to define because of >99.9% killing at 1 and 2 μg of vancomycin per ml but not above 2 μg of vancomycin per ml.

(iv) The use of logarithmic versus stationary-phase inocula or inocula with densities from 10^6 to 10^4 CFU/ml did not result in significant differences in the delta log CFU of four out of five randomly selected strains exposed to amoxicillin (Table 1). Only one strain, *S. mitis* 3, appeared to be less susceptible to killing when a concentrated inoculum was used.

(v) When we retested by the same method the susceptibility to amoxicillin and vancomycin of bacteria which had survived exposure to the antibiotic, we found the MIC and mean delta log CFU to be similar to those of the original inoculum (data not shown).

DISCUSSION

The use of the MBC and its derivative, the MBC/MIC ratio, to assess the bactericidal activity of antibiotics has been questioned by many investigators (12, 16, 22, 28)

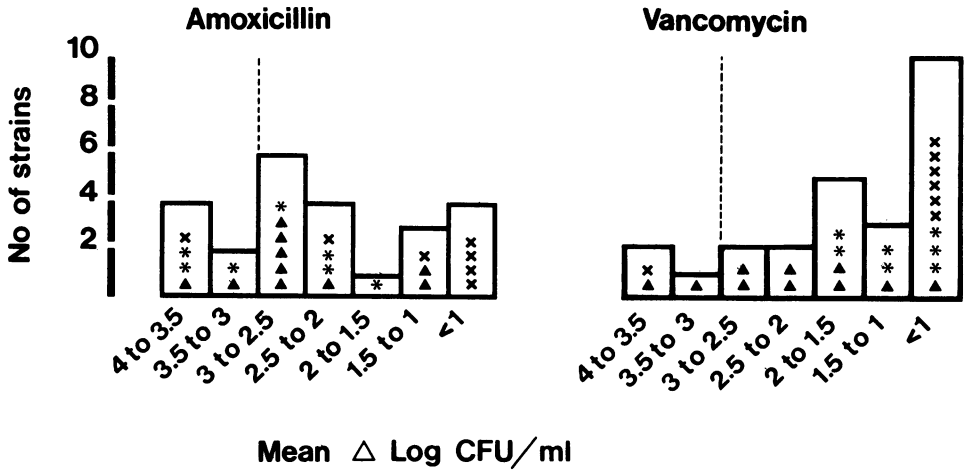

FIG. 4. Distribution of the mean delta log CFUs of the 24 strains of viridans group streptococci after testing with amoxicillin and vancomycin. The dashed lines indicate the cutoff value of 99.9% killing which defines the MBC and separates the strains into tolerant (high MBC/MIC ratio) and susceptible (low MBC/MIC ratio) strains. Symbols: ×, *S. sanguis*; ▲, *S. mitis*; *, other viridans group streptococcal strains.

because the values are based on arbitrary definitions and, perhaps more importantly, are often poorly reproducible. Indeed, the tube dilution method suffers several technical problems, such as carry-over of antibiotics (2, 4, 21, 28), bacteria splashed on the wall of the test tubes (15, 28), unbuffered medium (14, 29), or bacterial growth phase (12, 17), all of which have been shown to affect the number of CFU eventually recovered from the subculture plates. If the actual reduction in viable counts is close to 3 $\log_{10}$ (99.9% killing), slight variations in the number of CFU due to these technical problems may greatly affect the determination of the MBC and may account for its poor reproducibility, an observation reported frequently (4, 12, 21, 22, 28). Even under optimal statistical conditions for sampling and for counting survivors (21), subculture plates from the various tube dilutions may grow numbers of colonies slightly different from each other. Therefore, when the reduction of viable count is close to 99.9% it may be difficult to accurately determine the concentration of antibiotic that defines the MBC (7, 21).

Our observations may help uncover some of the reasons for these difficulties. First, we found that the percentage of viridans group streptococci which escaped killing by amoxicillin and vancomycin was rather constant above the MIC and did not decrease with increasing antibiotic concentrations. This important observation could be made only because stringent precautions were taken to avoid the carry-over effect, which may simulate an enhanced killing effect at high antibiotic concentrations. Almost 40 years ago, Eagle and Musselman (7) studied the bactericidal effect of penicillin against various species of gram-positive cocci, taking care to avoid the carry-over of antibiotics. They observed that penicillin exerted a paradoxically reduced killing effect at high concentrations against certain organisms (the so-called paradoxical Eagle effect). In addition, they also observed that penicillin had a rather constant bactericidal activity with increasing concentrations of antibiotics above the MIC. Recently, Goessens et al. (9, 10) made similar observations with *S. aureus*. This phenomenon might hamper an accurate MBC determination for strains that show upon antibiotic exposure a delta log CFU very close to 3, i.e., near the 99.9% killing endpoint. Indeed, for these strains, minimal variations of the delta log CFU from one tube dilution to the other along the whole range of antibiotic concentrations might result in erratic crossing above or below the 99.9% killing cutoff value. In addition, slight variations of the delta log CFU values from one experiment to another due to the aforementioned technical problems would explain the poor reproducibility of the MBC determination observed for some strains (4, 12, 21, 22, 28). These slight variations would, however, only slightly affect the delta log CFU. Furthermore, when studying the delta log CFU of five strains with inocula of different magnitude, we

TABLE 1. Effect of growth phase and inoculum density on the susceptibility of five strains of viridans group streptococci to amoxicillin

Strain and inoculum phase	Mean delta log CFU[a] at inoculum density (CFU/ml) of:		
	10^6	10^5	10^4
S. mitis 1			
Logarithmic	3.04[b]	2.79	2.74
Stationary	2.96[b]	2.79	2.78
S. mitis 2			
Logarithmic	3.30	3.52	3.22
Stationary	3.09	3.60	3.69
S. mitis 3			
Logarithmic	1.43	3.70[b]	3.68
Stationary	2.11	2.70[b]	3.30
S. sanguis II 1			
Logarithmic	1.48	1.60	<1
Stationary	<1	<1	<1
S. sanguis II 2			
Logarithmic	2.53	2.45	3.39
Stationary	2.52	2.95	3.22

[a] The mean delta log CFU was defined as the average of the delta log CFU values observed in each tube dilution at and above the MIC in a standard broth dilution susceptibility procedure.

[b] At these inoculum densities, the MBCs (defined by >3-$\log_{10}$ killing of the initial inoculum) of the logarithmic-phase versus stationary-phase inocula would be very different.

found that four of five strains displayed similar delta log CFU values.

Second, the traditional cutoff value of 99.9% killing used to define the MBC artificially divides bacteria into strains that are susceptible or resistant (tolerant) to the bactericidal effect of cell-wall-active antibiotics. This was obvious in our experiments in which viridans group streptococcus strains were either susceptible or tolerant to the bactericidal effect of amoxicillin or vancomycin as determined by the MBC, while more careful analysis of the lethal effect as determined by the actual reduction of the viable counts showed a regular distribution from the most to the least susceptible strains. Moreover, strains such as *S. mitis* 1 and *S. mitis* 2 (Fig. 2), which were classified as susceptible and tolerant, respectively, by the MBC, displayed very similar susceptibilities as determined by the mean delta log CFU.

Similar observations have been made by Goessens et al. (9, 10) for clinical isolates of *S. aureus*. These researchers showed that the percentage of *S. aureus* surviving after 24 h of exposure to antibiotic was an accurate and reproducible way to express the lethal effect of beta-lactams against this species. In addition, this percentage was not influenced by the growth phase of the bacteria used for the test, an observation also made in the present study with viridans group streptococcus strains. Recently, Handwerger and Tomasz (12) pointed out that time-kill studies remain the most reliable means to differentiate tolerant from nontolerant strains by their relative rate of killing during the first hours of exposure to antibiotic. Using this approach with viridans group streptococci, we found that time-kill studies gave results very similar to the reduction of viable count at 24 h. First, the reduction of bacterial counts upon exposure to antibiotics for 2 or 4 h correlated closely with the reduction observed after 24 h of exposure. Second, the maximal rate of bacterial killing upon antibiotic exposure was achieved by antibiotic concentrations very near the MIC and was not enhanced by increasing antibiotic concentrations. Third, the slopes of the time-kill curves showed a regular distribution from the most sensitive to the least susceptible (tolerant) strains (data not shown). Therefore, both the experiments by Goessens et al. on *S. aureus* and ours on viridans group streptococci suggest that the bactericidal effect of cell-wall-active antibiotics might be appropriately and simply determined using conventional broth dilution techniques, provided it is expressed by the actual reduction of bacterial counts rather than by the MBC. This method would be less tiresome than time-kill studies.

Tolerance to the bactericidal effect of cell-wall-active antibiotics has been reported with increasing frequency among clinical isolates of *S. aureus*, *S. epidermidis*, and various other streptococcal species (12). It has been difficult to establish a relationship so far between the tolerance of bacteria to cell-wall-active antibiotics as measured in vitro by the MBC/MIC ratio and the in vivo efficacy of antibiotic treatment (16). Our findings suggest that it would be worth investigating whether the actual reduction of viable counts of gram-positive cocci upon exposure to cell-wall-active antibiotics might yield a better correlation with the efficacy of in vivo treatment than the traditional MBC or the MBC/MIC ratio.

ACKNOWLEDGMENTS

We thank José Entenza and Marlyse Giddey for technical assistance and Sylviane Bovey for typing the manuscript.

P.R.M. was supported by grant 3.836.081 from the Swiss National Foundation for Scientific Research.

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