ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1982, p. 618–621
0066-4804/82/040618-04$02.00/0

Vol. 21, No. 4

# pH-Dependent Penicillin Tolerance May Protect Intraleukocytic *Staphylococcus aureus* from Killing by Cloxacillin

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Received 8 October 1981/Accepted 29 December 1981

Exposure of *Staphylococcus aureus* to cloxacillin at neutral pH rendered the bacteria more susceptible to subsequent lysis by lysostaphin. This sensitization effect also occurred at lower pH levels which were nonpermissive for the bactericidal action of cloxacillin, but the effect disappeared at pH 5.0. These findings elucidate previous observations on the protection of intraleukocytic *S. aureus* from killing by cloxacillin and indicate that low pH in the phagolysosomes may be involved.

Protection of viable intraleukocytic *Staphylococcus aureus* from killing by penicillins was previously attributed to an inability of penicillins to penetrate phagocytes (9). However, recent evidence suggests that penicillins do enter leukocytes but fail to kill intracellular staphylococci, possibly because of their very low intracellular growth rate (3; A. Scoging, V. R. Aber, and D. B. Lowrie, Soc. Gen. Microbiol. Q. 7:189, 1980). It was found that staphylococci exposed to cloxacillin while inside bovine polymorphonuclear neutrophil leukocytes (PMN) remained viable but were rendered more sensitive to lysis by lysostaphin after their release from within the phagocytes (3). This increase in sensitivity to muralytic enzymes was indicative of sublethal cell wall damage by penicillins, cell walls from penicillin-treated *S. aureus* being more sensitive to endogenous muralysin activity than normal cell walls (11). Although sublethal damage of intracellular *S. aureus* was seen with relatively low (5 μg/ml) concentrations of cloxacillin, a 40-fold increase in concentration still failed to reduce viability, indicating that the bactericidal effect was prevented, despite a sufficient antibiotic concentration (3).

The lethal action of penicillins is dependent on bacterial autolytic enzyme activity which may be impaired under conditions of low pH; this effect was described as pH-dependent penicillin tolerance (8). Staphylococci surviving within PMN are subjected to conditions within the microenvironment of the phagolysosomes, which include a fall in pH. In a previous study, we measured the pH of the phagocytic vacuole of bovine PMN, using yeast cells stained with pH indicator dyes; a fall in pH occurred in a substantial proportion (up to half) of the phagolysosomes, with falls to pH 5.4 or lower being maintained over 2-h incubation periods (N. Craven, Ph.D. thesis, University of Reading, Reading, U.K., 1981). Similarly, a minimum pH of 5.0 within a minority of phagocytic vacuoles of bovine PMN was reported by other workers (D. M. Reinitz and M. J. Paape, J. Dairy Sci., vol. 62, Abstr. P94, 1979). Thus, in our earlier study (3) many intracellular staphylococci experienced cloxacillin exposure at a low pH and may have been protected from lethal antibiotic action by pH-dependent tolerance. If so, then the observed increase in lysostaphin sensitivity of these intracellular staphylococci may be a general feature of *S. aureus* which results from exposure to cloxacillin at pH levels which inhibit the bactericidal effect. This hypothesis was tested in the present study, using *S. aureus* exposed to cloxacillin in broth media of differing pH values.

## MATERIALS AND METHODS

***S. aureus.*** The staphylococci used were the Oxford strain (NCTC 6571) and strain M60 (a penicillinase producer), which was originally isolated from a case of bovine mastitis. The 24-h minimal inhibitory concentrations of cloxacillin in Mueller-Hinton broth (Oxoid, Basingstoke, U.K. were 0.25 μg/ml for Oxford and 0.5 μg/ml for M60. The Oxford strain was highly sensitive to the bactericidal action of cloxacillin, whereas M60 showed a slower loss of viability and may be regarded as a moderately cloxacillin-tolerant strain (the mean survival after 24-h exposure to cloxacillin concentrations in excess of four times the minimal inhibitory concentration was 0.01% (±0.004 standard error) for Oxford and 0.28% (±0.09) for M60 (N. Craven, Ph.D. thesis).

**pH-buffered media.** Mueller-Hinton broth was diluted with 0.2 M phosphate buffer, pH 6.0 or pH 5.8, or 0.2 M citrate buffer, pH 5.0, using 1 part buffer

solution to 3 parts broth. Mueller-Hinton broth was similarly diluted with isotonic saline to produce an unbuffered diluted control (pH 7.6). The pH levels were rechecked after autoclaving.

**Preparation of cloxacillin-treated *S. aureus*.** *S. aureus* (ca. $10^7$ colony-forming units) was added to 10 ml of buffered broth and incubated for 45 min at 37°C to allow bacterial division to commence. A 4-ml portion of this suspension was then added to 4 ml of fresh buffered broth or buffered broth containing 5 μg of cloxacillin per ml. After a further 5 h of incubation, saline (0.25 ml) containing 25 IU of broad-spectrum *Bacillus cereus* β-lactamase (Whatman Biochemicals, Maidstone, U.K.) was added to each sample and incubated for a further 5 min to inactivate the cloxacillin. Tests for sensitivity to killing by lysostaphin were then made with samples of these broth cultures of *S. aureus*. The growth of staphylococci in the antibiotic-free broth and loss of viability in the presence of cloxacillin were monitored in a duplicate series of samples, by plating out suitable dilutions onto blood agar plates which had previously been treated with 3 IU of β-lactamase.

**Test of susceptibility to lysostaphin.** Duplicate series of 10-fold dilutions of *S. aureus* suspension were made in either phosphate-buffered saline at pH 6.8 or phosphate-buffered saline containing 0.15 μg of lysostaphin per ml (Sigma Chemical Co., St. Louis, Mo.). This concentration of lysostaphin at pH 6.8 had previously been found to produce maximum discrimination between normal and cloxacillin-treated *S. aureus* through preferential lysis of the latter; lower concentrations were ineffective, whereas higher levels caused extensive lysis of both normal and cloxacillin-treated cocci. After standing for 30 min at 25°C, four 20-μl samples of each dilution were dropped onto ox blood agar plates. After overnight incubation at 37°C, the number of colonies that grew from each dilution in each series was counted. The susceptibility to killing by dilute lysostaphin was estimated by comparing counts obtained from dilutions in phosphate-buffered saline only with counts from similar dilutions in phosphate-buffered saline with lysostaphin, and the percentage of survival was calculated.

## RESULTS AND DISCUSSION

Growth of both strains of staphylococci and killing by cloxacillin (Fig. 1) were most rapid at pH 7.6, whereas at pH 6.0, although growth was slightly reduced, the reduction in the bactericidal effect of cloxacillin was more marked, especially with the Oxford strain (i.e., pH-dependent tolerance occurred). This confirmed the findings of Goodell et al. (5). At pH 5.0, however, there was no growth or killing of *S. aureus*. Horne and Tomasz have noted that the increase in acidity

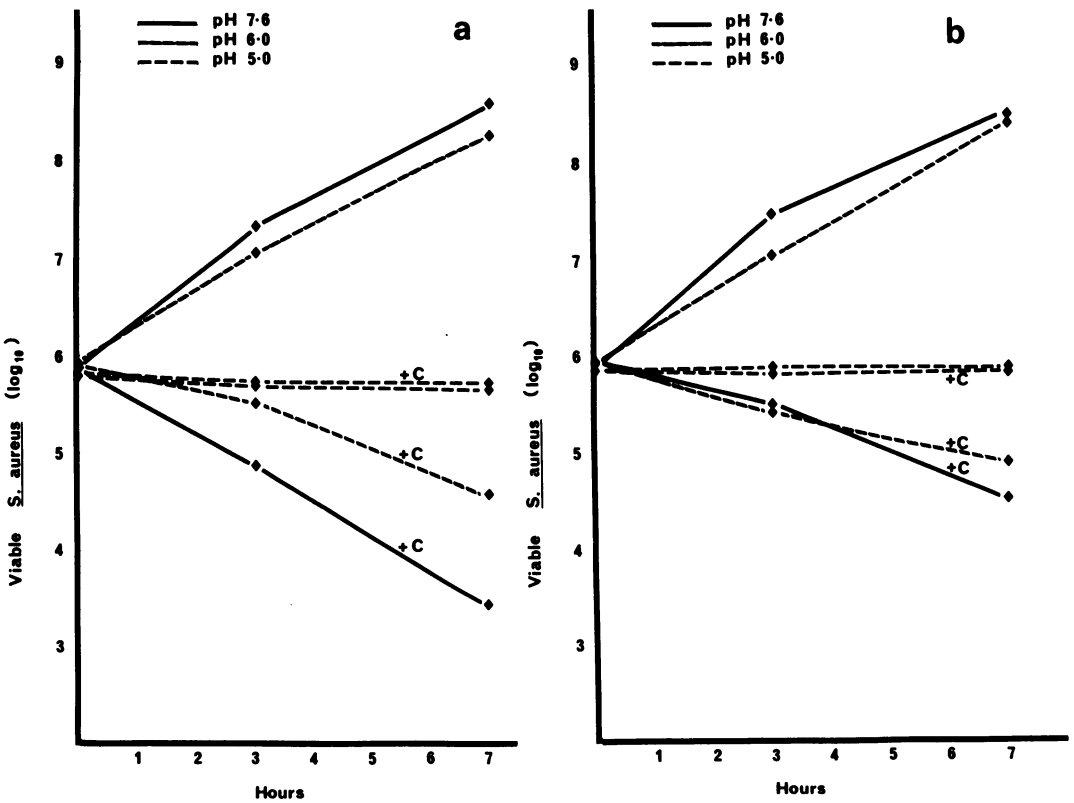


FIG. 1. Growth of *S. aureus* Oxford (a) and M60 (b) and killing by cloxacillin in broth buffered at different pH levels. +C = Samples containing 5 μg of cloxacillin per ml. Each point is the mean of two or more observations.

TABLE 1. Sensitivity to lysostaphin of *S. aureus* grown in broth at different pH levels

| *S. aureus* | % Survival[a] of *S. aureus* diluted in lysostaphin (0.15 μg/ml) at pH 6.8, after incubation in broth at pH: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Oxford | | | | M60 | | | |
| | 7.6 | 6.0 | 5.8 | 5.0 | 7.6 | 6.0 | 5.8 | 5.0 |
| Untreated control grown in medium for 5 h | 83 | 71 | 84 | 87 | 114 | 65 | 110 | 104 |
| Incubated with 2.5 μg of cloxacillin per ml for 5 h[b] | 0 | 1 | 18 | 97 | 2 | 13 | 71 | 77 |

[a] Values represent: (viable count in 0.15 μg of lysostaphin per ml, pH 6.8/viable count in phosphate-buffered saline, pH 6.8) × 100.

[b] Cloxacillin inactivated at time of testing.

of unbuffered nutrient medium, which resulted from growth of group B streptococci, was sufficient to protect these bacteria from the lethal action of added penicillin (7). In the present study there was no shift in pH in the broth media which contained cloxacillin, probably because the antibiotic was present throughout incubation and thus prevented significant bacterial growth. An increase in sensitivity of *S. aureus* to lysostaphin occurred after exposure to cloxacillin at pH 7.6 and 6.0. This effect was diminished after growth at pH 5.8 and was virtually absent at pH 5.0 (Table 1). Thus, between pH 6.0 and 5.0 *S. aureus* may show tolerance to the bactericidal action of cloxacillin but may still sustain cell wall damage, as shown by their greater susceptibility to muralytic enzymes after their return to a more neutral pH. At pH 5.0 no cell wall changes were detected, but this pH was itself bacteriostatic.

The slower bactericidal action of cloxacillin for M60 at pH 7.6 may indicate the relative tolerance of this strain when compared with the very sensitive Oxford strain. This inherent tolerance of some isolates of *S. aureus* has been extensively reported, and its clinical significance is uncertain. Our own work with strains of *S. aureus* from bovine mastitis suggests that there is a spectrum of response to the bactericidal effects of cloxacillin rather than a clear division between sensitive and tolerant strains, with no obvious correlation between tolerance in vitro and the response to therapy in vivo (N. Craven and J. C. Anderson, manuscript in preparation). One factor may be that strains which appear sensitive in vitro may demonstrate tolerance in vivo due to the conditions which prevail in infected tissues. In chronic mastitis, many viable staphylococci are found within phagocytic cells, and this location may be important in the frequent failure of therapy with cloxacillin (2, 3). The estimated pH within many phagocytic vacuoles of bovine PMN is within the pH range over which *S. aureus* showed tolerance to cloxacillin in vitro. Furthermore, although cloxacillin largely failed to kill staphylococci in this pH range in vitro, they were, nevertheless, sensitized by the antibiotic to subsequent killing by lysostaphin, as were intracellular staphylococci after exposure to cloxacillin (3) or other β-lactam antibiotics (unpublished data). This implies that the protection of intracellular *S. aureus* may be due, at least in part, to the low pH within phagolysosomes which imposes a tolerant response to penicillins.

We have previously shown that pretreatment with cloxacillin and other penicillins renders *S. aureus* (sensitive or tolerant) more susceptible to killing by bovine PMN, possibly through cell wall effects similar to those which influence lysostaphin sensitivity (4; N. Craven, M. R. Williams, and J. C. Anderson, Comp. Immunol. Microbiol. Infect. Dis., in press). This enhanced killing of penicillin-treated *S. aureus* may be mediated by neutrophil factors which operate at a neutral pH, since it has been observed in bacteria adhering to the surface of PMN in the absence of phagosome formation (10). After phagosome formation, internalized bacteria are subjected first to substances which are active optimally at neutral pH and then, as the pH progressively falls, to the acid hydrolases (1). This fall in pH may thus "switch off" the enhanced killing by the former lysostaphin-like systems, enabling some bacteria to persist. Such bacteria would also be protected by the acidity from the lethal consequences of further exposure to penicillins but may still be sensitized to cell wall lytic enzymes. It may therefore be argued that, after exposure of intracellular *S. aureus* to cloxacillin, the bacteria, when released (and thus returned to neutral pH levels), should show an increased susceptibility to killing by fresh bovine PMN. However, we were unable to demonstrate this, possibly because of experimental limitations (4). Recent observations on enhanced susceptibility of penicillin-pretreated streptococci to killing by human PMN (6) indicated that this sensitization still occurred after growth of the streptococci with penicillin at pH 5.5 (conditions which offer protection against the lethal effects of the drug). This implies that bacteria exposed to penicillins while protected inside phagocytes by a low

intravacuolar pH may in fact become more susceptible to subsequent encounters with PMN. It is perhaps worth noting that staphylococci within those phagolysosomes of bovine PMN, which show maximum pH depression to pH 5.0, would be completely protected by this pH from both bactericidal and sensitization effects of cloxacillin.


## ACKNOWLEDGMENT

N.C. acknowledges financial support from Beecham Pharmaceuticals Research Division.


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