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Tolerance Percentage as a Criterion for the Detection of Tolerant *Staphylococcus aureus* Strains

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In this study, the degree of tolerance was determined in several populations of *Staphylococcus aureus* isolates. The degree of tolerance of a staphylococcal strain can be established in a reproducible way by exposing the strain to increasing concentrations of a β-lactam antibiotic and determining the number of surviving bacteria at each concentration. The number of surviving bacteria was expressed as a fraction of the initial inoculum. By this technique, it appears that for each strain the value of the surviving fraction stabilized above a certain concentration of the antibiotic. This value was called the tolerance percentage of the strain. In 64 *S. aureus* strains isolated from blood cultures in 1982, the tolerance percentages, after exposure to methicillin, varied from ≤0.1 to 6; 28% of the strains showed a tolerance percentage of ≤0.1, and 12.5% showed a tolerance percentage of ≥2. Similar tolerance percentages were found with cloxacillin, nafcillin, cephalothin, and penicillin. Strains with a tolerance percentage of ≥2 showed slow killing and lysis in the presence of a high methicillin concentration. A tolerance percentage of 2 appeared to be the breakpoint between susceptible and tolerant strains. Older collections of *S. aureus* strains, dating from the years 1951 to 1953 and 1957 to 1958, also included strains with a survival percentage of ≥2, thus indicating that tolerance of *S. aureus* to β-lactam antibiotics is not a new phenomenon.

Sabath et al. (11) have described tolerance of *Staphylococcus aureus* to β-lactam antibiotics as a new type of resistance. This type of resistance has also been observed in other gram-positive bacteria (13). Tolerant strains show a normal MIC and a distinctly increased MBC (MBC:MIC ratio, ≥32) (11), with MBC defined as the concentration at which 99.9% of the inoculum is killed (1).

The efficacy of antimicrobial treatment in infections caused by staphylococci with a high MBC:MIC ratio was studied several times. A negative correlation between tolerance and antimicrobial response was demonstrated in some (3, 8, 10, 11) but not all (6, 7) cases. The diverse results obtained in these studies may be based on differences in laboratory conditions used to demonstrate the phenomenon. In a previous study, we have demonstrated that the percentage of surviving bacteria exposed to high concentrations of a β-lactam antibiotic is reproducible constantly within certain limits (5). In the present study, we attempt to indicate the threshold value between susceptible and tolerant strains by determining the tolerance percentage of a number of *S. aureus* strains. To investigate whether the prevalence of tolerance has increased in the last few decades, two collections of older strains were studied as well.

MATERIALS AND METHODS

Bacteria. The *S. aureus* strains used in this study are composed of the following collections: 64 strains isolated in 1982 from blood cultures of patients admitted to the Rotterdam University Hospital (group I); 29 strains from the collection of the Statens Seruminstitut, Copenhagen, Denmark, isolated during the period 1957 to 1958 from blood cultures of Danish patients (group II); 29 strains isolated from pus, sputum, or blood of patients admitted to the Leiden University Hospital, Leiden, The Netherlands, during the period 1951 to 1953 (group III).

All strains were identified as *S. aureus* on the basis of colony form, color, and a positive coagulase test. The strains of groups I and II were phage typed as well. All strains were catalase positive and were freeze-dried for storage.

Antibiotics. The antibiotics used in this study was cloxacillin and methicillin (gifts from Beecham Pharmaceuticals, Amstelveen, The Netherlands), nafcillin and penicillin (Gist-Brocades, Delft, The Netherlands), and cephalothin (Eli Lilly Nederland, Amsterdam, The Netherlands).

Estimation of tolerance percentage. Tolerance percentages were estimated as previously described (5). The method can be summarized as follows: estimations were carried out by incubation of an inoculum of 10^5 CFU/ml in serial twofold dilutions of cloxacillin, nafcillin, cephalothin, methicillin, and penicillin in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) for 24 h at 37°C. After incubation, 50 μl from the concentrations that suppressed visible growth was spread on nutrient agar (Oxoid Ltd., London, England) plates containing 0.15 U of β-lactamase I and 0.015 U of β-lactamase II per ml (Whatman Biochemicals Ltd.). After 48 h of incubation at 37°C, counts of viable bacteria were made and converted to percentages of the initial inoculum. At higher antibiotic concentrations, survival of the bacteria is no longer dependent on the antibiotic concentration. The plateau of survival level is a characteristic of the strain and is called the tolerance percentage.

Killing curves. For killing curves, 200 μl of a diluted 18-h culture of the strain to be tested was added to a number of tubes containing various concentrations of methicillin in 2 ml of Mueller-Hinton broth. This resulted in an inoculum of 10^5 CFU/ml. Antibiotic concentrations tested ranged from 512 to 0.5 μg/ml. Samples (100 μl) were taken after incubation at 37°C for 0, 3, 6, 12, and 24 h. They were carried through serial 10-fold dilutions in sterile saline, and 50 μl of each dilution was spread on nutrient agar plates containing β-

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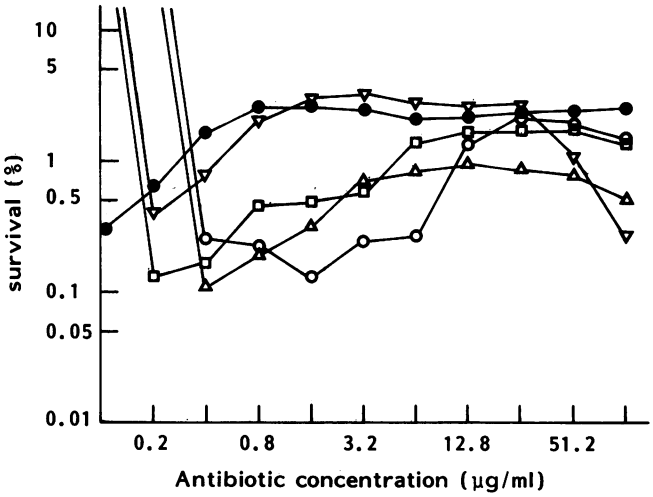

FIG. 1. Survival rates of an isolate of *S. aureus* 335 in the presence of penicillin (●), cloxacillin (□), nafcillin (△), cephalothin (▽), and methicillin (○). Serial concentrations were 0.1 to 102.4 μg/ml for the first four antibiotics. Due to a divergent susceptibility of the strains for methicillin, serial concentrations used in the presence of this antibiotic ranged from 0.5 to 512 μg/ml (data not shown).

lactamase (as stated above). After incubation for 48 h, CFU counts were made and converted to percentages of the initial inoculum.

Lysis curves. Lysis was measured in an MS-2 research apparatus (Abbott Laboratories, Diagnostic Division, Irving, Tex.). With this apparatus, light transmission is read in a multichamber cuvette, and optical densities are recorded at 5-min intervals on a magnetic tape. Analysis of the data by a microcomputer gives the growth curves on a screen (12).

RESULTS

MBCs of penicillins for *S. aureus* proved to be influenced by carry-over of the antibiotic (5). By adding β-lactamase to the subculture plates, carry-over of the antibiotic is prevented. 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. Figure 1 shows a tolerance percentage of 2 to 2.5% for strain 335, determined for five β-lactam antibiotics.

TABLE 1. Distribution of tolerance percentages[a] for 64 isolates of *S. aureus* isolated in 1982 from blood cultures

Antibiotic	No. of strains corresponding to the following ranges of tolerance percentages[b]							
	0–0.5	0.5–1	1–2	2–3	3–4	4–5	5–6	6–7
Methicillin	47 (5)	8 (1)	1	2 (1)	2	1	1	2
Cloxacillin	51 (5)	5 (1)	1	2 (1)	4		1	
Nafcillin	53 (6)	5 (1)		3	2		1	
Cephalothin	48 (5)	9 (1)	2	2	2 (1)	1		
Penicillin	(5)	(1)		(1)				

[a] Each tolerance percentage represents the mean of duplicate determinations.

[b] Numbers in parentheses denote the number of penicillin-susceptible strains.

TABLE 2. Distribution of tolerance percentages[a] for two groups of *S. aureus* isolates determined for methicillin

Group[b]	No. of strains corresponding to the following ranges of tolerance percentages[c]					
	0–0.5	0.5–1	1–2	2–3	7–8	10–20
II	25	1		1	1	1
III	23 (5)	5		(1)		

[a] Each tolerance percentage represents the mean of duplicate determinations.

[b] Group II consists of 29 strains isolated during the period 1957 to 1958; group III consists of 29 strains isolated during the period 1951 to 1953.

[c] Numbers in parentheses denote the number of penicillin-susceptible strains.

To study the distribution of tolerance percentages in a population of *S. aureus* strains, we determined these percentages for 64 *S. aureus* strains isolated in 1982 from positive blood cultures. The values of the tolerance percentages obtained with these strains are shown in Table 1. The majority of these strains (87.5%) had a methicillin tolerance percentage of ≤1; in 28%, this percentage was in fact ≤0.1 (Table 1). The tolerance percentage of the remaining eight strains (12.5%) varied from 2 to 6. A tolerance percentage of ≥2 to the other β-lactam antibiotics tested was shown by 10.9, 9.4, 7.8, and 14.3% of the 64 strains.

Tolerance was also looked for in *S. aureus* strains isolated a few decades ago. For this purpose, we used two collections of 29 strains each dating back to the years 1951 to 1953 (group III) and 1957 to 1958 (group II). Because strains tolerant to methicillin show cross-tolerance to other β-lactam antibiotics, strains of groups II and III were tested only in the presence of methicillin. Both collections show a broad range of tolerance percentages which vary from <0.1 to 12 for group II and from <0.1 to 2.5 for group III (Table 2).

Ten strains from the three collections were further studied on the basis of killing and lysis curves. A comparison of the killing curves of the five strains with high (≥2) tolerance percentages to those of five strains with low (≤0.1) tolerance percentages revealed that the tolerance percentage determined after 24 h was highly related to the killing rate (Fig. 2A). However, at relatively low methicillin concentrations (Fig. 2B) there were no longer any demonstrable differences in killing rate between strains with high and low tolerance percentages. That the killing of *S. aureus* strains is a result of lysis was demonstrated in experiments in which the lysis rate of the same strains in the MS-2 was followed spectrophotometrically during 24 h (Fig. 3). An unmistakable difference in lysis was found between strains with high and low tolerance percentages at a high antibiotic concentration (128 μg of methicillin per ml) (Fig. 3A). The difference in lysis was not demonstrable at a low antibiotic concentration (4 μg of methicillin per ml) (Fig. 3B).

DISCUSSION

In a previous study, we have demonstrated that the determination of MBCs for *S. aureus* showed marked differences, depending on the method of detection used. These differences resulted from carry-over of the antibiotic to the subculture plates (5). After minimalization of this carry-over effect, MBCs were no longer found. A certain percentage of the inoculum survived even very high concentrations of the β-lactam antibiotic. This survival percentage proved to be a

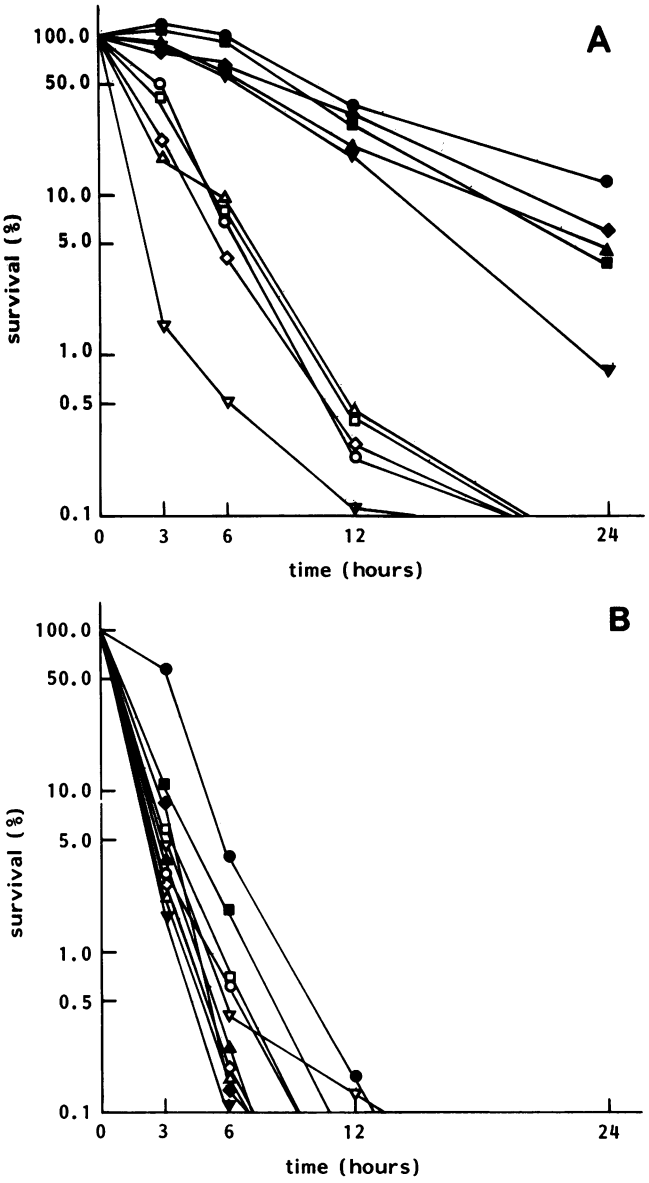

FIG. 2. Killing curves of 10 strains of *S. aureus* at two concentrations of methicillin: 128 μg/ml (A); 4 μg/ml (B). Closed symbols represent five different strains with tolerance percentages of ≥2; open symbols represent five different strains with tolerance percentages of ≤0.1 for methicillin.

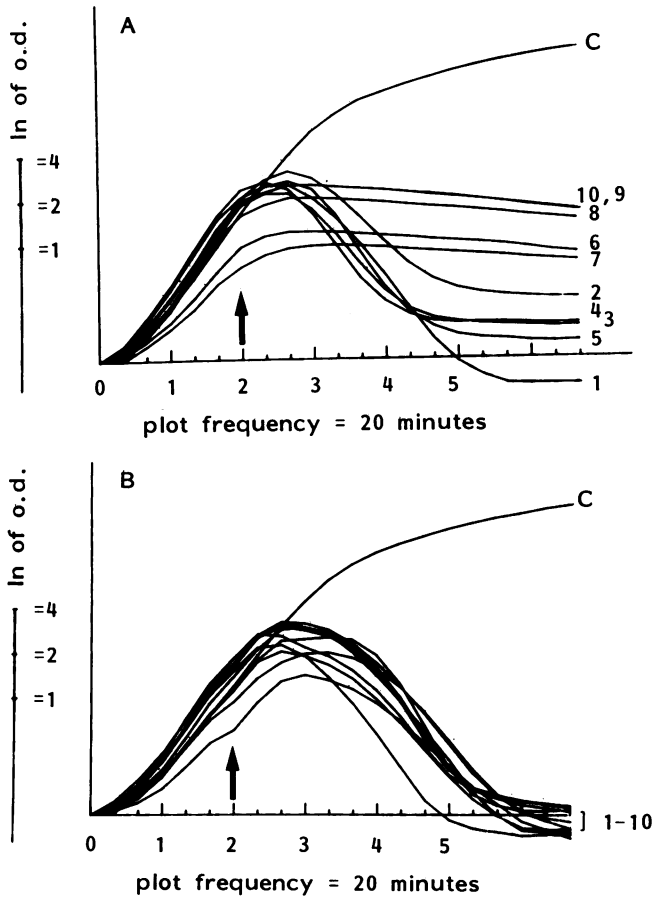

FIG. 3. Growth curves of five strains with tolerance percentages of ≥2 (numbers 6 to 10) and five strains with tolerance percentages of ≤0.1 (numbers 1 to 5) of *S. aureus*. After 2 h of incubation (arrow), methicillin, 128 μg/ml (A) or 4 μg/ml (B), was added to the log-phase cells. The optical density (o.d.) was measured in an MS-2 apparatus. C, Control.

characteristic of the strain and was called the tolerance percentage of the strain. To gain more insight into the distribution of tolerance percentages in a population of strains, these percentages were measured in 64 *S. aureus* strains isolated in 1982 from positive blood cultures from hospitalized patients. It is shown that the majority of the strains (87.5%) have methicillin tolerance percentages of ≤1. The remaining strains show survival percentages of ≥2. Estimations of the tolerance percentages of a strain to different antibiotics show that these are about the same. This means that cross-tolerance exists, even for penicillin-susceptible strains. On account of the results obtained, we conclude that, apart from a few exceptions, there is a breakpoint between strains with tolerance percentages of <2 and those with tolerance percentages of >2. The observations on two other collections of *S. aureus* strains dated from the periods 1951 to 1953 and 1957 to 1958 seem to corroborate the value accepted as breakpoint. In view of the results of these experiments in vitro, efforts are being made to determine the efficacy of antimicrobial treatment in experimental infections caused by tolerant and susceptible *S. aureus* strains.

Our *S. aureus* strains with tolerance percentages of >2 and ≤0.1 showed similar killing and lysis curves as those described by Mayhall et al. (9) and Best et al. (2). This implies that the distribution of tolerance percentages reveals a breakpoint which differentiates between susceptibility and tolerance. It should be borne in mind that the tolerance percentages discussed here were estimated in the presence of high antibiotic concentrations. Our results show, however, that at low antibiotic concentrations, susceptible and tolerant strains do not differ in lysis or killing rate. This so-called paradoxical zone phenomenon described by Eagle and Musselman (4) has been found to a varying extent for all strains tested so far, including those from groups II and III.

The presence of highly tolerant strains in the old collections indicates that tolerance is probably not a new type of resistance. However, the phenomenon has remained unnoticed because the effect of carry-over of antibiotic in estimat-

ing the bactericidal effect of a β-lactam antibiotic was not taken into account.

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LITERATURE CITED

1. **Barry, A. L., and L. D. Sabath.** 1974. Special tests: bactericidal activity and activity of antimicrobics in combination, p. 431–435. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
2. **Best, G. K., N. H. Best, and A. V. Koval.** 1974. Evidence for participation of autolysins in bactericidal action of oxacillin on *Staphylococcus aureus*. Antimicrob. Agents Chemother. **6**:825–830.
3. **Denny, A. E., L. R. Peterson, D. N. Gerding, and W. H. Hall.** 1979. Serious staphylococcal infections with strains tolerant to bactericidal antibiotics. Arch. Intern. Med. **139**:1026–1031.
4. **Eagle, H., and A. D. Musselman.** 1948. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J. Exp. Med. **88**:99–131.
5. **Goessens, W. H. F., P. Fontijne, and M. F. Michel.** 1982. Factors influencing detection of tolerance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **22**:364–368.
6. **Goldman, P. L., and R. G. Petersdorf.** 1979. Significance of methicillin tolerance in experimental staphylococcal endocarditis. Antimicrob. Agents Chemother. **15**:802–806.
7. **Guze, P. A., G. M. Kalmanson, and L. B. Guze.** 1982. The role of antibiotic tolerance in the response to treatment of pyelonephritis due to *Staphylococcus aureus* in rats. J. Infect. Dis. **145**:169–173.
8. **Hilty, M. D., J. S. Venglarcik, and G. K. Best.** 1980. Oxacillin-tolerant staphylococcal bacteremia in children. J. Pediatr. **96**:1035–1037.
9. **Mayhall, C. G., G. Medoff, and J. J. Marr.** 1976. Variation in the susceptibility of strains of *Staphylococcus aureus* to oxacillin, cephalothin, and gentamicin. Antimicrob. Agents Chemother. **10**:707–712.
10. **Rajashekaraiah, K. R., T. Rice, V. S. Rao, D. Marsh, B. Ramakrishna, and C. A. Kallick.** 1980. Clinical significance of tolerant strains of *Staphylococcus aureus* in patients with endocarditis. Ann. Intern. Med. **93**:796–801.
11. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcus aureus*. Lancet **i**:443–447.
12. **Spencer, H. J., J. Stockert, P. Welaj, R. Wilborn, and B. Price.** 1976. Automated antibiotic susceptibility testing with the MS-2 system, p. 272–275. *In* H. H. Johnston and S. W. B. Newsom (ed.), Second International Symposium on Rapid Methods and Automation in Microbiology. Learned Information, Europe Ltd., Oxford, England.
13. **Tomasz, A.** 1974. The role of autolysins in cell death. Ann. N.Y. Acad. Sci. **235**:439–447.