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Effect of Storage and Changes in Bacterial Growth Phase and Antibiotic Concentrations on Antimicrobial Tolerance in *Staphylococcus aureus*

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Forty clinical isolates of *Staphylococcus aureus* were tested for tolerance to oxacillin and cephalothin by broth dilution susceptibility tests and killing curves. Most experiments were carried out with stationary-phase inocula, but nine tolerant isolates were retested with log-phase inocula. All 40 isolates were retested in killing curves at double the antibiotic concentrations used in initial tests. Isolates were retested for tolerance to oxacillin after storage at −70°C for 1 year. In broth dilution tests, 23 of 40 (57.5%) and 20 of 40 (50%) isolates were tolerant to oxacillin and cephalothin, respectively. By killing curves, 25 of 40 (62.5%) and 22 of 40 (55%) isolates were tolerant to oxacillin and cephalothin, respectively. When nine tolerant isolates were retested with log-phase inocula, none manifested tolerance. Only 25 to 30% of the isolates were tolerant in killing curves performed with oxacillin and cephalothin at concentrations double those used in initial tests. After storage at −70°C for 1 year, only two-thirds of the isolates remained tolerant. In isolates that remained tolerant, the degree of tolerance diminished to about 25% of that observed in initial tests.

When bacterial cells incubated with clinically achievable concentrations of a bactericidal antibiotic remain viable, even though their growth is inhibited, they are said to be "tolerant" to that agent. The term "drug tolerance" was first used by Tomasz et al. (14) in a report of their experiments with pneumococci that were not killed by penicillin. The term drug tolerance was first applied to *Staphylococcus aureus* by Best et al. (1) in their description of an isolate that was inhibited but not killed by oxacillin. Since then, several reports have appeared in the literature that implicate tolerance in the failure of therapy of clinical infections treated with antibiotics that inhibit cell wall synthesis (4, 6–9, 13; M.D. Hilty, J.S. Venglarcik, and G.K. Best, Progr. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., Abstr. no. 205, 1978).

In the last 4 years several reports have described some characteristics of tolerant isolates of *S. aureus* in in vitro studies. Mayhall et al. (9) observed that tolerance was encountered frequently in clinical isolates of *S. aureus* tested for their antimicrobial susceptibility by a broth dilution technique. Using killing curve studies, they found that tolerance was due to slower killing of a minority of cells in the inoculum. Sabath et al. (13) confirmed that tolerance was encountered frequently when clinical isolates of *S. aureus* were tested with tube dilution susceptibility tests and that tolerance was due to slower killing of a minority of cells in these strains. Sabath et al. found no correlation between tolerance and phage type and noted that tolerance was unstable and diminished on laboratory storage.

Bradley et al. (3) confirmed that tolerance is encountered frequently in clinical isolates of *S. aureus,* that tolerance is due to slower killing of a minority of the cells in tolerant isolates, and that tolerant isolates cannot be differentiated from nontolerant isolates by phage typing. They demonstrated that tolerance is unrelated to production of L-forms, penicillinase, or deoxyribonuclease. Bradley et al. were also unable to differentiate tolerant from nontolerant isolates on the basis of carbohydrate utilization, growth in 15% NaCl broth or on 40% bile agar or by esculin hydrolysis. They noted no relationship between tolerance and production of pigment or hemolysis or clinical source of isolates.

In this study, 40 clinical isolates of *S. aureus* were tested by broth dilution tests and killing curves. Using log-phase inocula versus stationary-phase inocula and increasing the concentration of antibiotics in the incubation medium were also studied for their effects on tolerance. Finally, tolerant isolates were retested after storage in the laboratory for 1 year to examine the stability of antimicrobial tolerance.

MATERIALS AND METHODS

Bacteria. Forty clinical isolates of *S. aureus* were selected at random from among specimens processed

in the microbiology laboratories of the Medical College of Virginia Hospitals and the McGuire Veterans Administration Medical Center. Isolates were identified as *S. aureus* by colonial and microscopic morphology, by positive coagulase test, and by fermentation of mannitol.

Antibiotics. Antibiotic stock solutions were prepared in sterile distilled water at a concentration of 200 μg/ml and frozen at −20°C until used. Oxacillin was supplied as standard powder by Bristol Laboratories (Syracuse, N.Y.), and cephalothin was purchased as Keflin from Eli Lilly and Co. (Indianapolis, Ind.).

Broth dilution susceptibility tests. Broth dilution tests were performed in duplicate by a technique published previously (3) with the exception that minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined after 18 to 24 h of incubation only. All tests were performed by using the same lot of Mueller-Hinton broth (MHB) (Difco). Stationary-phase inocula were prepared by incubation of each isolate in MHB at 37°C for 18–24 h. Log-phase inocula were prepared by incubation in MHB at 37°C for 2 to 3 h. All tests were performed with stationary-phase inocula except where noted otherwise. When isolates were retested after storage at −70°C for 1 year, a glass bead from each of the storage vials was dropped into MHB and incubated overnight. The broth was subcultured to Trypticase soy agar (BBL Microbiology Systems) containing 5% sheep blood to check for purity of the isolates, and inocula for broth dilution test were prepared from these plates. The MIC was defined as the lowest concentration which yielded no visible turbidity after incubation for 18 to 24 h, and the MBC was defined as the lowest concentration that yielded no growth on subculture, i.e., ≥99.9% killing. Isolates were considered tolerant by broth dilution tests if the MIC was ≤1.5 μg/ml and the MBC was ≥25 μg/ml.

Killing curves. Killing curves were performed in duplicate by a method described previously (3). All tests were performed with the same lot of MHB used for broth dilution tests. Stationary and log-phase inocula were prepared in the same manner as those used in broth dilution tests. Stationary-phase inocula were used in all killing curves except as noted otherwise. Isolates were considered tolerant by killing curve tests when <99.9% of the colony-forming units (CFU) in the inoculum were killed by 24 h when the killing curves were performed at concentrations of 12.5 μg of oxacillin or cephalothin per ml.

Statistical methods. The differences between the percent decrease in CFU from zero time for tolerant and nontolerant isolates at various sampling times in killing curves were tested for significance by the Wilcoxon rank sum test.

RESULTS

Broth dilution susceptibility tests. Results of broth dilution susceptibility tests with oxacillin and cephalothin are shown in Table 1. All isolates had MICs of oxacillin and cephalothin ≤ 1.5 μg/ml. By broth dilution tests, 23 of 40 (57.5%) and 20 of 40 (50%) isolates were tolerant (MBC ≥ 25 μg/ml) to oxacillin and cephalothin, respectively. The 20 isolates tolerant to cephalothin were all tolerant to oxacillin.

Killing curves. Of 40 (62.5%) isolates, 25 were tolerant in killing curves performed with oxacillin, and 22 of 40 (55%) were tolerant to cephalothin. There were no significant differences in the concentrations of CFU in inocula at zero time between tolerant and nontolerant isolates tested with oxacillin ($P = 0.34$) and cephalothin ($P = 0.22$). For oxacillin, there was a significant difference between tolerant and nontolerant isolates at 6 h ($P = 0.001$) in the percent decrease in CFU from zero time, but not at 48 h ($P = 0.09$). With cephalothin there were significant differences at both 6 h ($P = 0.001$) and 48 h ($P = 0.04$). There was a spectrum of tolerance; between 0.13 and 4.4% of the CFU in the inoculum of isolates tolerant to oxacillin survived at 24 h; between 0.16 and 5.8% of the CFU of cephalothin-tolerant isolates survived at 24 h.

There was good correlation between broth dilution tests and killing curves in differentiating tolerant from nontolerant isolates. Test results with the two techniques were concordant for 38 of 40 (95%) isolates tested with oxacillin and 34 of 40 (85%) isolates tested with cephalothin.

Effect of growth phase of the inoculum on tolerance. The effect of inoculum growth phase on tolerance as determined by broth dilution tests with oxacillin and cephalothin is shown in Tables 2 and 3. Note that MICs for the isolates with stationary- and log-phase inocula were similar, whereas MBCs with log-phase inocula were markedly lower than those obtained with stationary-phase inocula, with one exception (isolate 29 tested with oxacillin). The last-mentioned isolate required an MBC of 50 μg/ml, but was nontolerant by killing curve determination.

The killing curves for nine isolates tested with oxacillin and cephalothin with both stationary- and log-phase inocula are shown in Fig. 1 and 2.

TABLE 1. *Results of broth dilution tests with oxacillin and cephalothin for 40 isolates of S. aureus*

Antibiotics	Isolates	No. (%)	Mean MIC (range) (μg/ml)	Mean MBC (range) (μg/ml)
Oxacillin	Tolerant	23 (57.5)	0.68 (0.2–1.5)	44.6 (25–100)
	Nontolerant	17 (42.5)	0.69 (0.4–1.5)	3.3 (0.4–12.5)
Cephalothin	Tolerant	20 (50)	0.76 (0.4–1.5)	52.5 (25–100)
	Nontolerant	20 (50)	0.58 (0.2–1.5)	4.3 (0.4–12.5)

TABLE 2. *Results of broth dilution tests for nine isolates of S. aureus performed with oxacillin using both stationary-phase and log-phase inocula*

Isolates	Stationary-phase inocula		Log-phase inocula	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
1	0.78	50	0.78	0.78
14	0.39	50	0.39	0.39
29	0.78	50	1.56	50[a]
30	0.78	25	0.78	3.13
32	0.78	25	0.78	1.56
33	0.78	50	0.39	1.56
34	0.78	50	0.78	3.13
36	0.39	50	0.39	0.78
38	0.78	50	0.78	0.78

[a] Nontolerant by killing curve determination.

TABLE 3. *Results of broth dilution tests for nine isolates of S. aureus performed with cephalothin using both stationary-phase and log-phase inocula*

Isolates	Stationary-phase inocula		Log-phase inocula	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
1	0.78	>100	0.78	0.78
14	0.78	50	0.39	3.13
29	0.78	50	1.56	12.5
30	1.56	50	0.78	12.5
32	1.56	25	0.78	1.56
33	1.56	50	0.78	0.78
34	0.78	12.5[a]	0.78	1.56
36	0.39	50	0.78	0.78
38	0.78	100	0.39	0.39

[a] Tolerant by killing curve determination.

Note that even though the concentrations of the log-phase inocula were slightly higher than those of the stationary phase inocula, each isolate was killed much more rapidly when tested with inocula, each isolate was killed much more rapidly when tested with inocula in the log phase. When killing curves were determined with log-phase cells, no isolate had more than 10 CFU/ml surviving at 24 h. The results when log-phase cells were used were similar with oxacillin and cephalothin.

Effect of doubling antimicrobial concentrations. When the 40 isolates were retested in killing curves with 25 μg of oxacillin or cephalothin per ml, 12 of 40 (30%) and 10 of 40 (25%) were tolerant, respectively. Of the latter isolates, 11 of 12 (91.7%) and 10 of 10 (100%) were also tolerant in killing curves performed with 12.5 μg of oxacillin and cephalothin per ml, respectively. All isolates tolerant to 25 μg of cephalothin per ml were also tolerant to 25 μg of oxacillin per ml. When isolates tolerant only at 12.5 μg/ml and those tolerant at 25 μg/ml were compared with respect to percent survival of CFU at 24 h in killing curves performed at 12.5 μg of oxacillin or cephalothin per ml, there was a significantly higher percent survival for isolates tolerant at 25 μg/ml compared with those tolerant only at 12.5 μg/ml (oxacillin, $P < 0.05$; cephalothin, $P < 0.01$). The MBCs for most isolates tolerant to 25 μg of oxacillin or cephalothin per ml in killing curves were ≥50 g/ml.

Stability of tolerance on storage. The 40 isolates were retested by broth dilution tests with oxacillin after 1 year of storage in the laboratory on glass beads at −70°C. Of 23 isolates that were tolerant on initial testing, only 15 (65.2%) remained tolerant when retested 1 year later. For the 15 isolates that remained tolerant, fewer CFU were recovered recently

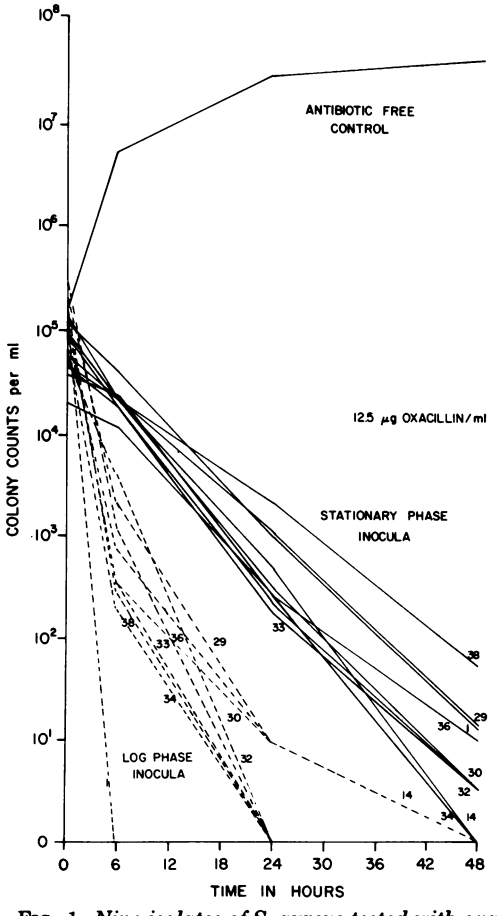

FIG. 1. *Nine isolates of S. aureus tested with oxacillin in killing curves using both stationary-phase* (——) *and log-phase* (- - -) *inocula.*

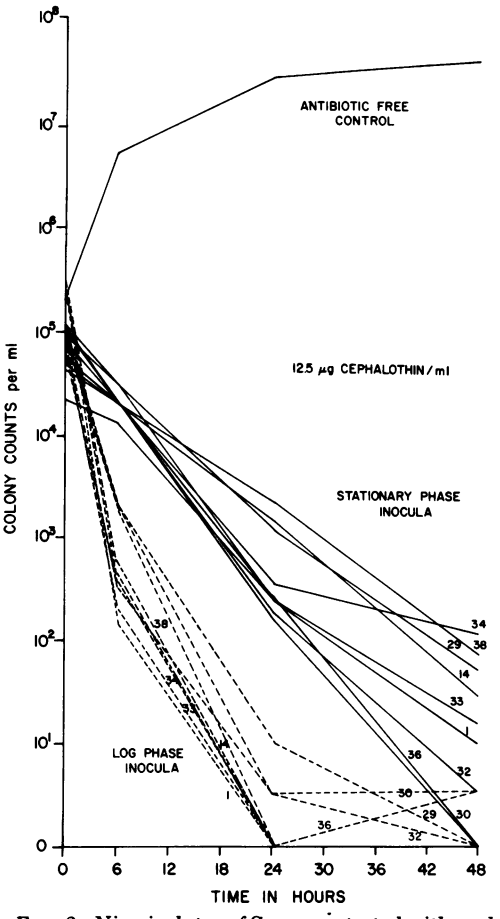

FIG. 2. *Nine isolates of S. aureus tested with cephalothin in killing curves using both stationary-phase* (——) *and log-phase* (- - -) *inocula.*

from subcultures of tubes containing ≥12.5 μg of oxacillin per ml than when the tests were performed 1 year earlier. Thus, on initial testing of isolates that remained tolerant after 1 year of storage, subcultures of duplicate sets of tubes containing ≥12.5 μg of oxacillin per ml yielded an average of 28.7 CFU compared with an average of 7.5 CFU on tests performed 1 year later. Of 17 isolates that were nontolerant on initial testing, two appeared tolerant in repeat tests, but there were only 4 and 2 CFU recovered from subcultures of duplicate sets of tubes containing ≥12.5 μg of oxacillin per ml, respectively.

DISCUSSION

As in our previous studies (3, 9), 50 to 60% of randomly selected clinical isolates of *S. aureus* were tolerant when tested in broth dilution tests and killing curves with oxacillin and cephalothin. Other investigators have also noted a high incidence of tolerance in isolates of *S. aureus* tested in vitro with antibiotics that inhibit cell wall synthesis. Reports of the incidence of tolerance varied between 44 and 100% (2, 12, 13).

We again observed that there was variation in degree of tolerance among tolerant isolates. In a previous study (3), we noted a spectrum of tolerance with between 0.2 and 42% of the CFU in the inoculum remaining viable at 24 h in killing curves. In this study, between 0.13 and 5.8% of CFU survived 24 h of incubation. This is similar to the observations of Sabath et al. (13) who noted that in tolerant isolates 7% or less of the organisms were tolerant.

Most tolerant isolates in this study showed cross-tolerance between oxacillin and cephalothin in both broth dilution and killing curve tests. Other investigators have also observed cross-tolerance between antibiotics that inhibit cell wall synthesis (3, 8, 9, 12, 13).

Good correlation was noted between broth dilution tests and killing curves in differentiating tolerant from nontolerant isolates. Thus, broth dilution tests which are easier to perform and less time consuming appear to be very effective in detecting tolerance in clinical isolates of *S. aureus.*

Our observations that tolerance cannot be demonstrated by using log-phase cells conflicts with the reports of other investigators. Best et al. (1) observed tolerance with log-phase cells, but it is difficult to compare our study with theirs, because they determined changes in CFU spectrophotometrically rather than by colony count and they did not provide information on inoculum concentration. Furthermore, they studied changes in cell concentration over much shorter periods of time compared with our killing curves, and they used a different culture medium which has been shown to effect killing of *S. aureus* by oxacillin (10). Sabath et al. (13) observed tolerance with log-phase inocula in both broth dilution tests and killing curves. However, the conditions used in their killing curves were, again, quite different from those of our studies. They measured changes in the concentration of organisms in their incubation medium by changes in optical density, and they did not report the concentration of CFU in their inocula. In addition, they used a much lower concentration of nafcillin and studied changes in cell concentration over a shorter period of time. Raynor et al. (11) studied tolerance in *S. aureus* with log-phase inocula, but they also used a lower concentration of oxacillin and a different incubation medium. Although the latter authors demonstrated tolerance in three isolates with log-phase inocula in killing curves with colony counts taken at several points in time over 24 h,

they did not perform killing curves with these same isolates with stationary-phase inocula. Thus, further studies will be needed to understand the influence of the growth phase of the organisms in the inoculum on antimicrobial tolerance.

When the 40 isolates of *S. aureus* were tested in killing curves with 25 μg of oxacillin or cephalothin per ml, only 25 to 30% were tolerant at the higher antibiotic concentrations. Only one of the isolates tolerant at 25 μg/ml was not tolerant at 12.5 μg/ml. Isolates tolerant at 25 μg/ml were significantly more tolerant at 12.5 μg/ml than the isolates tolerant only at 12.5 μg/ml. However, for isolates tolerant at both 25 and 12.5 μg/ml, the percent survival of CFU was always lower at 25 μg/ml than 12.5 μg/ml. Thus, some tolerant isolates appeared to have a heterogenous population of tolerant cells, with some tolerant only at 12.5 μg/ml and others tolerant at both 12.5 and 25 μg/ml. This observation makes it unlikely that tolerance is related to the phenomenon described by Eagle and Musselman (5). The latter authors noted that killing of *S. aureus* took place maximally at a certain optimal concentration of penicillin G and that higher concentrations resulted in no further killing. Thus, the phenomenon that they described differs from our observations in that additional killing took place in every tolerant isolate when the antibiotic concentration was doubled.

Sabath et al. (13) observed that tolerance in *S. aureus* was unstable and diminished on storage for 4 to 12 months in the laboratory. We confirmed and extended their observations. Of 23 tolerant isolates stored at −70°C for 1 year, only 15 remained tolerant, and the degree of tolerance in the latter diminished. It is possible that tolerance may diminish even more rapidly when isolates are stored on agar at room temperature. Thus, when studying antimicrobial tolerance in *S. aureus,* its instability during storage must be kept in mind, and only fresh clinical isolates should be used for laboratory investigations.

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