

SENSITIVITY OF COCCAL AND L FORMS OF *STAPHYLOCOCCUS AUREUS* TO FIVE ANTIBIOTICS

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ABSTRACT

KAGAN, B. M. (Cedars of Lebanon Hospital, Los Angeles, Calif.), SUSAN ZOLLA, R. BUSSER, AND SILVIJA LIEPNIKS. Sensitivity of coccal and L forms of *Staphylococcus aureus* to five antibiotics. *J. Bacteriol.* 88:630-632. 1964.—Antibiotics whose primary site of action is in the cell wall (penicillin and cephalothin) do not inhibit growth of L-phase organisms. In this study, kanamycin, neomycin, polymyxin B, lincomycin, and gentamycin were found to be more active against L-phase growth of *Staphylococcus aureus* in vitro than against the coccal forms. Therefore, their primary site of antimicrobial activity appears to be other than that involved in the synthesis or integrity of the cell wall.

Antibiotics whose primary site of action is in the cell wall (penicillin and cephalothin) do not inhibit growth of L-phase organisms (Wood and Archer, 1961; Davis and Feingold, 1962; Chang and Weinstein, 1963). However, those antibiotics which cause damage to the cytoplasmic membrane, or which inhibit cytoplasmic metabolic reactions, are generally more effective against L-phase growth than against growth of the coccal phases.

The present report is concerned with related studies of the mode of action of the following five antibiotics: kanamycin, neomycin, polymyxin B, lincomycin, and gentamycin.

MATERIALS AND METHODS

Five strains of coagulase-positive *Staphylococcus aureus* were studied. Four of the strains of *Staphylococcus* were human pathogens; the fifth was obtained from the American Type Culture Collection (strain 6538p). The coccal forms were maintained on Brain Heart Infusion agar slants (Difco; 5.2 g per 100 ml of distilled water).

The L-phase variants were induced and maintained on medium made of the following: Brain Heart Infusion agar (Difco), 6.0 g; sodium

chloride, 6.7 g; distilled water, 116 ml; and human serum, 30 ml. The serum was prepared as follows. Plasma from whole citrated blood was decanted and centrifuged at 2,500 rev/min for 15 min at 4 C. It was then inactivated at 56 C, frozen overnight at -20 C (to precipitate the fibrinogen), and reentrifuged. After passage through diatomaceous earth and Schleicher and Schuell analytical filter paper (no. 589 white ribbon, 604 and 602), it was passed in sterile manner through a Millipore HAWP filter of 0.45 μ pore size and stored in sterile vaccine bottles in a refrigerator until used.

To induce the L phases of each strain, methicillin was added and the transfers were made as previously described (Kagan, Molander, and Weinberger, 1962). All five strains were stable in their L phases in that, when placed on this medium without methicillin, none reverted to coccal form even after many transfers.

The antibiotics kanamycin, neomycin, polymyxin B, lincomycin, and gentamycin were tested in graduated concentrations for their effectiveness upon both the coccal and L phases of each of the five strains under study. Each antibiotic was in reagent form as provided for tube dilution testing and dissolved in sterile distilled water.

All culture plates were prepared and incubated overnight at 37 C before use. A generous loop from each slant of the coccal forms was suspended in 5 ml of sterile 5% saline. A 1 cm² block of agar containing the L-phase organisms was placed in 5 ml of sterile 5% saline and allowed to stand with periodic shaking for about 1 hr before plating. The media upon which these antibiotic assays were performed were of the same composition as the standard medium, with the addition of the particular antibiotic being tested. Each plate was inoculated with 0.05 ml of the saline suspensions described above, and the inoculum was spread over the surface of the agar with a sterile glass rod.

All plates were insulated with wide rubber bands to hold in the moisture; they were incubated under aerobic conditions upside down at 37 C for 56 hr.

A preliminary screening of strains 325 and 6538p was performed with each antibiotic to determine the range of concentrations of the drug to be studied. These preliminary assays were performed with only the coccal forms of the two strains. The method of culture was the same as above, with the exception that the coccal suspensions were plated on the medium without serum but containing various concentrations of one of the antibiotics being studied. From the effectiveness of the antibiotics in these preliminary runs, the range of concentrations for each antibiotic was chosen in each case to show a gradation in growth density. The range of concentrations finally used was extended lower if later experiments showed complete L-phase inhibition.

All results were recorded on a scale. The scale reflected a comparison of the growth density relative to the growth of the same strain on a control plate of the medium without antibiotic. Four classes of growth density were established to tabulate the results. Those plates with growth of equal density to that found on the control plate were considered as showing "no inhibition" as a result of the antibiotic they contained. Fairly good growth, but in considerably less-dense pattern compared with the control plate, was classified as "moderate inhibition." Those plates showing up to approximately 250 colonies (as compared with the several thousand colonies found on the plates with moderate or no inhibition) were judged to have "minimal growth." Finally, when the plates showed no growth at all, the results were classified as "complete inhibition" (Fig. 1).

RESULTS AND DISCUSSION

Kanamycin and neomycin are similar in their chemical and physical properties, in their broad spectrum of activity, in their pharmacological characteristics, and in their toxicity (Yow and Yow, 1961). This similarity is reflected in results of this study. In their effect on the coccal forms, these two antibiotics were quite similar, neomycin being perhaps slightly more effective. Both drugs were less effective ($\mu\text{g}/\text{ml}$) than lincomycin against the L forms. Their order of

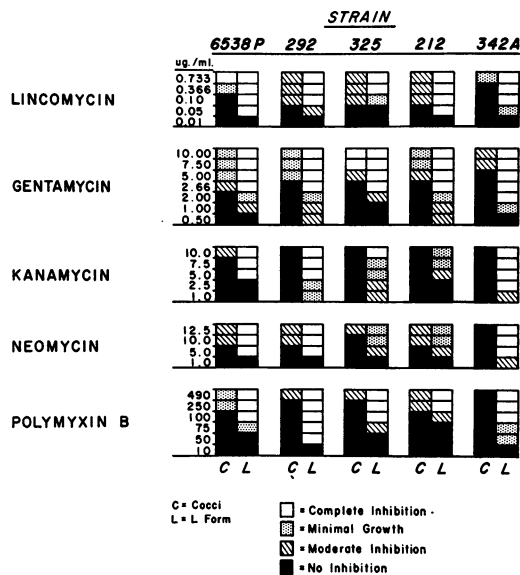


FIG. 1. Sensitivity of *Staphylococcus aureus* coccal and L-phase variants to antibiotics.

effectiveness against the L forms was similar to that of gentamycin. Kanamycin was somewhat less effective than gentamycin ($\mu\text{g}/\text{ml}$) against the coccal forms. These findings suggest that kanamycin and neomycin can be classified with lincomycin and gentamycin, in that their action is probably not primarily against cell-wall synthesis.

Studies reviewed by Newton (1956) strongly suggest that the bactericidal activity of polymyxin is due to its surface-active properties, which enable the compound to combine with the bacterial cell and disturb the cell's osmotic equilibrium. The selective activity of the drug may depend on the chemical composition and structure of the cell wall (Few and Schulman, 1953; Newton, 1954). The cell walls of organisms sensitive to polymyxin B have a much higher affinity for the drug than those organisms resistant to it. Newton (1954) suggested that it is the cell membrane rather than the capsule that absorbs the drug, and that the greater sensitivity of gram-negative organisms to polymyxin B as compared with gram-positive organisms may be due to the higher lipid phosphorus content found in the gram-negative bacterial cell membrane.

The cell membrane, which is largely phospholipid, is the exposed surface in L-phase organisms. The L forms of *S. aureus* are much more sensitive to polymyxin B than are the coccal

forms. The cell membrane is covered by a cell wall or capsule whose phospholipid content is much lower than that of the cell membrane (Salton, 1961); this could account for the greater resistance shown by the coccal forms.

Lincomycin is a bacteriostatic antibiotic which is quite effective in vitro in low concentrations against gram-positive organisms (Mason, Dietz, and Deboer, 1963). The L-phase variants were at least ten times more sensitive to lincomycin than were the coccal forms (Fig. 1); in most cases, their sensitivity was greater than tenfold. Lincomycin was the most effective of the five antibiotics studied in its in vitro effect against coccal forms, in that 0.4 $\mu\text{g}/\text{ml}$ inhibited four of the five strains. Because 0.1 $\mu\text{g}/\text{ml}$ or less inhibited all of the L forms, the data suggest that its primary site of action is not in the cell wall.

Gentamycin (Weinstein et al., 1963) is a "broad-spectrum" antibiotic that also showed a very wide differential effect, in that complete inhibition of the L forms was achieved in four of the five strains at a concentration of 2.66 $\mu\text{g}/\text{ml}$ or less, whereas the coccal forms remained unaffected at this concentration. Here, again, it would appear that the primary site of action of gentamycin is not in the cell wall.

It is of importance to note that both bacteriostatic and bactericidal agents are active against L forms as long as the primary site of action of the agents (such as kanamycin and neomycin) is intracellular or on the cell membrane rather than the cell wall.

The high degrees of sensitivity of the L forms, particularly to low concentration of lincomycin, suggest its possible use to control L-form growth in human tissues, should the latter prove of importance. That L-phase variants of staphylococci may account for persistor states or recurrence of staphylococcal disease in humans is a hypothesis currently being subjected to study in the department.

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