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# Preferential Inhibition of Penicillinase Induction by Oxytetracycline and Its Effect on the Penicillin Susceptibility of Staphylococci

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Exposure of penicillinase-producing staphylococci to a combination of penicillin and oxytetracycline resulted in a synergistic inhibitory activity of the antibiotics on the bacteria. Oxytetracycline was employed in concentrations having little or no effect on bacterial growth. It was found that the synergistic antibacterial effect was caused by the preferential inhibition of penicillinase induction by oxytetracycline, rendering the staphylococci more susceptible to penicillin.

The inhibition of protein synthesis by the bacteriostatic antibiotics has been shown recently to be quite selective. For example, induction of β-galactosidase in *Escherichia coli* was found to be inhibited by chloramphenicol at concentrations permitting exponential growth (11). The present study was designed to learn whether induction of penicillinase in staphylococci would also be inhibited by a bacteriostatic drug in the absence of substantial interference with bacterial growth. If so, then penicillinase-producing (penicillin-resistant) staphylococci should become more susceptible to penicillin in the presence of a bacteriostatic drug. Thus, a synergistic action between a bacteriostatic and a bactericidal drug would be achieved.

## MATERIALS AND METHODS

*Bacteria.* Coagulase-positive, penicillin-resistant strains of *Staphylococcus aureus* were isolated from throats of patients at the House of the Good Samaritan. Freshly isolated staphylococci were first tested for their resistance to penicillin and oxytetracycline by the disc method. Later, a more accurate determination of such resistance was made by the double-dilution technique in Antibiotic Medium 3 (Difco) broth.

*Antibiotics.* Penicillin G (Chas. Pfizer & Co., Inc., Brooklyn, N.Y.), oxytetracycline (Chas. Pfizer & Co., Inc.), and Dimocillin-RT (buffered methicillin sodium; E. R. Squibb & Sons, New York, N.Y.) solutions were prepared freshly in distilled water prior to each experiment.

*Penicillinase determination.* The amount of penicillinase produced was determined by the iodometric assay described in detail by Csanyi (1). Bacterial suspensions tested for penicillinase production were adjusted to contain 0.25 mg (dry weight) per ml.

*Measurement of antibiotic resistance.* Staphylococci were grown for 18 hr at 37 C in Todd Hewitt Broth (Difco). Samples of 1 ml were inoculated into tubes containing 20 ml of fresh broth and incubated until the growth reached $10^7$ cells per ml. A 0.1-ml amount of these growths was added to 2.0-ml samples of fresh Todd Hewitt Broth containing increasing concentrations of penicillin and oxytetracycline. The bacteria were exposed to the antibiotics separately and in combination. The turbidity of bacterial growth in the tubes was read and recorded after 18 hr of incubation.

*Measurement of oxytetracycline effect on penicillinase induction.* Samples (2-ml) of 18-hr cultures of staphylococci grown in Todd Hewitt Broth were transferred into 50 ml of fresh broth in Erlenmeyer flasks and agitated in a water bath at 37 C. After 2 hr of incubation, 10-ml samples were transferred into photometric tubes to which methicillin (0.5 μg/ml) was added, followed 30 min later by the addition of oxytetracycline in varying concentrations. Control tubes contained only methicillin or oxytetracycline. The tubes were incubated for 2 hr at 37 C, and then the bacteria were sedimented by centrifugation and resuspended in a phosphate buffer. Bacteria were adjusted to the same concentration in all tubes. The penicillinase determination of each one of these suspensions was performed by use of the procedure described by Csanyi (1).

## RESULTS

*Effect of penicillin and oxytetracycline on bacterial growth.* The following types of strains of *S. aureus* with different degrees of susceptibility to penicillin and oxytetracycline were isolated from

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throat cultures: (i) susceptible to penicillin and oxytetracycline, (ii) resistant to penicillin and susceptible to oxytetracycline, and (iii) resistant to penicillin and oxytetracycline. The resistance of all these strains to the antibiotics was determined as described in Materials and Methods. Table 1 shows results of experiments in which the bacteria were exposed to each antibiotic alone and in combination. Oxytetracycline was added in concentrations which, after 18 hr of incubation, showed no visible inhibitory effect on bacterial growth of the tested strain. Of a total of 60 coagulase-positive strains of *S. aureus* tested, 52 strains were penicillin-resistant (penicillinase-producing) strains. Of the penicillin-resistant strains, 65% were susceptible to oxytetracycline and the remaining strains were oxytetracycline-resistant. Table 1 shows the results obtained with nine strains of different types of antibiotic resistance; these results are representative for other strains.

Addition of oxytetracycline to penicillin-susceptible staphylococci did not alter bacterial susceptibility to penicillin. In contrast, exposure of penicillin-resistant (penicillinase-producing) strains of staphylococci to the combination of penicillin and oxytetracycline resulted in marked growth inhibition which was not obtained by penicillin alone. The increase in the growth inhibition by the combination of the two antibiotics was 10- to 60-fold. We also confirmed an observation reported by other investigators that the size of initial inoculum substantially affected the staphylococcal resistance to penicillin; when greater inocula were used, penicillin-resistant strains grew in the presence of higher concentrations of penicillin (2). Both oxytetracycline-susceptible and -resistant strains of penicillinase-producing staphylococci behaved similarly; when resistant strains were tested, more oxytetracycline had to be added in proportion to their antibiotic susceptibility.

Table 1. *Combined antibacterial activity of penicillin and oxytetracycline*

| Strain of *Staphylococcus aureus*[a] | Concn of oxytetracycline | Concn of penicillin (units/ml) capable of inhibiting bacterial growth | | | |
|---|---|---|---|---|---|
| | | $5 \times 10^5$[b] | $5 \times 10^4$ | $5 \times 10^3$ | $5 \times 10^2$ |
| | *μg/ml* | | | | |
| (A) | | | | | |
| D.B. | 0 | 0.05 | 0.05 | 0.05 | 0.05 |
| | 0.1 | 0.05 | 0.05 | 0.02 | 0.02 |
| G.T. | 0 | 0.8 | 0.8 | 0.8 | 0.8 |
| | 0.1 | 0.8 | 0.8 | 0.8 | 0.8 |
| J.G. | 0 | 0.8 | 0.8 | 0.4 | 0.1 |
| | 0.1 | 0.8 | 0.8 | 0.4 | 0.1 |
| (B) | | | | | |
| M.Q. | 0 | 250.0 | 75.0 | 25.0 | 0.8 |
| | 0.1 | 100.0 | 25.0 | 1.6 | 0.1 |
| T.M. | 0 | 100.0 | 8.0 | 0.8 | 0.4 |
| | 0.3 | 25.0 | 0.4 | 0.1 | 0.05 |
| M.N. | 0 | 800.0 | 12.5 | 1.6 | 0.4 |
| | 0.1 | 50.0 | 0.8 | 0.05 | 0.02 |
| A.S. | 0 | 1000.0 | 400.0 | 25.0 | 3.1 |
| | 0.1 | 100.0 | 50.0 | 3.1 | 0.2 |
| (C) | | | | | |
| 55-1-C | 0 | 2000.0 | 1000.0 | 100.0 | 3.1 |
| | 10.0 | 2000.0 | 250.0 | 2.5 | 0.16 |
| R.J. | 0 | 500.0 | 250.0 | 50.0 | 1.6 |
| | 25.0 | 100.0 | 50.0 | 0.8 | 0.08 |

[a] (A) Susceptible to penicillin and oxytetracycline; (B) resistant to penicillin and susceptible to oxytetracycline; (C) resistant to penicillin and oxytetracycline.

[b] Size of bacterial inoculum per tube.

*Effect of oxytetracycline on penicillinase induction.* Since subinhibitory concentrations of oxytetracycline were found to affect penicillin sensitivity of staphylococci, we investigated whether the bacteriostatic antibiotic at these low concentrations would reduce induction or production of penicillinase. Since penicillin G is rapidly hydrolyzed by penicillinase, it was a poor inducer. Therefore, methicillin, which is not attacked by the enzyme, was used for induction in these experiments. Amount of penicillinase produced by each tested strain was measured by a technique described in Materials and Methods. Table 2 shows the results of experiments in which oxytetracycline was added to exponentially growing staphylococci with methicillin present for induction of penicillinase. In these experiments, previously tested strains of bacteria were employed, and oxytetracycline substantially reduced penicillinase production through interference with the induction of the enzyme: the reduction in the induction varied from 80 to 95%. In additional experiments, it was determined that addition of oxytetracycline to a penicillinase producing culture did not affect the enzyme.

TABLE 2. *Effect of oxytetracycline on penicillinase induction*

| Strain of *Staphylococcus aureus* | Controls (no antibiotic) | Methicillin (0.5 μg/ml) | Methicillin (0.5 μg/ml) and oxytetracycline | Oxytetracycline |
|---|---|---|---|---|
| M.Q. | 2.5[a] | 32.0 | 3.0 (0.1)[b] | 2.5 (0.1) |
| T.M. | 2.0 | 25.0 | 3.0 (0.3) | 4.0 (0.3) |
| M.N. | 9.0 | 90.0 | 10.0 (0.1) | 9.0 (0.1) |
| A.S. | 5.0 | 80.0 | 25.0 (0.1) | 5.0 (0.1) |
| 55-1-C | 5.0 | 100.0 | 5.0 (10.0) | 7.5 (10.0) |
| R.J. | 2.0 | 60.0 | 10.0 (25.0) | 3.0 (25.0) |

[a] Results expressed as units of penicillinase per milliliter.

[b] Numbers in parentheses indicate micrograms of oxytetracycline per milliliter.

*Preferential inhibition of penicillinase.* After establishing that oxytetracycline affected induction of penicillinase, we wished to determine whether this enzyme was inhibited preferentially by the bacteriostatic drug. Oxytetracycline in increasing concentrations was added to cultures of penicillinase-producing staphylococci. All tubes contained methicillin (0.5 μg/ml) as an enzyme inducer. Details of the technique are described in Materials and Methods. Figure 1 shows the differential plots of enzyme production versus growth, both affected by increasing amounts of oxytetracycline. From these plots, it is evident that the amount of the antibiotic necessary to inhibit penicillinase production does not affect greatly the bacterial growth, indicating a case of preferential inhibition.

*Quantitative determination of the antibiotic activity on the staphylococcal growth.* The turbidimetric determination of growth inhibition by the antibiotics provided us with qualitative data, but it was desirable to obtain more exact information on the fate of staphylococci after exposure to penicillin and oxytetracycline. Tubes containing nutrient broth and the antibiotics were inoculated with $10^5$ penicillinase-producing staphylococci, and at time intervals samples were taken from the tubes and plated on agar to determine the number of surviving bacteria. (The inoculum was taken from a culture in the log phase of growth.) As shown in Fig. 2, exposure to penicillin or oxytetracycline alone in concentrations used in other experiments prolonged the lag phase of the bacterial growth. The combination of two antibiotics resulted in a bacteriocidal effect on the tested strain.

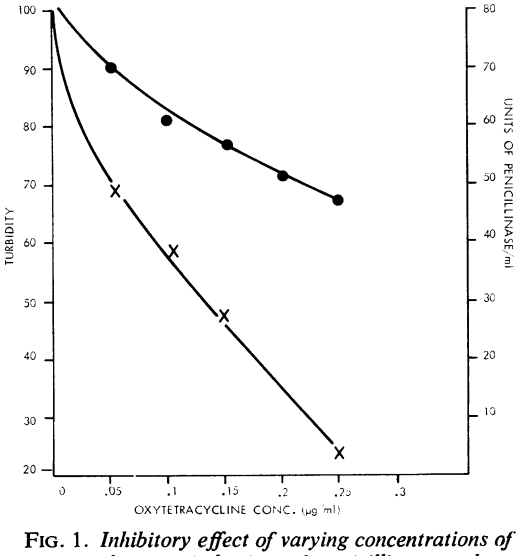


FIG. 1. *Inhibitory effect of varying concentrations of oxytetracycline on induction of penicillinase and on bacterial growth. Symbols: ●, bacterial growth measured by turbidity; ×, units of penicillinase per ml. Test strain A.S. was used.*

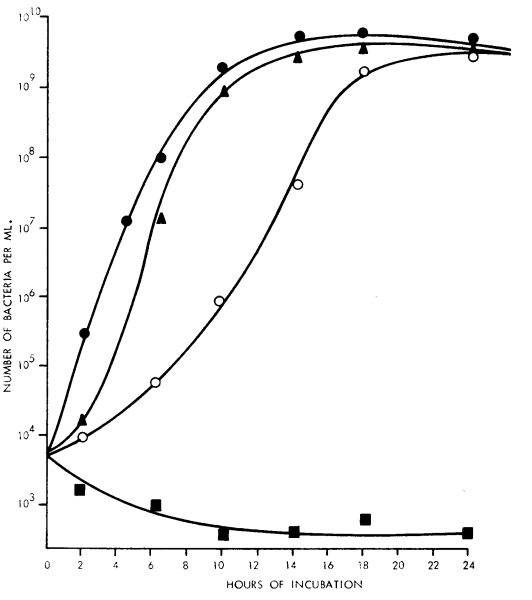


FIG. 2. *Effect of penicillin and oxytetracycline on growth of penicillin-resistant Staphyloccocus aureus strain T.M. Symbols: ●, control; ▲, penicillin G, 1 unit per ml; ○, oxytetracycline, 0.3 μg/ml; ■, penicillin G + oxytetracycline.*

## DISCUSSION

A combination of a bactericidal and a bacteriostatic drug is believed to result in antagonistic activity (4, 5). This antagonism is assumed to be caused by the differences in the mode of action of the drugs. Penicillin exerts its antibacterial activity through interference with the synthesis of the bacterial cell wall (6, 7), whereas the bacteriostatic drugs, such as oxytetracycline, inhibit protein synthesis, bacterial growth, and multiplication (3). It is therefore assumed that in the presence of a bacteriostatic drug, when growth of bacteria is arrested, penicillin will not interfere with the synthesis of the cell wall, and this results in reduction of the bactericidal activity of penicillin (3, 5).

Our present study has not been concerned with the growth-inhibitory activity of a bacteriostatic drug; instead, we were interested in determining whether subinhibitory concentrations of oxytetracycline have an effect upon penicillin susceptibility of penicillin-resistant staphylococci. The penicillin resistance of freshly isolated virulent staphylococci is caused by the production of an enzyme, penicillinase, which inactivates the drug (8). Thus, if subinhibitory concentrations of

oxytetracycline would suppress production of penicillinase, bacteria should be expected to become more susceptible to penicillin. Our experimental data indicate that, indeed, induction of penicillinase production is suppressed preferentially by the bacteriostatic drug and that this suppression results in increased penicillin susceptibility of penicillin-resistant staphylococci. The preferential inhibition of penicillinase induction by oxytetracycline was demonstrated both in oxytetracycline-susceptible and oxytetracycline-resistant bacteria. As would be expected, a greater amount of oxytetracycline was required to inhibit penicillinase induction in oxytetracycline-resistant strains than in oxytetracycline-susceptible strains.

The concept of selective suppression of enzymatic induction was recently suggested by Sypherd and Strauss, who showed that chloramphenicol may preferentially inhibit β-galactosidase synthesis when chloramphenicol is added to a growing culture of *Escherichia coli* (9, 10, 11). The authors postulated that the treatment with these antibiotics indirectly alters the regulative mechanism for inducible enzymes.

Pathogenic bacteria produce a wide variety of extracellular enzymes which may have important functions in their virulence. Presently, very little is known about whether these enzymes are inducible and which substances act as their inducers. It would be interesting to determine whether some of the bacteriostatic drugs might preferentially inhibit these enzymes, contributing to the decrease of the bacterial virulence and making the pathogens more susceptible to the destruction by the host defenses.

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