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Variation in the Susceptibility of Strains of *Staphylococcus aureus* to Oxacillin, Cephalothin, and Gentamicin

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Three cases of staphylococcal bacteremia caused by organisms that were inhibited by low concentrations of oxacillin, but were resistant to the killing effects of oxacillin, stimulated us to screen 60 clinical isolates of *Staphylococcus aureus* for their susceptibility to oxacillin, cephalothin, and gentamicin. All 60 strains were inhibited by low concentrations of the antibiotics. The antibiotics were bactericidal against 27 of the 60 strains and only bacteriostatic against the other 33 after 24 h of incubation. However, after 48 h of incubation, the antibiotics were also bactericidal against the latter group.

The introduction of the penicillinase-resistant semisynthetic penicillins has resulted in a significant improvement in the therapy of severe staphylococcal infections. However, in spite of the use of these effective antibiotics, the mortality of staphylococcal bacteremia remains high, approaching 50% in some series (17). This high mortality cannot be attributed to drug resistance because, unlike the European experience (1, 7), reports of methicillin-resistant staphylococci have been few in number in the United States (2, 15).

We have encountered three patients with staphylococcal bacteremias over the past 3 years who failed to respond to adequate dosages of oxacillin despite evidence of the organisms' susceptibility to this drug and required the addition of gentamicin to control the infections. Tube dilution susceptibility tests revealed that the oxacillin had a low minimal inhibitory concentration (MIC) but a very high minimal bactericidal concentration (MBC) against these organisms. The three cases stimulated an investigation of the frequency of the occurrence of this phenomenon in a large number of clinical isolates of *Staphylococcus aureus* picked randomly from isolates in the Barnes Hospital Microbiology Laboratory. The results of these studies are the subject of this report.

CASE REPORTS

Case 1. An 18-year-old man was admitted to Barnes Hospital with a 3-day history of chills, fever, and right-sided pleuritic chest pain. He was a frequent user of heroin intravenously and had administered his last dose 1 week before admission. On examination, he had a rectal temperature of 105°F (40.6°C). He had a pleuritic rub heard at the base of his right lung. No heart murmur was noted. Chest X-ray showed scattered nodular lesions in both lung fields. He was started on 2 g of oxacillin, given intravenously, every 4 h. Four blood cultures drawn before initiation of therapy grew *S. aureus*. In spite of the oxacillin therapy, he remained febrile and one of four blood cultures was positive for *S. aureus* 48 h after initiation of therapy. The organism was susceptible to methicillin and oxacillin by the disk diffusion method (3). An oxacillin tube dilution susceptibility test, using Mueller-Hinton broth, showed an MIC of 0.20 μg/ml and an MBC of 50 μg/ml. A serum specimen taken 1 h after a dose of oxacillin was bacteriostatic against his organism at a 1:8 dilution but was not bactericidal. The organism was susceptible to gentamicin by the disk diffusion method, and tube dilution tests showed gentamicin to be bacteriostatic at 1.5 μg/ml and bactericidal at 3.0 μg/ml. Gentamicin was added to his therapy at a dosage of 1 mg/kg intravenously every 8 h, and he defervesced 12 h after gentamicin was started; subsequent blood cultures were negative. Serum specimens were bactericidal against his organism at a dilution of 1:8 while the patient was on both antibiotics. He was treated with oxacillin and gentamicin for 5 weeks and recovered. Tests for synergism of oxacillin and gentamicin showed that the MBC of the combination against his organism was 0.5 μg of oxacillin plus 0.25 μg of gentamicin.

Case 2. A 67-year-old man with lymphosarcoma developed chills, fever, and mental obtundation. Three of three blood cultures were positive for *S. aureus*, and the organism was susceptible by disk diffusion to oxacillin and gentamicin. He was given 2 g of oxacillin intravenously every 4 h, but his temperature remained elevated, and blood cultures were still positive over the next 48 h. His serum, drawn 1 h after a dose of oxacillin, inhibited growth of the staphylococcus at a dilution of 1:16 but was not bactericidal. Gentamicin was added to his therapy and he defervesced in about 24 h; blood cultures became negative. The MIC of oxacillin against his

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organism was 0.20 μg/ml, and the MBC was 25 μg/ml. The MIC of gentamicin was 0.5 μg/ml, and the MBC was 4.0 μg/ml. The MBC of the combination was 0.5 μg of oxacillin per ml and 0.5 μg of gentamicin per ml.

Case 3. A 79-year-old man with cancer of the prostate and multiple metastatic lesions developed *S. aureus* bacteremia during his hospitalization. The organism was susceptible to oxacillin and gentamicin by disk. He failed to respond to 2 g of oxacillin intravenously every 4 h and remained febrile over the next 5 days. No follow-up blood cultures were drawn before 1 mg of gentamicin per kg every 8 h was added to his regimen. Twenty-four hours after gentamicin treatment was started he became afebrile and had negative blood cultures. The MIC of oxacillin against his organism was 0.20 μg/ml with an MBC of 25 μg/ml. The MIC of gentamicin was 1.5 μg/ml with an MBC of 6.0 μg/ml. The MBC of the combination was 1.0 μg of oxacillin per ml and 1 μg of gentamicin per ml.

MATERIALS AND METHODS

Bacteria. Sixty strains of *S. aureus* collected randomly from clinical specimens submitted to the Barnes Hospital Microbiology Laboratory over the period of 12 months were studied. All of the isolates were from different patients, and there was no consistent pattern of antibiotic susceptibility, except that all were penicillin resistant. All of the clinical isolates were identified as *S. aureus* on the basis of their gross and microscopic morphology, coagulase positivity, and ability to grow in 6.5% sodium chloride agar.

Antimicrobial agents. The following antimicrobial agents were tested: oxacillin, purchased as Prostaphlin from Bristol Laboratories; cephalothin, purchased as Keflin from Eli Lilly and Co.; and gentamicin, purchased as Garamycin from Schering Corp. All antibiotics were dissolved in sterile, distilled water and stock solutions were prepared at concentrations of 200 μg/ml and frozen at −10°C until used. All stock solutions were used within 1 month of preparation.

Antimicrobial susceptibility tests. Tube dilution susceptibility tests were performed on each organism in clear plastic tubes (22 by 77 mm) with Mueller-Hinton broth (Difco) prepared from the same batch of powder (603741). Antibiotic concentrations tested ranged from 0.1 to 100 μg/ml in a volume of 0.5 ml. Inocula were prepared from cultures on blood agar plates and transferred into tubes with 5 ml of broth. After incubation at 37°C for 18 h, turbidity was measured and the suspensions of organisms were diluted in broth to a concentration of 10^5/ml, and 0.5-ml portions were added to each tube containing the antibiotics. Control tubes without antibiotics were included in each series of dilutions; all tests were done in duplicate. After incubation for 18 to 20 h, the MIC, defined as the lowest concentration of antibiotic that prevented visible growth, was recorded. Broth was then removed from each clear tube by a calibrated platinum loop (0.03-ml) and streaked on blood agar plates. After overnight incubation, the MBC was determined; it was defined as the lowest concentration of antibiotic that resulted in less than two surviving colonies on the blood agar plates. This required a killing of 99.9% or greater of the initial inoculum by the bactericidal antibiotic. MICs were determined without shaking the tubes because our earlier studies had demonstrated that shaking caused significant numbers of organisms to be inoculated and to survive on the inside walls of the tubes above the incubation mixtures. Shaking the tubes when reading the MICs washed some of these viable organisms into the broth, and these grew on a subculture giving a spuriously high MBC.

Killing curves. Killing curves were performed in broth prepared from the same batch of powder as that used in the tube dilution susceptibility testing. Ten strains of *S. aureus* were chosen. Oxacillin had a high MBC against six strains and a low MBC against the other four strains. The effects of 25 μg of oxacillin per ml, 25 μg of cephalothin per ml and 6.25 μg of gentamicin per ml were measured on the colony counts of these organisms as a function of time. The appropriate dilution of each antibiotic, in Mueller-Hinton broth, was added to each tube in a volume of 2 ml. Inocula of 10^5 organisms/ml were prepared as described above, and 2 ml of each inoculum was added to each tube to bring the final volume to 4 ml.

The staphylococci were incubated with the antimicrobial agents at 37°C, and 0.5-ml portions were removed from each of the incubation mixtures at 4, 8, 12, 24, and 48 h. Several tests were performed at 30°C with comparable results. Each sample was carried through several 10-fold dilutions in sterile normal saline, and 0.1 ml of these dilutions was spread on the surface of blood agar plates with a glass rod. After incubation overnight, the colonies were counted and recorded. Killing studies were performed in duplicate on each organism.

RESULTS

Tube dilution susceptibility testing to oxacillin on the 60 strains of *S. aureus* revealed two distinct groups based on differences ($P < 0.001$) in the MBCs (Table 1). Oxacillin had similar MICs for both groups (0.33 ± 0.18 for group 1 and 0.30 ± 0.53 for group 2). For the organisms in group 2, the MBCs of oxacillin were close to

TABLE 1. *Minimal inhibitory and bactericidal concentrations (MIC, MBC) for the 60 isolates of Staphylococcus aureus*[a]

Isolates	Mean MIC (μg/ml)	MBC (μg/ml)
Group 1 (33)	0.33 ± 0.18[b] (0.1–0.80)[c]	>100[b] (50–>100)[c]
Group 2 (27)	0.30 ± 0.53 (0.1–1.60)[c]	2.2 ± 5.33 (0.20–12.5)[c]

[a] The values are the average of three tests run in duplicate.
[b] Standard deviation.
[c] Range.

the MICs and always at concentrations that were easily achievable in blood by normal therapeutic doses, 2.5 ± 5.33 μg/ml. The MBCs for group 1 (>100 μg/ml), however, were always markedly different from the MICs. The distribution of the MBCs of the organisms in both groups was clearly bimodal, with no overlap between the groups when 25 μg/ml was used as the division point. An MIC of 25 μg/ml has been the level usually used to differentiate methicillin-resistant and -sensitive strains (12).

There were 33 organisms in group 1 and 27 in group 2. Confluent growth was never seen on subcultures from the tubes with concentrations of oxacillin higher than the MICs of the organisms, and the survivors on subculture of the organisms in group 1 represented 0.1 to 10% of the initial inocula. In most strains, however (17 out of 33), survivors were approximately 1% of the initial inocula. Subcultures of the organisms in group 1, recovered from tubes with high concentrations of oxacillin, did not give rise to a homogeneous population of cells resistant to the bactericidal effects of oxacillin. On retesting, the bacteria derived from these subcultures behaved like the parent strains, with only a small percentage of the organisms resistant to killing by oxacillin. The ability of the group 1 strains to resist the oxacillin-induced killing was not influenced by inoculum size. Oxacillin was not bactericidal when 10^3 or 10^6 organisms per ml were used as the initial inoculum.

Ten organisms (six from group 1 and four from group 2, Table 2), were picked randomly from the two groups and the effects of oxacillin, cephalothin, and gentamicin on colony counts over a 48-h period were measured. Figure 1 shows the response of these 10 strains to 25 μg of oxacillin per ml. This concentration of oxacillin was used because it was the dividing point between the MBCs of group 1 and group 2 organisms (Table 1). At 24 h, oxacillin was bactericidal for the organisms in group 2, but only bacteriostatic for the organisms in group 1. The killing curves of the organisms in group 2 were steeper than the ones in group 1, and at every point measured, there was a statistically significant difference between the colony counts of the two groups ($P < 0.01$ by the analysis of variance). This confirmed our previous tube dilution studies on these organisms (Table 1). After 48 h of incubation, oxacillin became bactericidal for the organisms in group 1. It is also important to note that at 48 h of incubation, there was no regrowth of the organisms in group 2. In addition, growth rates of the organisms in group 1 were the same as those in group 2 (data not shown). Similar results were seen with 25 μg of cephalothin per ml (Fig. 2); there was a statistically significant difference between the killing curves and the colony counts of the organisms in groups 1 and 2 ($P < 0.01$, analysis of variance). Lower concentrations of oxacillin and cephalothin were bactericidal at 48 h for some of the organisms in group 1, but neither was effective below 10 μg/ml.

Figure 3 shows the responses of organisms in groups 1 and 2 to 6.25 μg of gentamicin per ml. This concentration of gentamicin was chosen

TABLE 2. *Characteristics of the 10 isolates on which the antibiotic bactericidal effects were analyzed*

Strains	MIC[b] (μg/ml)	MBC[b] (μg/ml)
Group 1		
WE 3395	0.80	>100
WE 3262	0.20	>100
3445	0.20	50
WE 185	0.40	>100
RE 658	0.80	>100
WE 409	0.20	>100
Group 2		
WE 3449	0.20	0.10
WE 3399	0.20	0.20
RE 966	0.40	0.40
RE 1532	0.20	0.20

[a] The values are the results of three tests run in duplicate.

[b] Oxacillin.

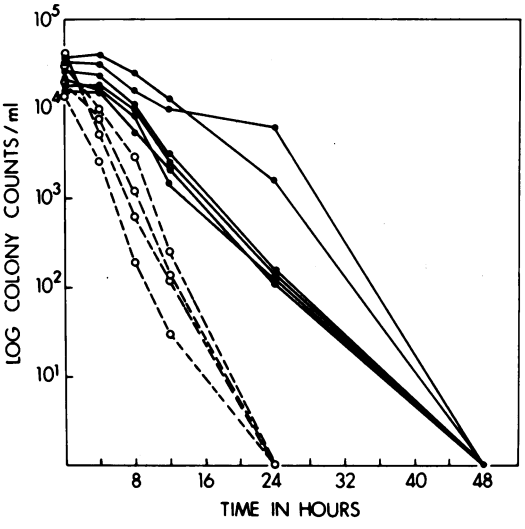

FIG. 1. *Killing curve of ten strains of Staphylococcus aureus incubated with 25 μg of oxacillin per ml. Inoculum was 5 × 10^4 organisms/ml. The curves are the averages of three tests run in duplicate. The base line for the colony counts was 2 colonies per ml. (- - - -) four strains with low MBCs; (——) six strains with high MBCs.*

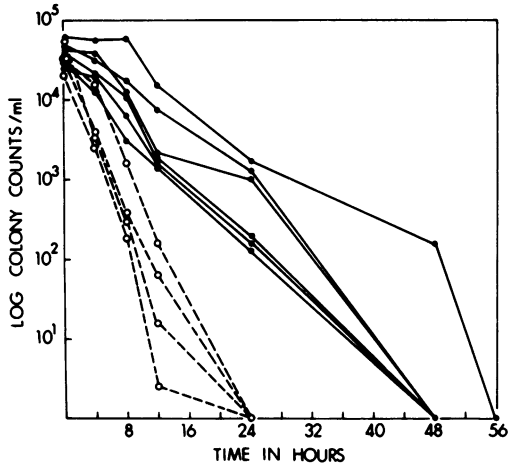

FIG. 2. *Same as Fig. 1. Incubation with 25 μg of cephalothin per ml.*

because it is a high therapeutic level of this drug and therefore comparable to the 25-μg/ml concentration of oxacillin and cephalothin used. Gentamicin killed the organisms in both groups more rapidly than oxacillin and cephalothin. Three of the organisms in group 2 were killed completely by 4 h, and one was killed by 8 h. It required 8 h to kill three of the organisms in group 1 and 12 h to kill the other three. Although there was some overlap in the responses of these organisms to gentamicin, the difference between the responses of the two groups was significant ($P < 0.01$, analysis of variance).

DISCUSSION

The observation that oxacillin was only inhibitory and not bactericidal for the three clinical isolates of *S. aureus* that caused the bacteremias which were unresponsive to oxacillin stimulated us to study the frequency of this susceptibility pattern in *S. aureus* isolated in Barnes Hospital.

All 60 of the strains selected at random from the Clinical Microbiology Laboratory were inhibited by low concentrations of oxacillin. This was not unexpected and was consistent with the susceptibility of the great majority of strains of *S. aureus* tested for susceptibility to methicillin and oxacillin at our hospital. Oxacillin had very high MBCs against 33 of the 60 strains, and these organisms resembled the strains isolated from our three patients. Only a small percentage of the staphylococci in cultures of these organisms resisted the killing effects of oxacillin and subcultures of these "resistant" bacteria behaved like the parent strains, with only a small percentage of cells resisting killing by oxacillin. In this respect, the pattern of resistance was similar to that found in true methicillin-resistant staphylococci (14).

The variability in response to the killing action of oxacillin that we have found confirms the report of a similar phenomenon described by Best et al. (5). The difference in the frequency of occurrence of relatively resistant organisms in their series and ours is probably due to differences in criteria for the MBC of oxacillin. In their study they defined a bactericidal effect of oxacillin as 99% killing, whereas we required 99.9% killing for our definition of a bactericidal effect.

When the time-dependent effects of the antibiotics at high therapeutic concentrations were studied, the organisms also segregated themselves into two distinct populations. It is clear that the responses to these concentrations of drugs were determined by the rates of bacterial killing. All of the organisms from group 2 were killed within 24 h by oxacillin, and the organisms from group 1 required 24 to 48 h of exposure to the drugs for complete killing to occur. Therefore, the organisms in group 1 were not truly oxacillin resistant.

Gentamicin killed all of the organisms more rapidly than oxacillin or cephalothin, but the organisms also segregated into two groups with the same populations in each on the basis of the rapidity of their response to the concentration of gentamicin used. Therefore, the differences in responses of group 1 and group 2 organisms were not specific to cell wall antibiotics. A very rapid killing effect of gentamicin compared

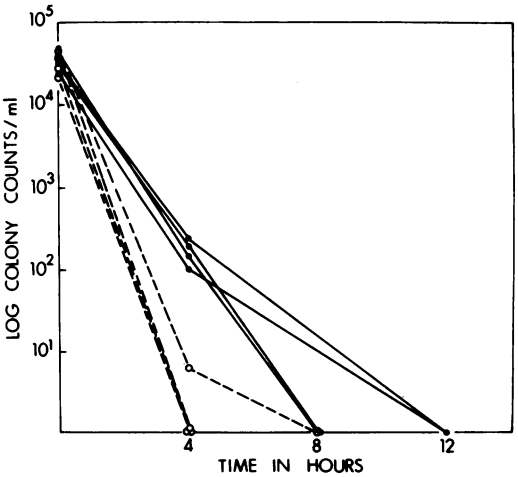

FIG. 3. *Same as Fig. 1. Incubation with 6.75 μg of gentamicin per ml.*

with penicillin has also been noted by others (13, 16), but in these studies there was eventual regrowth of the organisms after prolonged incubation with the drug. In our studies, regrowth was not seen. The difference between our results and others may be due to the lower initial inocula and higher gentamicin concentrations used in our studies.

The mechanism underlying the differences in the responses of the two groups of staphylococci to oxacillin, cephalothin, and gentamicin is unknown. There was no difference in growth rate of the two groups and the presence of L-forms or cell wall-deficient bacteria is unlikely because our media were not selective for L-forms and the response to gentamicin, an inhibitor of protein synthesis, was the same as the responses to the cell wall antibiotics. We did not perform phage typing of the organisms in groups 1 and 2, but antibiotic susceptibility patterns of the organisms within the groups were not uniform and there was no relationship between the antibiotic sensitivity patterns and the MBCs of oxacillin, cephalothin, and gentamicin.

Hobby et al. (11) have described so-called "bacterial persisters" in the presence of penicillin. Bigger (6) and Gunnison et al. (9) also carried out extensive studies on the action of penicillin on strains of *S. aureus* and observed that a small number of organisms survived incubation in bactericidal concentrations of penicillin for 24 or more h. They postulated that the persisting cells survived the action of penicillin because they were in a dormant state unable to initiate division or cell wall synthesis at the time of exposure to penicillin. More recently, Greenwood (8) has offered a hypothesis to explain the phenomenon of microbial persisters that is based on the observation of Hartmann et al. (10). The latter showed that in addition to its inhibitory effects on cell wall synthesis, penicillin also inhibited the action of two mucopeptide hydrolases in the cell wall of *Escherichia coli*. Greenwood has proposed that cells with complete cell envelopes at the time of penicillin exposure would remain intact because the penicillin would inhibit the activity of mucopeptide hydrolases. This hypothesis has received support from the studies of Best et al. (4), who noted a diminished level of cell wall autolysin activity of a tolerant strain of *S. aureus* when compared with a susceptible strain after exposure of both organisms to oxacillin. We cannot be certain, but we suspect, that our group 1 organisms are similar to the so-called bacterial persisters described by those previous workers. Further characterization of our organisms will have to be done to confirm this notion.

Any discussion of the clinical implications of our findings is speculative. Unfortunately, the organisms in our case reports were lost before killing curves could be performed so we do not know if they would have responded to oxacillin in a fashion identical to that of our group 1 organisms. The lack of clinical response of the patients to oxacillin is unusual in our experience, but the underlying disease in each of the patients may be responsible for this. At this point, we have no basis for stating that infections with group 1 organisms are more difficult to treat. Careful analysis of clinical cases of staphylococcal infection, their outcomes, and correlations with the MICs and MBCs of the organisms to the antibiotics used will have to be done before any such conclusions can be drawn.

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