Production of Polysaccharide by *Staphylococcus aureus*. IV. Correlation of Lytic Response with Penicillin Activity. (29681)

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A previous report(1) from this laboratory has shown that strains of *Staphylococcus aureus* grown in the presence of subinhibitory concentrations of benzylpenicillin or nafcillin produced a marked accumulation of polysaccharide component within the staphylococcal cell. It was also found that treatment with nafcillin rendered the normally lysozyme-resistant *S. aureus* susceptible to partial lysis by lysozyme, and that concomitant addition of trypsin resulted in further dissolution of the cell bodies(2). Although the precise cytologic action of subinhibitory levels of penicillin on the integrity of the cell wall of *S. aureus* is unknown, evidence supported the hypothesis that relatively low concentrations interfered with normal utilization of the polysaccharide by preventing its incorporation into the bacterial cell wall(1,2). We also suggested that the accumulation of polysaccharide within the cell might be closely correlated with ability of lysozyme and trypsin to penetrate the cell wall. More recent observations made with subinhibitory concentrations of semisynthetic penicillins nafcillin, oxacillin, and cloxacillin(3) have suggested quantitative differences in their ability to disorganize the cell wall structure of *S. aureus*.

The experimental design reported here was predicated on an initial assumption that changes in the integrity of the cell wall induced by a particular penicillin may fairly accurately reflect the antibacterial activity of that penicillin. Thus it is conceivable that impairment of the cell wall of *S. aureus* by subinhibitory levels of a penicillin may be correlated with the lytic behavior following treatment with lysozyme and trypsin. If this concept is substantiated, a model system for evaluation of the antibiotic activity of the penicillins could be developed in which pretreatment followed by lytic response would be utilized as a determinative method. As far as ascertained, no studies have been reported that quantitate penicillin activity as a function of the ability to influence enzymic lysis of cells.

We attempted in the present study (a) to determine the possible effects of subinhibitory concentrations of nafcillin, oxacillin, and cloxacillin on the enzymic lysis of a number of penicillin-sensitive and penicillin-resistant isolates of *S. aureus* and (b) to correlate differences in susceptibility to lysis and sensitivity to penicillins. Since lysozyme is present in secretions and certain cells of man and animals, the study may contribute toward a better understanding of the differences observed between the therapeutic action of nafcillin and the isoxazole penicillins in experimental infections in mice(4).

Materials and methods. Clinical isolates of *S. aureus* were obtained from several sources. Minimal inhibitory concentrations (MIC) were determined in brain heart infusion (BHI) by the tube serial dilution technic of Knox(5). Benzylpenicillin, nafcillin, oxacillin, and cloxacillin were procured from commercial sources. Stock solutions (1 mg/ml) were prepared in distilled water, sterilized by passage through Millipore HA filters, and stored at —20°C. Suitable concentrations of the penicillins were prepared in BHI, and 10 ml quantities were added to 250 ml Erlenmeyer flasks containing 90 ml of BHI. The flasks were seeded with 5 ml of 10^{-2} dilution of an 18-hr culture and were shaken at 85 rev/min on a rotary shaker at 37°C. Turbidities were measured at a 1:10 dilution in standardized 18 × 150 mm cuvettes in a Bausch and Lomb spectrophotometer (Spectronic 20) at 580 mu. Cells were harvested by centrifugation, washed twice with distilled water, suspended in 0.1 M phosphate buffer (pH 7.0), and standardized at 20% transmission. The lytic action of crystalline egg white lysozyme (Armour Laboratories) and

crystalline trypsin (Worthington Laboratories) was studied with the standardized suspensions of bacteria grown in the presence or absence of subinhibitory levels of the penicillins. Appropriate controls were included. For the turbidimetric study of lysis, 0.25 ml portions of buffered solutions of lysozyme and/or trypsin at concentrations of 800 and 400 μg/ml, respectively, were added to 10 ml aliquots of the suspensions in standardized cuvettes. The contents were well mixed, incubated for 3 hours in a 37°C water bath, and read spectrophotometrically. The percentage of lysis was calculated as described by Kohn(6):

$$\text{Percent lysis} = \frac{\text{OD (time 0)} - \text{OD (time X)}}{\text{OD (time 0)}} \times 100$$

OD = optical density, 0 = zero time,
X = end of incubation

Results. Penicillin sensitivity. Fig. 1 illustrates the percentage distribution of MIC values of nafcillin, oxacillin, and cloxacillin required for 14 clinical isolates of penicillin-resistant *S. aureus*. The resistance of the coagulase- and penicillinase-positive cultures to benzylpenicillin varied from 1.95 to > 250 μg/ml. It can be noted that the majority of the cultures were inhibited by the same quantities of each agent. Most strains were sensitive to 0.488 to 0.976 μg/ml of the semisynthetic penicillins.

Effect of penicillins on enzymic lysis. Since nafcillin, oxacillin, and cloxacillin demonstrated essentially identical inhibitory activity against the various isolates, it was of interest to examine the percentage lysis following concomitant action of lysozyme (20 μg/ml) and trypsin (10 μg/ml) on cells grown in subinhibitory concentrations of the penicillins. The distribution of the percentages is shown in Fig. 2 and 3. The cultures showed variation in susceptibility, some strains being somewhat resistant to lysis and others being markedly susceptible. Treatment with nafcillin resulted in significant lysis of cells upon the addition of the enzymes; the average decrease in turbidity was 74.4%. By contrast, treatment with identical concentrations of oxacillin and cloxacillin resulted in relatively poor lytic response; the average drop in optical density observed for cell suspensions treated with oxacillin and cloxacillin was 24.1 and 25.2% respectively.

The comparative lysis of *S. aureus* 3519 by lysozyme, trypsin, and a combination of both is shown in Fig. 4. The organism was grown in the various semisynthetic penicillins at 0.1 μg/ml. No change in turbidity resulted from 3-hour incubation of the control suspensions with buffer, lysozyme, trypsin, or mixtures of the enzymes. Similar incubation of oxacillin- and cloxacillin-treated cells resulted in poor lytic response, the extent of lysis not being appreciably different from that observed with buffer alone. However, suspensions of nafcillin-treated cells which had been incubated with lysozyme or trypsin showed an appreciable lysis, and a marked enhancement of lysis resulted from the combined action of the enzymes. The average percentage lysis achieved for the nafcillin-treated cells following the simultaneous action of the enzymes was 77%, compared to 43% for lysozyme, 48% for trypsin, and 22% for buffer.

Repeated tests in our laboratory have fairly well established that the lysis of staphylococcal cells by lysozyme and trypsin is dependent upon the exposure of a growing population to levels of penicillin that depress the growth rate. Since the percentage lysis of the penicillin-treated cells increases progressively with the concentration of the penicillin short of the MIC, it was of interest to study the effect of various subinhibitory concentrations on the extent of lysis of cell suspensions incubated with lysozyme and trypsin. Table I shows that the percentage

TABLE I. Comparative Growth and Lysis of *Staphylococcus aureus* 3519 Grown in Varying Concentrations of Semisynthetic Penicillins.

Drug concentration, μg/ml	Nafcillin		Oxacillin	
	Transmission*	Lysis	Transmissions*	Lysis
	%		%	
.2	82	75.3	58	31.8
.1	70	74.4	52.5	36.3
.05	55	42.1	42	27.0
.025	43	30.8	38	26.0
0	29	20.9	31	25.0

* Reading of 1:10 dilution made after 18-hr growth at 37°C. Lytic system: Lysozyme (20 μg/ml) + trypsin (10 μg/ml); time = 3 hr.

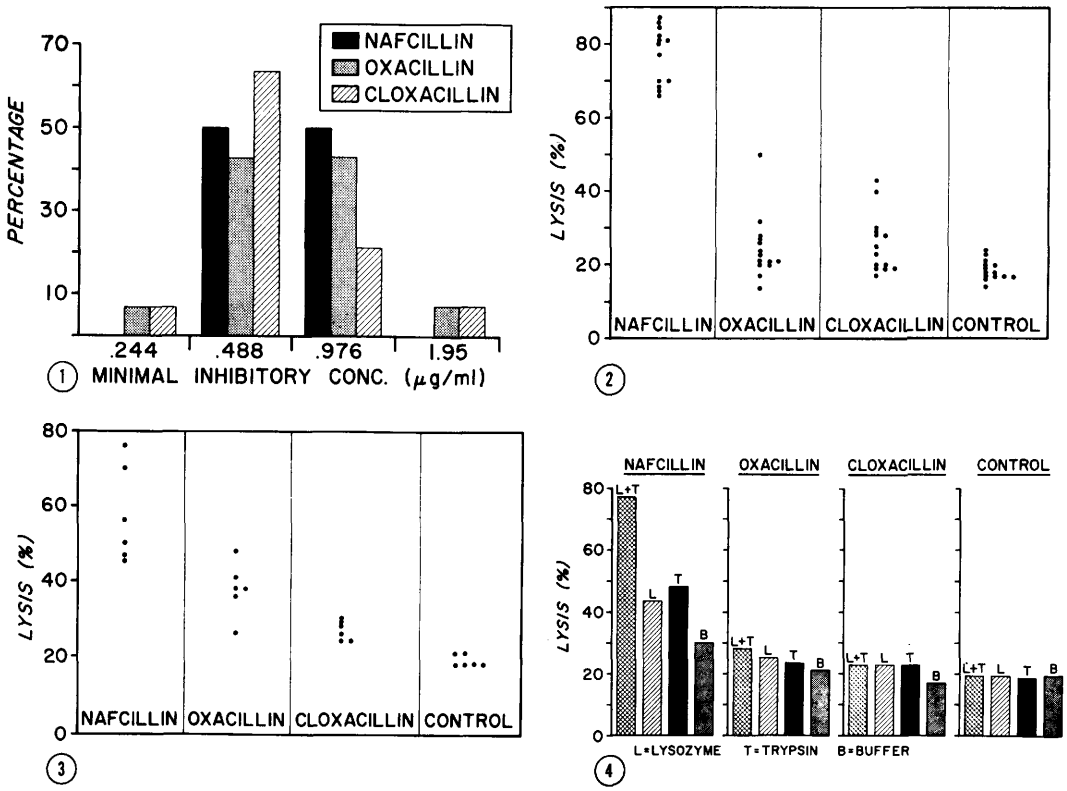

FIG. 1. Distribution of staphylococci (14 strains) according to sensitivity to penicillins.

FIG. 2. Distribution of percentage lysis by concomitant action of lysozyme (20 μg/ml) and trypsin (10 μg/ml) on penicillin-resistant staphylococci (14 strains) grown in semisynthetic penicillins at 0.1 μg/ml.

FIG. 3. Distribution of percentage lysis by concomitant action of lysozyme (20 μg/ml) and trypsin (10 μg/ml) on penicillin-sensitive staphylococci (6 strains) grown in semisynthetic penicillins at .05 μg/ml.

FIG. 4. Comparative lysis of *Staphylococcus aureus* 3519 grown in nafcillin, oxacillin, or cloxacillin at 0.1 μg/ml. L = lysozyme; T = trypsin; B = buffer.

lysis of *S. aureus* 3519 increased progressively with nafcillin concentration and attained a peak lysis of 75.3% at 0.2 μg/ml. No appreciable lytic response occurred with oxacillin at the same concentrations. Treatment of *S. aureus* with 0.1 μg/ml oxacillin resulted in only a 31.8% decrease in turbidity, and no further lysis occurred at the higher penicillin level (*i.e.*, 0.2 μg/ml).

Discussion. The observations made with a number of recent clinical isolates of penicillin-sensitive and penicillin-resistant *S. aureus* show that subinhibitory concentrations of nafcillin may have profound effects on the integrity of the cell wall. These effects, which vary from strain to strain, suggest an increase in accessibility of the cell wall and protoplasmic constituents to the action of lysozyme and trypsin, respectively. The observation that these enzymes, alone or in combination, cause only moderate lysis of some strains and marked lysis of other strains remains unexplained. It is conceivable that resistance to lysis may be governed by either the nature of the cell wall or by suppression of the synthetic activity of the bacterial cell by small amounts of the penicillin.

While nafcillin, oxacillin, and cloxacillin do not differ significantly in quantitative *in vitro* effectiveness against the staphylococci, cell wall alteration or disorganization appears to be accomplished more easily with nafcillin. The varying responses to the penicillins indicate basic differences within staphylococcus

in the sensitivities of various reaction systems to antibiotic activity. Since nafcillin and the isoxazole penicillins have little structural resemblance except for the 6-aminopenicillanic acid moiety, their effects on mucopeptide synthesis may involve somewhat different mechanisms and/or primary sites of action.

Preliminary experiments in our laboratories reveal that subinhibitory concentrations of nafcillin also reduce the lysozyme and trypsin resistance of streptococci. Small amounts of nafcillin probably alter the streptococcal wall structure. Michael *et al*(7) recently demonstrated that sub-lethal concentrations of benzylpenicillin bring about a loss in Group A streptococci of group-specific carbohydrate and type-specific M proteins—antigens known to be components of the cell wall.

Alterations in staphylococci resulting from subinhibitory amounts of nafcillin are transient, since continued incubation of the organism at the subinhibitory level or the transfer of the organism to penicillin-free media results in a resistance to enzymic lysis (1). However, the loss of resistance to lysis with lysozyme and/or trypsin undergone on exposure to small concentrations of nafcillin raises the question of possible *in vivo* activity of the enzymes on bacterial cells. Since lysozyme is known to be present in body fluids and in rather high concentration in leukocytes, it may alter staphylococcal cells previously exposed to small amounts of the penicillin. Although our studies in themselves do not prove that lysozyme and trypsin are involved in the destruction of staphylococci in infectious processes, other tissue enzymes may be involved. The question still arises whether a subinhibitory level of a penicillin, *e.g.* nafcillin, can render the cells susceptible to such enzymes and produce a favorable interplay between the tissue environment and the parasite.

The relatively large number of semisynthetic penicillins now available makes objective selectivity difficult, particularly when the greatest degree of protection is necessary. The described results suggest the exploration of this lytic system as a method for differentiation of the activities of penicillins that are otherwise identical *in vitro*. Such experiments are now being carried out in our laboratories.

Summary. Studies on the effects of subinhibitory concentrations of nafcillin, oxacillin, and cloxacillin on *S aureus* supported the view that nafcillin and the isoxazole penicillins may induce important changes in the cell wall during growth that result in increased susceptibility to lysis by lysozyme and trypsin. The observations suggested that significant quantitative differences exist between the ability of nafcillin and the other semisynthetic penicillins to render *S. aureus* susceptible to enzymic action. The significance of the data is discussed, with particular reference to the possible *in vivo* effects of subinhibitory concentrations of the penicillins in mediating a favorable interplay between the tissue environment and the parasite.

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