Development of Resistance to Novobiocin, Tetracycline, and a Novobiocin-Tetracycline Combination in Staphylococcus aureus Populations

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The antibiotic sensitivity of the individual organisms of a bacterial population was determined to study the comparative rates of development of resistance of Staphylococcus aureus to novobiocin, tetracycline, and to a combination of these antibiotics. Serial subculture of S. aureus with the combination of novobiocin-tetracycline (N-T 2.5:1; the ratio in serum of patients dosed with Panalba) showed a significant retardation of resistance outgrowth compared with subculture in the presence of the antibiotics individually. Increase in organisms resistant to novobiocin seen after one N-T subculture was related to the "concentration gap" between novobiocin and tetracycline. Two additional subcultures with N-T caused little or no increase in organisms resistant to novobiocin, tetracycline, or to the combination. The data suggest that the retardation of further development of resistance was the result of tetracycline inhibition of novobiocin-resistant strains and vice versa.

For several years, differences of opinion have been expressed concerning the usefulness of fixed antibiotic combinations in the treatment of infectious diseases. Despite the controversy, there appears to be general agreement that antibiotic combinations are effective in delaying the emergence of antibiotic-resistant organisms (1, 3, 4). In previous in vitro attempts to test this theoretically sound hypothesis, broth dilution techniques were used (2, 5-7). In these studies, bacterial cultures were subcultured in the presence of antibiotics singly and in combination, and changes in antibiotic sensitivity were determined by use of conventional minimal inhibitory concentration (MIC) procedures. Although these studies showed that development of resistance was suppressed by paired antibiotics, a definitive evaluation of this suppression was impossible because of limitations in the experimental techniques. The broth dilution procedure will only detect major changes in antibiotic resistance occurring within the bacterial population. Furthermore, this method is based on the operational assumption that each organism in the culture has a common MIC.

In the present study, Staphylococcus aureus was subcultured with novobiocin and tetracycline singly and in combination, and comparative rates of development of resistance were determined with techniques involving the fractionation of a bacterial population according to the antibiotic resistance of its individual members. Since this technique permits evaluation of the antibiotic sensitivity of each organism within the culture, development of resistance could be accurately quantitated. This technique also provides information (unavailable from MIC techniques) on two factors of major clinical importance: (i) the rate of increase of the small percentage of resistant organisms normally found in a bacterial population, and (ii) the level of resistance of individual organisms that survive high antibiotic concentrations. Success or failure in the treatment of serious bacterial infections will often depend on both of these parameters, since organisms that survive antibiotic therapy represent the inoculum for potential clinical relapse.

MATERIALS AND METHODS

The organism used in these studies was S. aureus OSU 284. This organism, sensitive to both novobiocin and tetracycline, was purified by single-colony
The procedure was identical for each subculture. In parallel experiments, *S. aureus* OSU 284 was subcultured three times serially in the presence of (i) novobiocin, (ii) tetracycline, and (iii) novobiocin-tetracycline (2.5:1). This ratio of novobiocin-tetracycline was chosen as representative of the ratio found in the blood serum of patients dosed orally with Panalba [Panalba is the Upjohn registered trademark for the combination of novobiocin (125 mg) and tetracycline (250 mg)]. For the first subculture, 0.1 ml of 1:100 dilution of an overnight culture was used to inoculate a series of broth tubes containing concentrations of the antibiotic (or the combination) that increased in twofold increments. The final volume of each tube was 10 ml. This procedure is identical to a standard broth dilution MIC determination. Tubes were incubated at 37 °C for 48 hr to allow maximal growth. The second and third subcultures were carried out in an identical manner with organisms from the previous subculture as inoculum. For this purpose, a 1:100 dilution was made from the tube containing the highest antibiotic concentration that still showed visible growth of the organism. This inoculation rate represents addition of approximately 10⁸ organisms per tube.

**Population sensitivity.** To estimate the antibiotic sensitivity of the individual organisms in each of the subculture populations, each subculture was plated out in agar medium containing different concentrations of the antibiotic. This was done in the following manner. To a series of test tubes, antibiotic was added in increasing twofold concentrations. The antibiotic solutions were added at 10 times the desired test concentration, with each tube receiving 1.0 ml of the appropriate concentration. The tubes were placed in a water bath at 45 °C, and 8.5 ml of BHI agar was added rapidly to obtain mixing of the contents. A 0.5-ml amount of an appropriate inoculum dilution was added to each of the tubes, and the contents were poured immediately into petri plates. Plates were rocked continuously to insure uniform suspension of the organisms in the agar. Five replicate plates of each test mixture were prepared. Plates were incubated at 37 °C for 2 to 3 days, after which time counts were made of the organisms that survived and grew to form a visible colony. Results of these experiments were expressed as population sensitivity curves by plotting the titer of surviving organisms versus the antibiotic concentration for each subculture. With the combination novobiocin-tetracycline subcultures, population sensitivity was determined both against the combination itself and against each of the component antibiotics. As a final step, individual clones were picked from the plates for subsequent MIC determination by use of standard agar dilution methods.

**RESULTS AND DISCUSSION**

**Serial subculture and MIC.** Figure 1 summarizes the subculture history of *S. aureus* OSU 284, and Table 1 lists the tube dilution MIC for each of the respective subcultures. Three serial subcultures with novobiocin resulted in a 125-fold increase in novobiocin resistance as measured by MIC (control = 0.4, N = 50 μg of novobiocin per ml). One subculture with tetracycline (T1) gave a fourfold increase in tetracycline MIC (0.1 to 0.4 μg/ml). Two additional tetracycline subcultures gave no further change in MIC. One subculture with the N-T combination (NT1) resulted in a twofold increase in MIC (0.2 to 0.08 to 0.4:0.16 μg/ml) and no further change after two subsequent N-T subcultures.

**Population sensitivity.** Analyses of population and sensitivity of the individual sub-
cultures are given in Fig. 2 through 6. Mean titer and the 99% confidence interval are plotted for each antibiotic concentration. During subculture with novobiocin (Fig. 2), rapid outgrowth of resistant organisms occurred. Of the $10^6$ organisms per milliliter in the control culture (before subculture with novobiocin), all but $10^4$ organisms per milliliter were inhibited by 0.2 \( \mu g \) of novobiocin per ml. The remaining organisms formed a “resistant plateau” that was not totally eradicated until a novobiocin concentration of 1.6 \( \mu g/ml \) was reached. The plateau organisms undoubtedly grew out upon subsequent novobiocin subculture (N1, N2, and N3). Both the concentration at which inhibition begins (drop-off) and slope of the inhibition curve were affected by subculture with novobiocin. The drop-off concentration increased with each successive antibiotic subculture from 0.1 \( \mu g/ml \) (control culture) to 12.8 \( \mu g/ml \) (N3). Novobiocin resistance gained during antibiotic subculture was a stable characteristic of the cultures. Subsequent growth in the absence of antibiotic had no effect on the novobiocin resistance of N1, N2, or N3.

Analysis of population sensitivity of the tetracycline subculture series is given in Fig. 3. Although development of tetracycline resistance was not as rapid as that seen with novobiocin, definite emergence of resistant organisms did occur during subculture with tetracycline. After one tetracycline subculture (T1), the drop-off concentration increased from 0.1 \( \mu g/ml \) (control culture) to 0.4 \( \mu g/ml \) where it remained through two more antibiotic passages. With each successive tetracycline subculture, the slope of the inhibition curve became more shallow, indicating a decreasing uniformity in the susceptibility of the surviving organisms.

Analysis of population sensitivity of S. aureus OSU-284 subcultured with the N-T combination is given in Fig. 4, 5, and 6. Figure 4 describes inhibition of the subcultures by the N-T combination, and Fig. 5 and 6 describe inhibition of the same subcultures by novobiocin.
and tetracycline individually. Analysis of these subcultures against all three antibiotic entities permits investigation of the influence exerted by one antibiotic on development of resistance to the other. After one subculture with novobiocin-tetracycline (2.5:1), organisms with increased resistance to the combination emerged (NT1, Fig. 4). An additional slight increase in resistance was seen after the second subculture (NT2), with no additional increase after the third subculture with the combination (NT3). Figure 6 shows that, during the three subcultures with the combination, no change in tetracycline resistance occurred. On the other hand, one subculture with the combination resulted in an increase in organisms resistant to novobiocin (NT1, Fig. 5). Two additional subcultures with the combination (NT2 and NT3) gave no further increase in novobiocin resistance. From these data, therefore, it is apparent that the increase in resistance to the combination seen in the first N-T subculture is the result of an increase in resistance to the novobiocin component only. In fact, comparison of the population sensitivity curve for the novobiocin subculture series (Fig. 2) with those for the N-T subculture series (Fig. 5) shows that the novobiocin sensitivity of N1 is identical to that of NT1. In other words, during the first subculture with the combination, novobiocin resistance increased as though no tetracycline were present.

The increase in resistance seen during the first NT subculture (and, to a lesser extent, the second NT subculture) appears related to the concentration differential between novobiocin and tetracycline in the combination. The 2.5:1 concentration "gap" between the component antibiotics allows a fixed amount of novobiocin resistance to occur before the concentration of the tetracycline is sufficient to inhibit the organisms. After the second NT subculture, the total increase in resistance permitted by the concentration "gap" was obtained and no further increase in resistance either to the combination or to novobiocin was seen. This does not necessarily mean that the population is completely stabilized with respect to the development of antibiotic resistance. The data do suggest, however, that further development of resistance would be extremely slow, with tetracycline inhibiting further emergence of novobiocin-resistant organisms and vice versa. It is significant to note that although a plateau of novobiocin-resistant organisms was seen after the first subculture with the combination (NT1, Fig. 5) it did not grow out during subsequent NT subcultures. Outgrowth of these novobiocin-resistant organisms was apparently prevented by the tetracycline component in the combination.

Confirmation of the population sensitivity results has been obtained by determining the MIC of large numbers of colonies selected from the population sensitivity plates. In these experiments, the MIC of each clone was determined simultaneously against the combination

**Fig. 5.** Population sensitivity of Staphylococcus aureus OSU 284 subcultured once (NT1), twice (NT2), and three times (NT3) with the combination novobiocin-tetracycline (2.5:1). Titer of surviving organisms at different concentrations of novobiocin alone.

**Fig. 6.** Population sensitivity of Staphylococcus aureus OSU 284 subcultured once (NT1), twice (NT2), and three times (NT3) with the combination novobiocin-tetracycline (2.5:1). Titer of surviving organisms at different concentrations of tetracycline alone.
and against both of the component antibiotics. Based on the MIC data thus obtained, an evaluation could be made as to which of the antibiotics in the combination was inhibitory when a strain was inhibited by the combination. The results showed that prior to antibiotic exposure (control culture) the majority of the organisms in the culture were inhibited by the novobiocin component. Tetracycline inhibited only those members of the population that were normally resistant to novobiocin (novobiocin-resistant plateau). After one subculture with the N-T combination (NT1), inhibition of members of the population was divided between the two antibiotics (with novobiocin inhibiting a somewhat greater percentage of the organisms). This would be expected since the population susceptibility data indicated emergence of some novobiocin-resistant organisms. Strains obtained from the second and third NT subcultures were inhibited by both components (with the majority of strains inhibited by tetracycline).

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LITERATURE CITED