Significance of Methicillin Tolerance in Experimental Staphylococcal Endocarditis

PEGGY L. GOLDMAN AND ROBERT G. PETERSDORF*

Department of Medicine, School of Medicine, University of Washington, Seattle, Washington 98195

Received for publication 23 March 1979

Methicillin-tolerant staphylococci are organisms that are inhibited by low concentrations of the drug but are resistant to its bactericidal effects. The clinical significance of this in vitro phenomenon is unknown. An experimental model of staphylococcal endocarditis in rabbits was used to determine whether methicillin-tolerant staphylococci are particularly difficult to eradicate from infected heart valves. In vitro sensitivity testing was used to identify a tolerant and a nontolerant strain of *Staphylococcus aureus*. Rabbits prepared by the insertion of a polyethylene catheter into the left ventricle and injected with a tolerant strain survived significantly longer than those injected with a nontolerant strain. No significant differences were demonstrated in the prevention of endocarditis with a single prophylactic dose of methicillin, or in the treatment of established endocardial infection with multiple doses of methicillin. In this study, methicillin had the same activity against experimental endocarditis caused by a tolerant and a nontolerant strain of *S. aureus*.

Staphylococcal endocarditis remains a life-threatening problem with a high mortality despite the fact that the causative organisms are usually susceptible to semisynthetic penicillins (19). Some patients who have responded poorly to antibiotic therapy have been infected with organisms that have a low minimum inhibitory concentration (MIC) but a very high minimum bactericidal concentration (MBC) (13). Such staphylococcal strains are "tolerant" to the bactericidal action of semisynthetic penicillins (16). Despite similar growth rates, tolerant strains require 24 to 48 h of incubation in vitro with therapeutic concentrations of oxacillin for complete loss of viability, whereas nontolerant strains are completely killed within 24 h (13).

The clinical significance of methicillin tolerance is unknown, but infections with organisms that are killed slowly might be expected to require more intensive antimicrobial management. Theoretically the importance of antibiotic tolerance should be magnified in situations in which bactericidal therapy is thought to be necessary, as in the prevention and treatment of bacterial endocarditis (9). To test the hypothesis that tolerant staphylococci are particularly difficult to eradicate from endocardial infections, we compared the prophylactic and therapeutic activity of methicillin against incipient or established infections with a tolerant strain of *Staphylococcus aureus*, to the activity of this agent against similar infections with a nontolerant strain.

MATERIALS AND METHODS

Organisms. An isolate from blood cultures of a patient with bacterial endocarditis, numbered strain 72, was obtained from Leon Sabath, and a second strain, numbered 68, was obtained from the University of Washington Clinical Microbiology Laboratory. Cultures were maintained on Columbia (Difco) blood agar plates at 2°C.

Antibiotic susceptibility tests. Staphylococcal inocula were prepared by adding five bacterial colonies from a plate culture to 5 ml of Trypticase soy broth (TSB) and incubating for 18 h at 37°C. Each inoculum was quantitated by adjusting the broth culture to an appropriate optical density using a Coleman Junior spectrophotometer and subculturing 0.1-ml portions of 10-fold dilutions.

For determination of the MIC, 0.5 ml containing bacteria was added to 0.5 ml of serial twofold dilutions of methicillin in broth. The tubes were mixed and then incubated for 8, 18, 24, and 48 h at 37°C. The MIC was read as the lowest concentration of methicillin required to inhibit visible growth. For determination of the MBC, 0.01 ml removed from each clear tube at 8, 18, 24, and 48 h was streaked on Columbia agar plates, which were incubated for 24 h at 37°C. The MBC was calculated to be the lowest antibiotic concentration necessary to achieve 99.9% killing. The MIC and MBC were performed in both TSB and Mueller-Hinton broth (MHB), using inoculum sizes from 5×10^4 to 5×10^7 bacteria per ml. In vitro tests were repeated using MHB and 10^5 organisms per ml approximately every 2 weeks.

Killing curves. The appropriate amount of methicillin needed to yield a final concentration of 25 µg/ml was added to glass tubes containing 4 ml of MHB. Inocula containing 10^5 organisms in 1 ml were pre-

pared as described above and added to each tube. Staphylococci were incubated with methicillin at 37°C in a water bath shaker, and 0.5-ml portions were removed at 3, 18, 24, and 48 h. Samples were diluted in sterile normal saline, and 0.1 ml was plated in Columbia agar pour plates. After incubating overnight, the colonies were counted, and the number of viable organisms was calculated.

Production and evaluation of bacterial endocarditis. New Zealand white rabbits weighing 1.5 to 2.5 kg, obtained from Lab Associates-Totem Farms, Kirkland, Wash., were prepared by the insertion of a polyethylene catheter into the left ventricle as previously described (6). Rabbits were injected intravenously with staphylococci, which reliably colonized valve vegetations. To quantitate the extent of infection, animals were necropsied or sacrificed by injecting 150 mg of pentobarbital. The hearts were removed aseptically, and endocardial vegetations were excised, weighed, and homogenized in glass with Teflon tissue grinders in 1 ml of TSB. The number of bacteria per gram of vegetation was calculated after subculturing 0.1-ml portions of 10-fold dilutions of the ground tissue. Vegetations were considered sterile when no growth was recovered from 0.1 ml of undiluted tissue homogenate.

In vivo studies. On the basis of in vitro testing (see Results section) the following staphylococcal inocula were chosen for animal experiments: strain 72 incubated for 24 or 8 h before injection into animals, and strain 68 incubated for 24 or 8 h before injection. The first inoculum was nontolerant (NT; MBC against methicillin equal to the MIC), and the other inocula were tolerant (T; MBC greater than 25 times the MIC). Preliminary experiments demonstrated that 10^6 bacteria infected at least 90% of the animals when vegetations were examined 48 h after injection.

To evaluate the relative virulence of each inoculum, rabbits were injected intravenously with 10^5 organisms on day zero and were left undisturbed. The vegetations were cultured quantitatively for evidence of infection when animals were necropsied, or on day 6 when the surviving rabbits were sacrificed.

The ability of a single dose of methicillin to prevent endocardial infection was evaluated. Using the agar well diffusion method with *Bacillus subtilis* as the test organism (1), serum levels were determined in three normal rabbits, after an intramuscular dose of methicillin (300 mg/kg), to be 190 ± 5.39 , 21.13 ± 2.82 , 1.51 ± 0.81 , and 0.47 ± 0.01 $\mu\text{g/ml}$ at 0.5, 2, 6, and 24 h, respectively. The dose of methicillin in animal experiments was chosen to yield serum levels above the MIC and MBC for the NT inoculum, but below the MBC of the T inocula. Rabbits were injected intravenously with methicillin (300 mg/kg) 24 h after placement of intracardiac catheters. After 0.5 h, 10^5 bacteria were injected intravenously. The endocardial vegetations were cultured quantitatively after 48 h, but were all sterile except for those obtained from controls not receiving methicillin. Thereafter 10^6 bacteria were injected intravenously 0.5 h after administration of methicillin. Endocardial vegetations were cultured quantitatively after 48 h.

The effect of prolonged antibiotic treatment on endocardial infection was examined. At 24 h after

placement of intracardiac catheters, rabbits were injected intravenously with 10^6 bacteria. After 24 h, methicillin (300 mg/kg) was injected intramuscularly every 12 h. Groups of animals were sacrificed on day 2, 3, 5, and 6, at which time the vegetations were cultured quantitatively. Two untreated rabbits in each experiment served as controls.

Statistical analysis. In vivo results are expressed in two forms: the proportion of infected animals in each group and the number of bacteria recovered per gram of vegetation. Chi-square analysis and the two-tail Fisher exact test were used to compare the proportion of animals with sterile and infected vegetations after antibiotic prophylaxis or treatment. The Student *t* test was used to determine whether differences in the mean $\log_{10}$ of bacteria recovered per gram of vegetation were statistically significant.

RESULTS

In vitro studies. (i) Time dependence of tolerance. Table 1 summarizes the time dependence of tolerance determined for strains 68 and 72. At 8 h, the MBC was greater than 1,000 times the MIC for both strains. When measured at 18 and 24 h, the MBC equaled the MIC for strain 72, but the MBC was still substantially greater than the MIC for strain 68. At 48 h, the MBC was equal to the MIC for both strains. If tolerance is defined as a ratio of MBC to MIC greater than 25 to 1, at 8 h both strains were T; at 18 and 24 h 68 was T and 72 was NT; and at 48 h both were NT. MIC and MBC data were reproducible within one tube dilution throughout the duration of storage for both strains.

(ii) Effect of incubating medium and inoculum size. To determine whether tolerance is a microbial characteristic manifested under a wide variety of conditions in vitro, the effect of the incubation medium and the size of the inoculum on the MBC were studied for strains 68 and 72. These experimental manipulations did not affect the MIC of methicillin for either strain. In contrast, the MBC of methicillin at 18 h was 100-fold higher in TSB than in MHB for strain 72 but not for strain 68. Inocula of 10^4 bacteria per ml lowered the MBC 100-fold for both strains when compared to 10^6 and 10^7 bacteria per ml. These data indicate that, simply by changing the broth, strain 72 changed from NT

TABLE 1. Time course of methicillin tolerance^a

Strain	8 h		18 h		24 h		48 h	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
68	2	2,048	4	500	4	256	8	8
72	2	2,048	4	4	4	4	8	8

^a Results are expressed as micrograms of methicillin per ml, in MHB, using 5×10^5 bacteria.

to T in TSB at inoculum sizes greater than 10^4 bacteria per ml, and that strain 68 changed from T to NT when the MBC was measured in either TSB or MHB at inocula of 10^4 bacteria per ml.

(iii) **Killing curves.** Figure 1 is a composite graph of four determinations of the effect of 25 μ g of methicillin per ml in the killing of both strains of *S. aureus*. Bacterial inocula were passaged over 2 weeks to determine whether results were reproducible. Methicillin reduced consistently and more rapidly the number of viable bacteria for NT strain 72 than for T strain 68. These data correlate well with the results of tube dilution susceptibility tests.

In vivo experiments. (i) **The course of experimental infection.** Figure 2 presents life-table survival curves for rabbits with untreated staphylococcal endocarditis. The data represent the results of five experiments in which the cultures implanted at one time were randomized among the various strains. Animals infected with strain 72 incubated for 24 h before injection (NT) had a median survival of 2 days, whereas those animals infected with strain 68 incubated for 24 h before injection (T) had a median survival greater than 6 days ($P < 0.001$, Fisher two-tail exact test). Animals infected with strain 72 incubated for 8 h (T) and strain 68 incubated for 8 h (T) had similar survival curves, with a median of 3 days each. Greater than 10^9 bacteria per g of vegetation was recovered from all rabbits that were evaluated after being found dead in the cage during the observation period. At the end of 6 days, the remaining rabbits were sacrificed. The only surviving rabbit injected with strain 72 (NT) and two rabbits injected with strain 68 (T) had sterile vegetations. The mean $\log_{10} \pm$ standard deviation bacteria recovered from surviving animals was as follows: $9.84 \pm$

FIG. 1. Killing curve of two strains of *S. aureus* incubated with 25 μ g of methicillin per ml. The inoculum consisted of 10^5 organisms per ml. The curves are the averages $\pm$ standard deviation of four tests. (----) Strain 72 with low MBC; (—) strain 68 with high MBC.

FIG. 2. Life-table analysis of survival of rabbits with untreated staphylococcal endocarditis. Rabbits with intracardiac catheters were injected with 10^4 organisms and followed. The figure is a composite of four experiments: (—) and (---), strain 72 incubated for 24 and 8 h, respectively; (- - -) and (.....), strain 68 incubated for 24 and 8 h, respectively. Only strain 72 incubated for 24 h was NT; all other strains were T. See text for interpretation.

0.34 for rabbits injected with strain 72 incubated for 8 h (T), $N = 2$; 8.86 ± 0.49 for rabbits infected with strain 68 incubated for 8 h (T), $N = 3$; and 4.95 ± 0.08 for rabbits infected with strain 68 incubated for 24 h (T), $N = 17$ ($P > 0.05$ for all comparisons).

(ii) **Antibiotic prophylaxis.** The ability of a single dose of methicillin to prevent experimental staphylococcal endocarditis is summarized in Table 2. A total of 14 of 43 (33%), 4 of 23 (17%), and 8 of 20 (40%) of animals injected with strain 72 (NT), 72 (T), and 68 (T) had 1.73 ± 0.48 , 0.97 ± 0.48 , and 2.74 ± 0.82 mean $\log_{10} \pm$ standard deviation bacteria per g of vegetation recovered, respectively, at autopsy. One untreated control per experiment had greater than 10^9 bacteria per g of vegetation recovered. Significantly fewer bacteria were recovered from animals injected with strain 72 (T) than with strain 68 (T) ($P < 0.05$). The importance of this finding is unclear.

(iii) **Treatment of experimental endocarditis.** The effect of methicillin therapy on established experimental infection caused by strain 72 (NT) and strain 68 (T) is presented in Table 3. One rabbit infected with strain 72 and one infected with strain 68 died after 5 and 4 days, respectively, of therapy, but both had sterile vegetations. These deaths are unexplained. All rabbits except one that received methicillin for 6 days also had sterile vegetations, but appeared well at time of sacrifice. The mean $\log_{10} \pm$ standard deviation bacteria per gram of vegetation recovered from animals receiving strain 72 (NT) and 68 (T) was 5.97 ± 1.33 and 3.96 ± 1.65

TABLE 2. Prevention of endocarditis with a single dose of methicillin (300 mg/kg) intramuscularly 30 min before inoculation with 10^6 *S. aureus*

Strain	No. of rabbits	Percent infected	Mean log ₁₀ bacteria per g of vegetation $\pm$ SD ^a
72 ^b	43	33	1.73 $\pm$ 0.48
72 ^c	23	17	0.97 $\pm$ 0.48
68 ^c	20	40	2.74 $\pm$ 0.82

^a SD, Standard deviation.

^b NT.

^c T.

TABLE 3. Treatment of experimental endocarditis^a

Day	Strain 72 ^b	(N)	Strain 68 ^c	(N)
2	5.97 $\pm$ 1.33	(5)	3.96 $\pm$ 1.65	(4)
3	2.26 $\pm$ 0.81	(7)	4.14 $\pm$ 1.51	(5)
5	2.16 $\pm$ 0.77	(10)	2.15 $\pm$ 0.80	(6)
6	0.79 $\pm$ 0.79	(8)	0.00 $\pm$ 0.00	(6)

^a Twenty-four hours after injection of 10^6 bacteria, rabbits with intracardiac catheters received methicillin (300 mg/kg) intramuscularly twice daily, starting on day 1. Results are expressed as the mean log₁₀ bacteria $\pm$ standard deviation per gram of vegetation recovered at autopsy.

^b NT.

^c T.

(day 2), 2.26 $\pm$ 0.81 and 4.14 $\pm$ 1.51 (day 3), 2.16 $\pm$ 0.77 and 2.15 $\pm$ 0.80 (day 4), and 0.79 $\pm$ 0.79 and 0.00 (day 6), respectively. These differences were not statistically significant.

DISCUSSION

Isolates of *Staphylococcus aureus* are known to resist β -lactamase antibiotics in two ways. The most common is based on the production of penicillinase (β -lactamase I), which catalyzes the hydrolysis of penicillins (12). Another type of resistance, called "intrinsic" methicillin resistance, is poorly understood, but probably depends upon alteration of the bacterial membrane rather than production of an enzyme (7). Antibiotic resistance affects the minimum inhibitory concentration in vitro. In contrast, methicillin tolerance is not true drug resistance, because serum inhibitory levels are in the susceptible range, although bactericidal levels are higher than can normally be achieved in serum (3).

The mechanism of staphylococcal tolerance is unknown. Although Best et al. (2) have proposed that deficient or absent autolytic enzyme activity may be important in the resistance of staphylococci to the bactericidal effects of penicillins, as occurs with pneumococci (17), Chatterjee et al. (5) have isolated a staphylococcal mutant deficient in autolytic activity which remained susceptible to the lethal effect of penicillin G. Alternatively, tolerant strains may exhibit the

zone phenomenon (8) or may have a high proportion of bacterial persisters (11). The importance of these observations awaits experimental proof.

In vitro conditions may alter autolytic activity, β -lactamase production, or other membrane or growth characteristics of the organisms which could affect the bactericidal capacity of an antibiotic. The MBC for our staphylococcal strains was extremely dependent upon the time at which it was measured, as well as on the size of the inoculum and the type of medium, as noted in other laboratories (14). Such information indicates the need for clarification of the criteria required to identify tolerant organisms correctly. Killing curves may be a better way of identifying tolerance (4). But whether in vitro bactericidal activity, as measured by either method, significantly alters the outcome of infection is not known.

An analysis of the course of experimental endocarditis suggests that inocula of mature, tolerant staphylococci may cause less severe infection in rabbits than nontolerant ones. The factors which promote adherence to the heart valve, or which affect opsonization, phagocytosis, chemotaxis, and susceptibility to intracellular killing in the vegetations, could be inversely related to the capacity of the bacterial strain to withstand the bactericidal action of some antibiotics in vitro. This hypothesis is similar to one suggesting that multiply drug-resistant *Enterobacteriaceae* are less virulent than drug-susceptible strains (10). No explanation exists for the observation that animals infected with log-phase bacterial inocula of strain 72 had enhanced survival, whereas the converse was found with strain 68.

In experiments studying the prevention of endocardial infection, we examined the in vivo consequences of the observation that staphylococci become nontolerant when incubated for more than 24 h. After 8 and 24 h of incubation, corresponding to in vitro determinations suggesting tolerance and nontolerance respectively, we injected the same number of organisms of strain 72 into rabbits with intracardiac catheters. A single dose of methicillin prevented infection in 19 of 23 (83%) and 29 of 43 (67%) of animals injected with the T and NT inocula, respectively. This result was not statistically significant. However, the quantity of bacteria recovered from vegetations of animals given tolerant organisms was significantly less than that recovered from animals given nontolerant organisms. A partial explanation of this phenomenon may be that tolerant organisms were in an exponential phase of growth during which immature production of membrane constituents or

extracellular proteins may have yielded organisms that were more easily phagocytized or killed in vivo. Alternatively, the antibacterial action of methicillin may have been greater on actively dividing cells in vivo.

The ability of drugs to prevent experimental endocarditis has, in general, paralleled in vitro susceptibility tests. In our studies, no significant difference in the ability of a single dose of methicillin to prevent endocardial infection was demonstrable using two strains of staphylococci with an MIC in the susceptible range, even though the serum level of antibiotic was substantially less than the MBC for the tolerant strain. Similarly, no significant differences were noted in the response to prolonged treatment of established endocarditis produced by tolerant and nontolerant *S. aureus*. Our result corroborate the observations of Watanakunakorn (18) and Reymann et al. (15), who found that antibiotic tolerance did not determine the response to antimicrobial therapy of patients with staphylococcal endocarditis. The dose of methicillin employed in our study may be greater than the least that could prevent the development of endocarditis or treat the established disease effectively. Therefore, small differences between tolerant and nontolerant strains may have been obscured. However, the chosen doses yielded serum levels approximately those achieved in humans and are more relevant to clinical situations in which large doses of semisynthetic penicillins are used in the treatment of serious staphylococcal infections.

Our conclusions therefore are (i) that, although a variation in the rate of bacterial killing of staphylococci (i.e., tolerance) has been noted in vitro, this phenomenon is extremely variable and depends on a number of factors such as length of incubation, inoculum size, and type of medium; and (ii) that methicillin tolerance does not significantly alter the response to antimicrobial therapy of experimental staphylococcal endocarditis.

ACKNOWLEDGMENTS

We thank J. David Sanchez for technical assistance.

This investigation was supported by Public Health Service grant HL-17751-03 from the National Institutes of Health.

LITERATURE CITED

- Bennett, J. V., J. L. Brodie, E. J. Benner, and W. M. Kirby. 1966. Simplified accurate method for antibiotic assay of clinical specimens. *Appl. Microbiol.* 14: 170-177.
- 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.
- Best, G. K., A. V. Koval, and N. H. Best. 1975. Susceptibility of clinical isolates of *Staphylococcus aureus* to killing by oxacillin. *Can. J. Microbiol.* 21:1692-1697.
- Bradley, J. J., C. G. Mayhall, and H. P. Dalton. 1978. Incidence and characteristics of antibiotic-tolerant strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 13:1052-1057.
- Chatterjee, A. N., W. Wong, F. E. Young, and R. W. Gilpin. 1976. Isolation and characterization of a mutant of *Staphylococcus aureus* deficient in autolytic activity. *J. Bacteriol.* 125:961-967.
- Durack, D. T., P. B. Beeson, and R. G. Petersdorf. 1973. Experimental bacterial endocarditis. III. Production and progress of the disease in rabbits. *Br. J. Exp. Pathol.* 54:142-151.
- Dyke, K. G., M. P. Jevons, and M. T. Parker. 1966. Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* i:835-838.
- 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.* 88:99-130.
- Finland, M. 1954. Medical progress: treatment of bacterial endocarditis. *N. Engl. J. Med.* 250:372-380.
- Garrod, L. P., and F. O'Grady. 1971. Antibiotics and chemotherapy, p. 243. E. & S. Livingstone, London.
- Gunnison, J. B., M. A. Fraher, and E. Jawetz. 1963. Persistence of *Staphylococcus aureus* in penicillin in vitro. *J. Gen. Microbiol.* 35:335-349.
- Kayser, F. H., E. J. Benner, R. Troy, and F. D. Hoeprich. 1971. Mode of resistance against β -lactam antibiotics in staphylococci. *Ann. N.Y. Acad. Sci.* 12: 106-117.
- 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.
- Peterson, L. R., D. N. Gerding, W. H. Hall, and E. A. Shierl. 1978. Medium-dependent variation in bactericidal activity of antibiotics against susceptible *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 13: 665-668.
- Reymann, M. T., H. P. Holley, Jr., and C. G. Cobbs. 1978. Persistent bacteremia in staphylococcal endocarditis. *Am. J. Med.* 65:729-737.
- 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.
- Tomasz, A., A. Albino, and E. Zanati. 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. *Nature (London)* 227:138-140.
- Watanakunakorn, C. 1978. Antibiotic-tolerant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 4:561-568.
- Watanakunakorn, C., J. S. Tan, and J. P. Phair. 1973. Some salient features of *Staphylococcus aureus* endocarditis. *Am. J. Med.* 54:473-481.