Oxacillin-Induced Lysis of *Staphylococcus aureus*

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Received for publication 18 May 1979

Six clinical isolates of *Staphylococcus aureus* were compared for their relative susceptibilities to the killing effects of oxacillin. Three of the strains had minimum bactericidal concentrations which were >10 times the minimum bacteriostatic concentration for this antibiotic and were designated tolerant (Tol⁺). The other strains had minimum bactericidal concentrations which were comparable to the minimum bacteriostatic concentration (Tol⁻). Lysis curves of these strains revealed that the Tol⁺ strains exhibited a diminished rate of lysis when inhibited by oxacillin. This reduced rate of lysis was reflected also in a reduced rate of viability loss when the cells were exposed to oxacillin. During log growth the uptake of [¹⁴C]glycerol by Tol⁺ cells was 1.5-fold greater than that by Tol⁻ cells. Glycerol-labeled cells of each phenotype secreted radioactivity when inhibited by oxacillin. However, the Tol⁺ strains released over twice as much label as the Tol⁻ strains. No difference in the proportion of lipid secreted by the two phenotypes was found. The behavior of 60 to 65% of the labeled material released by inhibited cells during both sodium dodecyl sulfate gel electrophoresis and Sepharose 6B chromatography corresponded to that of lipoteichoic acid. When the major component of secreted material was added to oxacillin-inhibited Tol⁻ strains, an inhibition of the lytic response was observed. These results suggest that oxacillin tolerance in *S. aureus* could be related to the enhanced secretion of an autolysin inhibitor, such as lipoteichoic acid.

Since the original reports from this laboratory (2, 3), others have described clinical isolates of *Staphylococcus aureus* which are tolerant to β-lactam and other cell wall antibiotics (5, 11, 12). These strains (Tol⁺) have a low minimum inhibitory concentration (MIC) for these antibiotics, but the minimum bactericidal concentration (MBC) is from 10- to over 100-fold greater than the MIC.

Since routine disk susceptibility tests on agar do not reveal Tol⁺ strains, subcultures of tubes in an antibiotic dilution series are necessary to establish which isolates resist the killing effects of drug levels approximating the MIC. This technique suffers from the necessity of having to wait at least 48 h to make this determination. In addition, there is no commonly accepted relationship of MIC to MBC to define tolerance. Sabath et al. (12) used an MBC which was 32 times the MIC as their criterion for tolerance; and Bradley et al. (5) defined tolerance as the presence of ≥100 colony-forming units (CFU) per ml after a 24-h incubation of 10⁵ CFU/ml in 12.5 μg of antibiotic per ml.

Since a given strain could be designated Tol⁺ or Tol⁻ depending on the definition given the trait, we have attempted to develop a rapid means to identify Tol⁻ strains. Our efforts were initially directed toward an observation made in our earlier report (2) that actively growing cells of *S. aureus* Tol⁺ strains do not lyse as readily as nontolerant (Tol⁻) strains when inhibited by a cell wall antibiotic. In addition, we previously reported that autolytic enzyme activity associated with cell walls of Tol⁺ strains decreased relative to that in Tol⁻ strains after inhibition by drugs such as oxacillin (2).

In this report, using different Tol⁺ and Tol⁻ strains, we present evidence that the lysis response of growing cells can rapidly indicate antibiotic tolerance in fresh clinical isolates. In addition, we show that Tol⁺ strains excrete larger quantities of a lysis inhibitor than do Tol⁻ strains. Finally, we demonstrate that the inhibitor is probably lipoteichoic acid (LTA) and that this substance can delay the onset of lysis by Tol⁻ cells inhibited with oxacillin.

**MATERIALS AND METHODS**

**Bacterial strains.** The *S. aureus* strains used in this study were fresh clinical isolates from blood cultures. Routine laboratory procedures showed that each isolate produced coagulate and fermented mannitol. All cultures were maintained on tryptic soy agar (TSA) slants (Difco Laboratories, Detroit, Mich.) at 4°C for 2 to 4 weeks and then replaced by lyophilized stock cultures of the original isolate.
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For experiments requiring radioactively labeled cells, the isolates were inoculated into tryptic soy broth (TSB) containing 0.5 μCi of [1,3-14C]glycerol (New England Nuclear Corp.) per ml and grown to the log phase (six to eight generations).

Antibiotic susceptibility tests. Antibiotic tolerance was assessed initially by adding 3 x 10^6 CFU of log-phase cells to 0.5 ml of TSB to each of a series of tubes containing twofold dilutions of oxacillin (Bristol Laboratories, Syracuse, N.Y.) in TSB. After 24 h at 37°C, the tubes were inspected visually to determine the lowest concentration of oxacillin precluding growth (MIC). A 0.1-ml amount from each clear tube was spread on TSA plates and incubated for 24 h. Those antibiotic levels which reduced the original population 99.9% were considered bactericidal. Strains of S. aureus with an MBC >10-fold the MIC were considered to be tolerant.

Lysis of growing S. aureus strains. The lytic response of log-phase cultures to oxacillin was determined spectrophotometrically. Actively growing cells (2 x 10^8 CFU/ml, final concentration) were added to TSB containing 5 μg of oxacillin per ml and incubated with shaking at 37°C. Spectrophotometric readings (540 nm) were made periodically over a 6-h incubation period. In some experiments, samples of cells were removed, diluted appropriately with sterile physiological saline, and plated with TSA to correlate viability with optical density.

Release of glycerol-labeled material by oxacillin-inhibited cells. Cells of S. aureus which had grown for six to eight generations in [14C]glycerol were pelleted by centrifugation, washed three times in phosphate-buffered saline, and resuspended in isotope-free TSB to a suspension density of 2 x 10^9 CFU/ml (absorbance at 540 nm, 0.40). Oxacillin was added to a final concentration of 5 μg/ml, and the cells were incubated with shaking at 37°C. Samples (1 ml) were removed from the flasks at 0, 30, 60, and 90 min and centrifuged at 10,000 g for 5 min. All radioactivity measurements were made by using a toluene-based scintillation fluid containing 30% Triton X-100, 0.5% 2,5-diphenyloxazole, and 0.013% POPOP [1,4-bis(5-phenyloxazolyl)benzene] and a model LS-230 liquid scintillation spectrometer (Beckman Instruments).

Characterization of glycerol-labeled cells and secreted radioactivity. Cells which had been labeled with [14C]glycerol as described above were prepared to an absorbance of 0.4 (540 nm) and grown in a medium containing 5 μg of oxacillin per ml for 75 min. At this time the cells were pelleted by centrifugation, and the resulting supernatant solution was dialyzed for 24 h against three changes of distilled water at 4°C. The dialysate was concentrated to a volume of about 10 ml by rotary evaporation at 37°C and extracted with an equal volume of 80% phenol at 4°C for 1 h.

The aqueous layer was removed, and the phenol layer was washed with an equal volume of distilled water. The combined aqueous layers were dialyzed for 24 h against distilled water to remove the phenol and then concentrated to a volume of about 2 ml by rotary evaporation. The sample was applied quantitatively to a column (2.5 by 50 cm) of Sepharose 6B (Pharmacia Fine Chemicals). The column was equilibrated and eluted with 0.2 M ammonium acetate containing 0.01% sodium azide (pH 6.9). Fractions (3 ml) were collected at a rate of 15 ml/h and analyzed for radioactivity, phosphate (6), and nucleic acids (absorption at 260 nm). This procedure has previously been employed by others to isolate and characterize LTA from other bacteria (1, 8).

In other studies, smaller quantities of cells were labeled by inoculation into 2.5 ml of TSB containing 2 μCi of [1,3-14C]glycerol per ml. After growth to the end of log phase, the cells were pelleted by centrifugation and washed twice in isotope-free media. Cultures (10 ml) were prepared to an absorbance of 0.4 (540 nm). After inhibition with 5 μg of oxacillin per ml for 70 min, the cells were separated by centrifugation.

Cell pellets were digested by incubation in 2 ml of lysostaphin (1 U/ml in phosphate-buffered saline; Schwarz/Mann, Orangeburg, N.Y.) for 30 min at 37°C. The relative amounts of lipid in glycerol-labeled material were estimated from the proportions of radioactivity that partitioned into the chloroform layer after extraction of the cell pellets and supernatant fractions by the method of Bligh and Dyer (4). Samples of the aqueous layers were counted directly in the Triton X-100-based scintillation cocktail, whereas samples of the chloroform layers were evaporated to dryness under N2 before counting. Samples of the supernatant solutions were taken at different stages during the isolation of the major component of the glycerol-labeled material and were subjected to sodium dodecyl sulfate (SDS) electrophoresis by the method of Weber and Osborne (15). The gels were sliced at 2-mm intervals, and the slices were extracted with 0.3 ml of 1% SDS overnight at room temperature before counting with Triton X-100-based scintillation cocktail. Recoveries of the radioactivity applied to the gels ranged from 65 to 70%.

Deacylation of LTA. Material from the Sepharose 6B column which eluted in fractions 20 to 30 (see Fig. 4) was deacylated in 10 volumes of methanolic KOH (0.2 M) at 37°C for 15 min. The treated material was then recovered after passage through a Dowex 50 column (7) by dialyzing 24 h against several changes of distilled water and concentrating by rotary evaporation.

RESULTS

Each of the strains used in this study was obtained from a recent survey of 65 S. aureus strains isolated from blood cultures. Of these strains, 38% were tolerant (MBC ≥ 10×MIC) to oxacillin, and none was oxacillin resistant. From this survey the six strains listed in Table 1 were selected at random for further study. As indicated, the Tol+ strains had MBCs which were 16- to 65-fold greater than the MIC. The Tol− strains had MBCs for oxacillin which were no more than twice the MICs.

Oxacillin-induced lysis of the six strains was measured by using log-phase cells growing in TSB containing 5 μg of oxacillin per ml. As Fig. 1 shows, the Tol+ isolates were readily distinguished from Tol− isolates by the rates at which the cells lysed when grown in oxacillin. Both the
invariably lyse to an absorbance below that of the initial inoculum in 4 to 5 h and that Tol+ strains do not.

Figure 2 shows that the diminished lysis by Tol+ strains growing in oxacillin was accompanied by an enhanced survival of the tolerant populations under these conditions.

In an effort to determine whether the Tol+ cells were resisting the lytic and killing effects of oxacillin through the release of an inhibitor of autolytic enzymes, we grew the six strains in TSB containing radioactive glycerol and inoculated the labeled cells into TSB with and without 5 μg of oxacillin per ml. Samples were removed at suitable intervals and centrifuged, and the radioactivity in the supernatant solution was determined. The results (Fig. 3) show that neither Tol+ nor Tol− strains growing in the absence of oxacillin released appreciable amounts of radioactivity. However, the inhibited strains differed significantly in the amount of radioactivity released. As Fig. 3 shows, the Tol+ strains excreted about twice the amount of label as the Tol− strains did after 30 min.

In an attempt to account for the difference in onset of lysis and the initial rates of turbidity loss were slower with Tol+ strains. In numerous experiments with these and other strains, we have observed that actively growing Tol− strains
After a 75-min incubation, the cells were removed by centrifugation, the supernatant solutions were extracted with phenol as described above, and the extracted material was chromatographed on Sepharose 6B. The elution profile of the radioactivity recovered after phenol extraction is shown in Fig. 4. The main peak of radioactivity (fractions 20 to 30; referred to as peak 1) was observed in the position of LTA, as found by others (1, 8), and failed to absorb ultraviolet light at 280 nm. This was followed by a smaller peak (fractions 35 to 45; referred to as peak 2), which has been previously reported to be deacetylated LTA (1, 8). We also observed that treatment of peak 1 material with methanolic KOH (7) quantitatively converted it to a form which eluted exclusively in the position of peak 2.

When the migration of glycerol-labeled material secreted by oxacillin-inhibited cells was determined by SDS electrophoresis, no differences between Tol+ and Tol- strains were observed with respect to either the distances migrated or the relative proportions of the bands of radioactivity (Table 4). When samples of the growth media were run directly, two bands of label were observed. The minor bands were found to contain up to 30% of the label applied to the gel. If the media were dialyzed against water before electrophoresis, the amount of label in the minor bands was reduced to 10 to 15% of the amount applied. Extraction of the dialyzed

### Table 2. Incorporation of [14C]glycerol by Tol+ and Tol- strains of S. aureus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amt of radioactivity incorporated into:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole cells (cpm/mg of dry wt, \times 10^6)</td>
</tr>
<tr>
<td>Bogdan (Tol+)</td>
<td>496 ± 20</td>
</tr>
<tr>
<td>L ance (Tol-)</td>
<td>323 ± 27</td>
</tr>
<tr>
<td>Tol+/Tol-</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*a Each strain was inoculated into 2.5 ml of TSB containing 2.0 μCi of [1,3-14C]glycerol and incubated at 37°C with shaking. At the end of log growth (absorbance at 540 nm, 1.00), the cells were removed by centrifugation, washed with phosphate-buffered saline, and resuspended in phosphate-buffered saline containing lysostaphin (0.5 U/ml). After 30 min at 37°C, the radioactivity was measured as described in the text. The values given are the means ± standard errors of determinations from separate experiments.

8 Samples of the lysates described above were extracted with chloroform (4), and the proportion of lipid in each strain was determined from measurements of the extractable radioactivity. The values given are the means ± standard errors of determinations from separate experiments.
medium with chloroform–methanol eliminated the minor band. The migration of the major bands increased from an $R_f$ of 0.49 to an $R_f$ of 0.66 after chromatography on Sepharose. Both Sepharose 6B peak 2 and deacylated peak 1 migrated with the tracking dye.

When a sample of peak 1 containing over 6.8 $\times 10^6$ cpm was extracted with chloroform–methanol by the method of Bligh and Dyer (4), less than 0.2% of the radioactivity was chloroform soluble (data not shown). Based on these results, we tentatively conclude that peak 1 material contains LTA.

It was of interest to determine whether the material in peak 1 affected the lytic response of oxacillin-inhibited cells of *S. aureus*. This was done by adding peak 1 material to Tol$^-$ cells inhibited by oxacillin (Fig. 5). As indicated, the material in peak 1 (at 0.75 nmol/ml) had no effect on uninhibited cells. However, when it was added to the culture at the same time as oxacillin or within 60 min of this time, there was a demonstrable effect on cellular lysis. It is also apparent from Fig. 5 that the effect was primarily one in which the onset of lysis was delayed. After 3 to 4 h of growth in the oxacillin, the rates of turbidity loss by the cultures were the same. Neither peak 2 material nor peak 1 material treated with methanolic KOH affected the lytic response to oxacillin (data not shown).

### DISCUSSION

Although the clinical significance of antibiotic tolerance in *S. aureus* is yet to be established, there are reports of infections by such organisms which fail to respond to cell wall antibiotics alone (11). Routine disk susceptibility tests do not permit the detection of tolerant strains which apparently do not have any other distinguishing phenotypic property (5). Our finding that Tol$^+$ cells lyse less readily than Tol$^-$ cells when grown in the presence of oxacillin provides a rapid and convenient means of detecting tolerance. However, additional isolates should be examined, and a routine procedure should be developed to evaluate the usefulness of lysis curves for detecting tolerance. Since organisms such as *Streptococcus sanguis* are well known to be killed by the $\beta$-lactams with minimal lysis (10) and since viability determinations are much more sensitive than absorbance measurements, it might be expected that lysis curves will produce some false positives when used to screen for tolerance. We have not encountered Tol$^-$ *S. aureus* strains which were rapidly killed but not rapidly lysed, however.
In our earlier studies on the physiological basis of tolerance in S. aureus (2), we found that the specific activity of autolysins extracted from cells subsequent to oxacillin inhibition of cell wall biosynthesis was much higher in a Tol− strain than in a Tol+ strain. At that time we noted that tolerance might be explained in part by differences in the cell wall composition or structure such that the autolysins might not associate as effectively with the damaged murein of the Tol+ strain as with that of the Tol− strain. We also found that the specific autolytic enzyme activity of extracts obtained from Tol+ cultures grown in the presence of oxacillin was dependent on the density of the cultures. High-density cultures yielded extracts of lower specific activity than low-density cultures did. This difference prompted us to explore the alternate possibility that Tol+ strains resist the lytic and killing effects of cell wall inhibitors via an autolysin inhibitor. The previous demonstrations of inhibition of lytic activity by LTA’s in pneumococci (9) and streptococci (7) suggested that LTA might be involved in staphylococcal tolerance.

Membrane teichoic acids in those gram-positive organisms which have been studied are covalently linked to a glycolipid moiety in the plasma membrane and are always polymers of glycerol phosphate (16). Waks and Tomasz (14) demonstrated the release of material similar to LTA into the medium of penicillin-treated pneumococci and showed that this macromolecule inhibited autolysin activity and protected penicillin-inhibited cells from lysis. Cleveland et al. (7) have shown that LTA also inhibits autolysin activity in Streptococcus faecalis. Our results

### Table 4. Electrophoretic migration of secreted radioactivity on SDS-acrylamide gels

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sample Description</th>
<th>No. of bands</th>
<th>Major Rf</th>
<th>Minor Rf</th>
<th>Major Rf/Minor Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium, Tol+ oxacillin+</td>
<td>2</td>
<td>0.49</td>
<td>0.74</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>Medium, Tol+ oxacillin−</td>
<td>2</td>
<td>0.49</td>
<td>0.75</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>Dialyzed medium, Tol+ oxacillin+</td>
<td>2</td>
<td>0.48</td>
<td>0.75</td>
<td>5.4</td>
</tr>
<tr>
<td>4</td>
<td>Dialyzed medium, Tol− oxacillin−</td>
<td>2</td>
<td>0.49</td>
<td>0.76</td>
<td>5.6</td>
</tr>
<tr>
<td>5</td>
<td>Sample 3 after CHCl3-methanol extrac</td>
<td>1</td>
<td>0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Peak 1, Sepharose 6B column+</td>
<td>1</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Peak 2, Sepharose 6B column+</td>
<td>1</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Sample 6, deacylated with NaOH+</td>
<td>1</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

* Samples containing from 5 x 10^6 to 9 x 10^6 cpm in 100 μl were electrophoresed on SDS-10% acrylamide gels at pH 7.4 by the method of Weber and Osborne (15). The Rf values indicated represent the migration of radioactivity relative to the tracking dye (bromophenol blue) and represent the average of values from two to four preparations.

* Represents the relative ratio of radioactivity in the major and minor bands.

* Medium after incubation for 90 min in TSB containing 5 μg of oxacillin per ml.

* After incubation for 90 min in TSB containing 5 μg of oxacillin per ml, the media were dialyzed for 24 h against several changes of distilled water (4°C).

* The radioactivities of peaks 1 and 2 (Fig. 4) were pooled, dialyzed, concentrated, and applied to the gels.

* Sample 6 was deacylated with 0.2 N NaOH as described in the text.

Fig. 5. Effect of secreted material on oxacillin-induced lysis of a Tol− S. aureus strain. The radioactive material eluting fractions 20 to 30 (see Fig. 4) was pooled, dialyzed, and concentrated by rotary evaporation. The concentration of this material was determined by phosphate measurements as described by Cleveland et al. (7). Its effect on a Tol− strain growing in TSB containing 5 μg of oxacillin per ml was determined by adding 0.75 nmol/ml (final concentration) at zero time (□), 30 min (△), and 60 min (○). Also shown are lysis controls (●) to which no secreted material was added and growth controls (●) to which neither oxacillin nor secreted material was added. The secreted material had no effect on culture turbidity in the absence of oxacillin.
indicate that upon oxacillin inhibition, *S. aureus* also secretes a glycerol polymer whose behavior on both SDS-acrylamide electrophoresis and Sepharose 6B chromatography corresponds to that of LTA (8). The inhibition of cell lysis by this material and its loss of such activity upon treatment with methanolic KOH suggest that the material is LTA.

Any firm conclusion regarding the secretion of LTA and its relation to antibiotic tolerance must await further studies. LTA has been proposed to function in normal, growing cells as a regulator for murein hydrolases (9). Its location at the cell surface (16) and its ability to inhibit autolysin activity (7) make LTA an ideal candidate for this role. Tol+ strains of *S. aureus* could be regulatory mutants which produce more LTA than other (Tol−) strains since they are labeled more extensively by glycerol (Tables 2 and 3). However, Tol+ organisms also release a greater proportion of the water-soluble material labeled during growth in [14C]glycerol, and this could reflect a difference in the physiological responses to oxacillin inhibition by the two phenotypes, which is not understood.

If LTA normally functions as a regulator of autolysin activity, its enhanced release after oxacillin inhibition of Tol+ strains compared with its release by Tol− strains could appear paradoxical. One might expect that the strains which resist lysis upon exposure to oxacillin (Tol+) would retain LTA rather than secrete it. Resolution of this problem may depend on studies which can distinguish between LTA secreted from the wall and LTA released from the cytoplasmic membrane. If, for example, oxacillin inhibition of Tol+ strains results in the loss of more wall LTA and less membrane LTA than in Tol− strains, the differential effect on lysis and cell killing could be explained by damage to the cytoplasmic membrane of Tol− strains.

A primary obstacle facing critical structure-function experiments is the necessity to use pure LTA. The recently reported purification of streptococcal LTA by using phosphatidyl choline vesicles (13) may eliminate this problem and permit further studies into the relationship of LTA to tolerance.

ACKNOWLEDGMENTS

We are grateful to Norma H. Best and Frances Garner for excellent technical assistance with various phases of this investigation. We also appreciate the assistance and cooperation of Milo D. Hilty in acquiring the clinical isolates used in this study.

This work was supported by a grant from the Georgia Heart Association, by Public Health Service grant AI-14656-01 from the National Institutes of Health, and by funds from the National Institutes of Health (FR-S365).

LITERATURE CITED