INDUCTION BY ANTIBIOTICS AND COMPARATIVE SENSITIVITY OF L-PHASE VARIANTS OF STAPHYLOCOCCUS AUREUS

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ABSTRACT

Molander, C. W. (Cedars of Lebanon-Mount Sinai Hospitals, Los Angeles, Calif.), B. M. Kagan, H. J. Weinberger, E. M. Heimlich, and R. J. Busser. Induction by antibiotics and comparative sensitivity of L-phase variants of Staphylococcus aureus. J. Bacteriol. 88:591-594. 1964.—The penicillins, cephalothin, vancomycin, and bacitracin were found to be less inhibitory to the L-phase variants than to their respective parent bacteria. Those antibiotics not considered to be primarily inhibitors of cell-wall synthesis were, in general, somewhat more inhibitory to the L form than to their parent bacteria. Only the penicillins and cephalothin readily induced L-phase variation. Novobiocin induced pleomorphic growth resembling the earliest stages of L-phase transformation. Failure of observable induction by bacitracin and vancomycin suggests that these two antibiotics affect cell-wall synthesis in a manner different from the penicillins, or that L-phase transformation may require more than “penicillin-like” interference with cell-wall synthesis.

There have been relatively few studies comparing in vitro sensitivities of bacteria and their L-phase variants to antibiotics. The in vitro antibiotic sensitivities of several strains of streptococci, a diphtheroid, several species of gram-negative bacilli, and their respective L-phase variants were reported by Ward, Madoff, and Dienes (1958). The L-phase variants in all of the species studied were considerably more resistant to penicillin than were the parent forms. The sensitivities of the L forms to the other antibiotics studied were comparable to those of their parent bacteria, with the exception of the L forms of the streptococci and one vibrio strain which were less sensitive to bacitracin.

Shockman and Lampen (1962) reported that protoplasts of streptococci were highly resistant to penicillin and to cycloserine, but that they and their parent forms had comparable sensitivities to other antibiotics.

Observations on the induction of L-phase variants of penicillin-sensitive staphylococci by penicillin G (Marston, 1961; Prozorovskii, 1959; Schonfeld, 1961) and of penicillin G-sensitive and -resistant staphylococci by methicillin (Kagan, Molander, and Weinberger, 1962) were described previously. In these studies, the L-phase variants were resistant to concentrations of these antibiotics far above those which inhibited their bacterial parents.

Godzieski, Brier, and Pavey (1962) recently reported that all antibiotics presently in clinical use are capable of inducing L-phase transformation in various bacterial species, including Staphylococcus. It was furthermore concluded by these authors that no antibiotic inhibited L-phase growth.

The purpose of the present investigation was to study further L-phase induction by various antibiotics and to determine the comparative sensitivity of several strains of coagulase-positive S. aureus to antibiotics with different modes of action. It was postulated that these observations might help clarify the mechanisms responsible for L-phase transformation and possibly the modes or sites of action of certain antibiotics.

MATERIALS AND METHODS

Strains and cultural methods. Five strains of coagulase-positive S. aureus were maintained on Brain Heart Infusion Agar (Difco). Four were isolated from human sources and the fifth was obtained from the American Type Culture Collection (strain 6538p). Strains 292, 325, and 6538p (Fig. 1) were penicillin G-sensitive, and the remaining two strains were penicillin G-resistant.

The L forms of these staphylococci were induced and maintained on Brain Heart Infusion
Agar containing 20% inactivated filtered human serum, 5% NaCl, and 500 μg/ml of methicillin as previously described (Kagan et al., 1962). They were further subcultured and maintained as "stable" L forms for more than 20 passages on the same medium containing no antibiotic.

Determination of L-form induction. Antibiotic-containing plates which had been inoculated with staphylococci as described below were examined for a period of 7 days by the Dienes staining procedure for L-type colonies.

**Determination of antibiotic sensitivities.** Staphylococcus suspensions were prepared in 5% NaCl from 24-hr cultures grown on antibiotic-free maintenance medium. Similar suspensions of their stable L forms were prepared from cultures of these forms grown on the same medium for 5 days; 0.05 ml of the L form and of the bacterial suspensions was spotted on maintenance medium plates containing increasing concentrations of the antibiotics under study. Plates containing no antibiotics served as controls. L-form suspensions contained from 10<sup>6</sup> to 10<sup>8</sup> viable organisms per ml as determined by plate count procedures. Bacterial suspensions of differing dilutions were inoculated. In this manner, one set of bacterial cultures similar in inoculum size to their L forms was available for accurate comparison of antibiotic sensitivity.

After incubation at 37 C, the bacterial cultures were observed at 24 hr and the L-form cultures at 48 hr. Inhibition of growth was evaluated by comparing the amount of growth on the antibiotic-containing plates with that on the control plates.

**Antibiotics.** The antibiotics listed in Fig. 1 were tested in the concentrations indicated. Cephalothin is the sodium salt of 2-(thiophene-2-acetamid) cephalexoramic acid. It is a semisynthetic compound, the basic structure of which resembles that of penicillanic acid (Chang and Weinstein, 1963). The other antibiotics tested were in reagent form as used for standard tube dilution testing. Dilutions were prepared in sterile distilled water and were added to autoclaved medium which was cooled to 50 C immediately prior to preparation of the plates.

**RESULTS**

The antibiotics listed in Fig. 1 were divided into two groups. Group a included those which inhibit cell-wall synthesis, and group b included those thought to have other modes of action (Davis and Feingold, 1962; Perkins, 1963).

**L-phase induction.** Among those antibiotics listed in group a, methicillin, oxacillin, and cephalothin readily induced characteristic L-phase growth in all five staphylococcal strains. Penicillin G induced L transformation in all but the two penicillin G-resistant strains (212 and 342A). Induction was not observed with bacitracin or vancomycin.

Novobiocin induced pleomorphic growth in all
strains at concentrations of antibiotics just inhibitory to bacterial growth. This growth resembled the earliest stages of L-phase transformation. However, no further development or survival of this growth was observed.

The antibiotics included in group b did not induce L-phase growth in any of the strains studied.

Comparative sensitivities. The penicillins and cephalothin did not inhibit any of the L-phase variants, even at extremely high concentrations. With the exception of the two penicillin-resistant strains (212 and 342A), the bacterial parents were highly sensitive to all of these antibiotics.

Bacitracin and vancomycin were also found to be much less inhibitory to the L-phase variants than to the parent bacteria. However, high concentrations were slightly to moderately inhibitory to some of the L strains.

The sensitivities of the L-phase variants to novobiocin and to those antibiotics included in group b (erythromycin, oleandomycin, chloramphenicol, tetracycline, and streptomycin) were either comparable to, or greater than, those of their bacterial parents.

Discussion
It has been suggested that agents or influences having an untoward effect upon the parent bacteria may induce transformation into the L phase, in which form the bacteria may continue to survive and multiply when suitable environmental conditions are provided. Upon removal of the untoward influence, reversion to the parent bacteria has been observed in many species (Dienes and Weinberger, 1951; Marston, 1961; Kagan et al., 1962). Exposure of bacteria to tap water, antagonistic strains, antiserum, various chemicals, and certain antibiotics has resulted in L-phase transformation in some species. L-phase transformation has also been observed to occur spontaneously in various bacterial species, including staphylococci (Dienes and Weinberger, 1952; Mattman, Tunstall, and Rossmore, 1961). A common mechanism may not be responsible for L-phase induction in all of these situations.

A major difference between the L-phase variant and the parent bacterium is the absence of a normal cell wall in the L form (Sharp, 1960). Since penicillin, an inhibitor of cell-wall synthesis, most readily induces L-phase variation in bacteria, the possibility was suggested that induction of L-phase variants results from exposure of the bacteria to agents primarily affecting cell-wall synthesis. Survival and further growth in the L phase after induction would require that the L-phase variants be more resistant to the inducing agent than their parent bacteria.

In the current studies, not all antibiotics considered to affect cell-wall synthesis primarily were capable of inducing L-phase transformation. Thus, penicillin G failed to induce L transformation in the penicillin-resistant strains. However, this was probably owing to its inactivation by penicillinase. Bacitracin and vancomycin also failed to induce L-phase growth, yet they are known to affect cell-wall synthesis. This could not be attributed to inhibition of the growth and survival of the L-phase variants, because these antibiotics did not inhibit growth of L-phase variants derived by the action of methicillin. These observations suggest that bacitracin and vancomycin affect cell synthesis in a manner different from penicillin or cephalothin. It may also be that induction of the L phase requires more than interference only with cell-wall synthesis.

Novobiocin has been shown to affect cell-wall synthesis (Strominger and Threnn, 1950). However, this effect is probably a consequence of its effect on the cell membrane (Shockman and Lampen, 1962; Davis and Feingold, 1962). As an inhibitor of cell-wall synthesis, it resembled the penicillins in inducing the early phases of L transformation. Its effect on the cytoplasmic membrane may have resulted in inhibition of the early L-phase growth that was induced, because the sensitivity of the L-phase variants to novobiocin was roughly comparable to that of the parent bacteria.

The failure of group b antibiotics to induce surviving L-phase variants may relate to the generally greater sensitivity of the L-phase variants to these antibiotics as compared with the parent bacteria. Perhaps by using a greater number of different concentrations within a narrower range at the critical level of inhibition of bacterial and L-phase growth, one might observe L-phase induction with the antibiotics in this group. This, along with possibly other differences in method and strain variability, may explain the discrepancy between these observations and those of Godzeski et al. (1962), who reported L-phase induction in staphylococcal and other species of bacteria by some antibiotics in both groups.
Although resistance of the L-phase variant to the antibiotic is necessary for survival, it is clear that this characteristic alone does not explain L-phase induction. Early L-phase induction was observed with novobiocin, although the parent and L form had comparable sensitivities. L-phase transformation did not occur with bacitracin and vancomycin, even though all of the L-phase variants studied were significantly more resistant to those antibiotics than were their parent bacteria.

The resistance of L-phase variants to all of those antibiotics listed in group b tend to support the view that their primary mode of action is in inhibition of cell-wall synthesis. The generally greater sensitivity of the L forms to group b antibiotics can perhaps be explained by more ready penetration due to absence of the usual cell wall.

It is intriguing to speculate that “persistor states,” clinical relapses, and bacterial resistance in patients treated with certain antibiotics may relate to the L transformation phenomenon in vivo. Staphylococci in the L form might be highly resistant to penicillin in vivo, for example, although the parent bacteria seemed to be sensitive to the antibiotic in vitro. Reversion to the bacterial form could thus occur after antibiotic treatment was discontinued. Conclusive evidence for in vivo L-phase induction of staphylococci is still lacking, although there are recent reports suggesting this possibility (Wittler et al., 1960; Kagan, 1962; Braude, Siemienski, and Jacobs, 1961; Mattman et al., 1961).

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LITERATURE CITED


