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Factors Influencing Detection of Tolerance in *Staphylococcus aureus*

WIL H. F. GOESSENS,* PETER FONTIJNE, AND MARC F. MICHEL

Department of Clinical Microbiology and Antimicrobial Therapy, Erasmus University Rotterdam, 3000 DR Rotterdam, The Netherlands

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The phenomenon of tolerance to cloxacillin and methicillin was studied in *Staphylococcus aureus*. It was demonstrated that the minimal bactericidal concentrations showed marked differences, depending on the method of detection used. These differences resulted from carry-over of the antibiotic to the subculture plates. When carry-over of the antibiotic was prevented by the addition of β-lactamase to the nutrient medium, the antibiotics were no longer bactericidal. At a certain antibiotic concentration and at higher concentrations, however, each strain showed a certain survival percentage after 24 h. The tolerance percentage was determined for 15 strains. The values found for the individual strains ranged from <0.1 to 11% for cloxacillin and methicillin. Since these percentages were reproducible within narrow limits, they could be regarded as a characteristic of the strains. The tolerance percentage was independent of the growth phase of the initial cultures.

Reports on the phenomenon of tolerance to penicillins, studied mainly in *Staphylococcus aureus* and *Streptococcus* species, have been regularly published in the past few years. The tolerance phenomenon was first described by Tomasz (12) in pneumococci. He observed that, in vitro, tolerant strains were not killed by penicillin in the conventional bactericidal concentrations. Subsequently, Best et al. (2) observed the same phenomenon in *S. aureus*. By using killing curves, they demonstrated that bacteria of certain strains were inhibited, but not killed, by oxacillin. The phenomenon was found to be based on delayed killing of a minority of bacteria in the inoculum. Mayhall et al. (9) and Sabath et al. (11) subsequently attempted to estimate the degree of tolerance. For this purpose, they used the ratio between the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC). Tolerant strains have a normal MIC but a markedly increased MBC of penicillins. In these studies, the MBC was defined as the concentration at which 99.9% of the original inoculum was killed after 24 h of incubation (1). Sabath accepted a ratio of 32 as borderline between susceptible and tolerant strains.

Several studies have related the tolerance of organisms causing infections to the failure of antimicrobial therapy with penicillins (3, 6, 11). Differences in the technique of determining the MBC make it difficult to determine whether tolerance is clinically relevant. Data from the literature indicate that the MBC varies with the medium in which it is determined (10) and also with the growth phase of the culture (7, 8). Factors such as subculture volume, duration of incubation of the subculture plates, and, possibly, density of the inoculum used may also influence these MBCs.

In the present study, the variables in the test methods were critically analyzed. Next, an attempt was made to evolve, by standardization, a reproducible method of determining the degree of tolerance in *S. aureus*.

MATERIALS AND METHODS

Bacteria. The staphylococcal strains used in this study were obtained from the bacteriological laboratory of the University Hospital Rotterdam/Dijkzigt, The Netherlands. The organisms were isolated from 15 patients with a positive blood culture and were identified as *S. aureus* on the basis of colony form and color, the presence of grapelike clusters in Gram stain, and a positive coagulase test. In addition, all strains were phage typed at the National Institute of Public Health. Of the 15 strains, 13 were susceptible to phages of the *S. aureus* phage-typing set. All strains were catalase positive. The strains were stored in freeze-dried form.

Antibiotics. The antibiotics used in this study were cloxacillin and methicillin (Beecham Pharmaceuticals, Heppignies, Belgium).

Estimation of MIC and MBC. Estimates of MIC and MBC were made in twofold serial dilutions of the antibiotic in glass tubes containing 2 ml of Mueller-Hinton broth (Difco Laboratories). The serial concentrations were 0.1 to 102.4 μg/ml for cloxacillin and 0.5 to 512 μg/ml for methicillin. For determination, we

used logarithmic and stationary-phase cultures of bacteria (see figure legends). These were obtained by suspending 1 loopful from a blood plate (Oxoid Ltd.) in 25 ml of Mueller-Hinton broth and incubating the suspension at 37°C for 4 and 18 h, respectively, on a shaking device. After incubation, the required density of the suspensions was ensured by densitometry at 660 nm in a photometer (Vitatron). The final concentration in the tubes was about 10^5 colony-forming units (CFU) per ml; the correct value was determined by viable counts in duplicate. After addition of the inoculum, the tubes were incubated at 37°C for 24 h without shaking, whereupon the MIC was read. The MIC was defined as the concentration that caused no visible turbidity after 24 h of incubation. The MBC was determined by taking 50 μl from those tubes that showed no growth. Before sampling, the tubes were shaken. The 50-μl volumes were spread on one-third (±20 cm^2) of the surface of nutrient agar (Oxoid) plates which contained 0.15 U of β-lactamase I and 0.015 U of β-lactamase II per ml (Whatman Biochemicals Ltd.). Plates with β-lactamase were used in all experiments unless otherwise stated. After 48 h of incubation at 37°C, the CFU counts were made and converted to percentages of the initial inoculum. The MBC was defined as the concentration that killed 99.9% of the original inoculum (1). All MIC and MBC estimates were made in duplicate.

RESULTS

In the early phase of this study, rejection values were used to determine the MBC. Given an inoculum of 10^5 CFU/ml and a subculture volume of 200 μl, this value was 20 CFU. At this value, 99.9% of the original inoculum was killed (MBC definition). By using this method, it was found that the MBC/MIC ratio for 15 strains did not exceed 4, and consequently, none of these strains were tolerant according to the definition of Sabath et al. (11). When the subculture volume was reduced from 200 to 50 μl, however, the MBCs for these strains increased. They increased further when the period of incubation of the nutrient plates was increased from 24 to 48 h. With a subculture volume of 50 μl and an incubation period of 48 h, the MBC of methicillin for strains 3 and 11, for example, was 512 μg/ml (Fig. 1A). Since the MICs of this antibiotic for these two strains are 1 and 2 μg/ml, respectively, their MBC/MIC ratios are 512 and 256, respectively. Both strains became highly tolerant after the abovementioned modifications were made. Table 1 shows that the MBCs for 15 different strains tested ranged from 0.4 to 102.4 μg of cloxacillin per ml and from 2 to 512 μg of methicillin per ml and that most of the strains tested showed tolerance as a general phenomenon, with the MBC/MIC ratios exceeding 32 according to the modified method. In two strains (9 and 13) the ratio was much lower than 32. Since the results obtained by the modified test method demonstrated that the MBCs were profoundly influenced by carry-over of the antibiotic, β-lactamase was added to the subculture plates in subsequent experiments. Carry-over of the antibiotic was thus prevented. The results of these experiments are presented in Fig. 1B. The antibiotic proved to be no longer bactericidal, and therefore MBCs could not be determined. To indicate the degree of tolerance nevertheless, a different parameter was introduced: the number of viable bacteria expressed as a percentage of the original inoculum after 24 h of incubation. This survival percentage is the plateau value attained when the antibiotic concentration is increased and is called the tolerance percentage of a strain. The tolerance percentage does not change, even in the presence of very high antibiotic concentrations (10 mg/ml). Figure 1B shows tolerance percentages of 1.5 for strain 11 and 6.0 for strain 3.

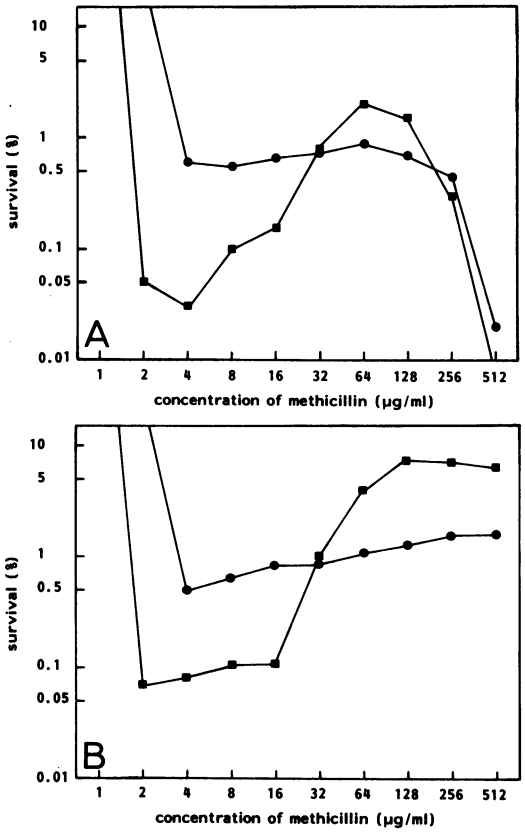

FIG. 1. Survival rates of two isolates of *S. aureus*, strain 3 (■) and strain 11 (●), at various concentrations of methicillin. Stationary-phase cultures were used as inocula. (A) Nutrient agar without β-lactamase. (B) Nutrient agar which contained β-lactamase.

The tolerance percentage can be regarded as a strain characteristic only if the values found are reproducible within narrow limits. This reproducibility of tolerance percentages was studied

TABLE 1. Estimation of MIC, MBC, and survival percentage for cloxacillin and methicillin in 15 strains of *S. aureus* with inocula in logarithmic and stationary phases[a]

Strain	Cloxacillin						Methicillin					
	Logarithmic phase			Stationary phase			Logarithmic phase			Stationary phase		
	MIC (μg/ml)	MBC (μg/ml)	Survival (%)	MIC (μg/ml)	MBC (μg/ml)	Survival (%)	MIC (μg/ml)	MBC (μg/ml)	Survival (%)	MIC (μg/ml)	MBC (μg/ml)	Survival (%)
1	0.2	25.6	3.0	0.2	25.6	6.0	1	512	7.0	1	512	8.0
2	0.1	25.6	0.5	0.1	25.6	1.0	2	256	1.0	1	128	2.0
3	0.2	25.6	0.4	0.2	51.2	6.0	2	256	0.9	1	256	6.0
4	0.2	25.6	1.2	0.2	12.8	0.8	2	256	1.7	1	256	2.0
5	0.2	12.8	0.3	0.2	12.8	0.15	2	32	0.5	1	256	0.3
6	0.2	25.6	0.3	0.2	25.6	0.9	2	256	0.3	1	512	1.5
7	0.1	3.2	0.12	0.1	1.6	<0.1	2	64	0.1	2	64	<0.1
8	0.2	25.6	0.4	0.2	25.6	0.3	2	256	0.25	2	128	0.6
9	0.2	0.4	0.12	0.2	25.6	0.4	4	512	0.25	2	256	0.6
10	0.2	12.8	0.12	0.1	6.4	0.15	4	64	0.15	2	128	0.2
11	0.2	12.8	0.5	0.2	25.6	1.0	2	128	0.3	2	512	1.5
12	0.2	51.2	9.0	0.2	51.2	3.0	2	256	11.0	1	512	7.5
13	0.2	0.4	<0.1	0.2	3.2	0.2	1	2	0.1	1	16	0.6
14	0.4	102.4	11.0	0.4	102.4	10.0	2	512	11.0	2	512	10.0
15	0.2	25.6	1.5	0.2	6.4	0.6	1	256	2.8	1	256	2.0

[a] The inocula used for estimation of the MICs were 10^5 CFU/ml. MBCs were obtained by subculturing after 24 h on nutrient agar without β-lactamase. Survival percentages were obtained by subculturing after 24 h on nutrient agar with β-lactamase.

by testing two strains four times with methicillin and cloxacillin. Figure 2 shows that the methicillin tolerance percentages of the two strains (14 and 15) are reproducible from a concentration of 128 μg/ml (mean ± standard deviation; 7.7 ± 3.1 and 0.62 ± 0.45 μg/ml, respectively); the cloxacillin tolerance percentages are reproducible from a concentration of 6.4 μg/ml (mean ± standard deviation; 7.6 ± 1.6 and 0.25 ± 0.08 μg/ml, respectively).

Figures 1 and 2 show that there is an optimal antibiotic concentration for the killing effect. This so-called zone phenomenon was observed by Eagle and Musselman (4) as early as 1948. Comparison of Fig. 2A with 2B reveals, moreover, that the killing effect of methicillin exceeds that of cloxacillin.

The results discussed so far were all obtained by proceeding from inoculation of stationary-phase cultures (18 h). In an effort to establish whether the MBCs obtained with a stationary-phase culture differ significantly from those obtained with logarithmic cultures, the cloxacillin and methicillin tolerance percentages of 15 strains were determined with 4-h and 18-h cultures. Table 1 shows that the tolerance percentages of the various strains in 18-h cultures were sometimes higher and sometimes lower than those obtained in 4-h cultures of the same strains.

Our results in terms of tolerance percentages can be plotted, not only against the antibiotic concentration, but also against sampling time (Fig. 3). The curves thus obtained provide more exact information on the degree of bacterial killing at the various concentrations of the antibiotic used. Figure 3 shows, however, that strain 3, with a methicillin tolerance percentage of 6, was killed more rapidly at methicillin concentrations of 4, 8, and 16 μg/ml than strain 2, which has a tolerance percentage of 0.45. These experiments thus demonstrate that a substantial difference in tolerance percentage is not necessarily accompanied by a difference in lysis rate.

DISCUSSION

Previous studies have shown that different techniques are being used to detect tolerance in *S. aureus*. Best et al. (2) and Sabath et al. (11), for example, studied the decrease in CFU with time spectrophotometrically and were thus able to demonstrate differences between susceptible and tolerant organisms. A variant used by such authors as Mayhall et al. (9) is the killing curve technique to study the decrease in CFU with time. The most widely used technique, however, is the tube dilution method. Several studies have shown that such factors as the medium used and the age of the initial culture influence the MBCs obtained (7, 8, 10).

We attempted to obtain more detailed information on the causes of these differences in results. It was found that reduction of the subculture volume and prolongation of the period of incubation each led to an increase in the MBC. A common factor in both modifications of the technique is the reduction of the carry-over of antibiotics to the subculture plates. When carry-

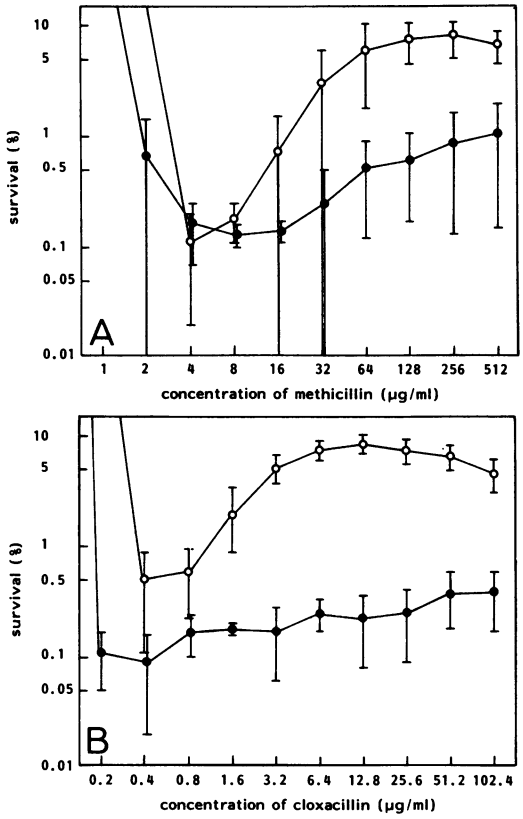

FIG. 2. Tolerance percentages for two isolates of *S. aureus*, strain 14 (○) and strain 15 (●), at various concentrations of methicillin (A) and cloxacillin (B). Stationary-phase cultures were used as inocula. Each point represents the mean of four determinations (± standard deviation).

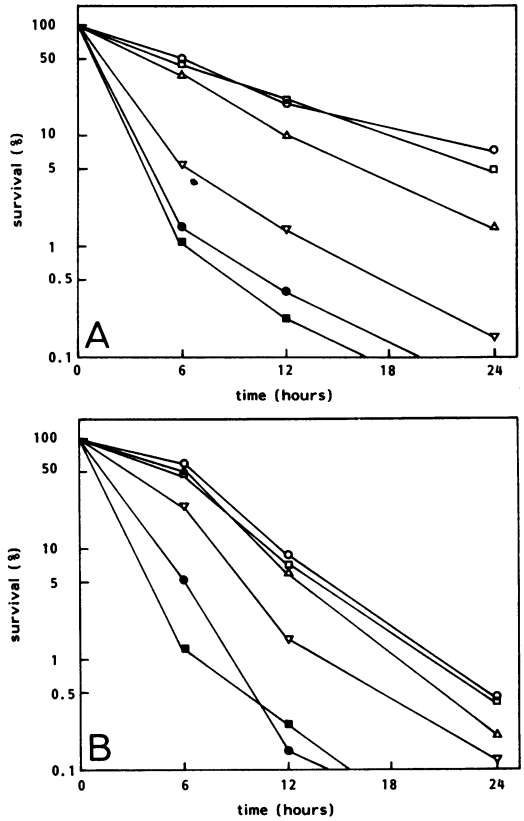

FIG. 3. Killing curves of two strains of *S. aureus* at various concentrations of methicillin with stationary-phase cultures as inocula. (A) Strain 3 has a tolerance percentage of 6.0. (B) Strain 2 has a tolerance percentage of 0.45. Drug concentrations were 4 (■), 8 (●), 16 (▽), 32 (△), 128 (○), and 256 (□) μg/ml.

over of the antibiotic is prevented by adding β-lactamase to the subculture plates, methicillin and cloxacillin are no longer bactericidal. The curves depicted in Fig. 2 were described by Eagle and Musselman as early as 1948 (4). The course of the latter part of such curves indicates that it is not possible to determine MBCs at high concentrations of these antibiotics. Since in this situation the MBC/MIC ratio can no longer be used as a measure of tolerance, efforts were made to find a different parameter with which the behavior of bacterial strains in response to high concentrations of β-lactam antibiotics could be characterized. This strain characteristic, which we define as tolerance percentage, is the number of bacteria expressed as a percentage of the original inoculum. The tolerance percentage is determined by the lowest concentration at which bacterial survival is stabilized. For each strain, the tolerance percentage is reproducible within narrow limits. Moreover, very high antibiotic concentrations fail to influence this characteristic. The age of the initial culture has no systematic effect on results in terms of tolerance percentage. The observations reported by Mayhall and Apollo (8) and Kim and Anthony (7) could therefore not be confirmed.

The tolerance percentage described in the present study can be explained as the result of the killing of the majority of the bacterial population. The phenomenon is not due to regrowth of part of the population, as described by Gwynn et al. (5). This was concluded from the fact that the number of CFU after 48 h never exceeded the number of CFU after 24 h. Inactivation of the β-lactam antibiotic during the process of subculturing, as first described by Eagle and Musselman (4), allows us to devise a reproducible parameter for estimation of the degree of tolerance. Construction of the entire survival curve is too cumbersome for use as a routine procedure. It is therefore advisable to determine the tolerance percentage at only one particular concentration: 25.6 μg of cloxacillin and 128 μg of

methicillin per ml. At these concentrations, the tolerance percentage was found to be stabilized. Survival percentages thus determined may contribute to a better understanding of the clinical relevance of the tolerance phenomenon.

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