BACTERIAL RESISTANCE TO ANTIBIOTICS IN VIVO

I. INCIDENCE OF RESISTANCE

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In studies of the interactions of antibacterial agents, it became important to try to elicit bacterial resistance to antibiotics in the experimental animal. Since this proved to be unexpectedly difficult, attention was directed to the process by which bacteria acquire resistance during exposure in vivo. The present paper is concerned with the incidence of streptomycin resistance in strains of staphylococci from mice infected with sensitive strains.

METHODS

Strains. Two strains of coagulase positive Staphylococcus aureus were used. Strain Zeut, recovered about 20 years ago from the sputum of a patient with a lung abscess, is sensitive to penicillin and streptomycin, the minimal inhibitory concentrations of the two agents being approximately 0.06 and 12.5 μg/ml, respectively. Strain 284, isolated from urine in 1951, produces a moderate amount of penicillinase and is resistant to penicillin in amounts ranging from 100 to 1200 μg/ml; it is inhibited by 31 to 125 μg/ml streptomycin.

Identification. The identity of substrains stemming from 284 is checked by tests for penicillinase. For those from Zeut, pigmentation was the only clue in the preliminary experiments. This seemed inadequate so, with the help of Dr. Robert Wise of Jefferson Medicine College, a collection of typing phages was started. Zeut is lysed by phage 44 A alone of the 18 phages prepared to date. This fortunate circumstance has made the identification of its substrains clear-cut. Strain 284 was found by Dr. John Blair to belong to the 6-7-47 group.

Antibiotics. Streptomycin sulfate (Parke, Davis and Company) and crystalline sodium penicillin G (kindly supplied by Merck, Sharp and Dohme)

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were used. Solutions, in water or broth, were stored in the freezing compartment of a refrigerator, until used.

Tests in vitro. Sensitivity tests were usually performed by the serial 2-fold dilution technique in broth, with a final volume of 1 ml. The medium was heart infusion broth (Difco) to which 0.07 per cent glucose and 2.0 per cent rabbit's blood were added. The standard inoculum was 0.5 ml of a 1:100 dilution, in blood broth, of an 18-hr blood broth culture. Tests were read after 17 to 20 hr incubation at 37 C. Occasionally, sensitivity and other tests were made on blood agar containing streptomycin. In this case the inoculum was limited to 0.1 ml of the appropriate dilution to avoid excess moisture and consequent coalescent growth. With plates it was found necessary to postpone the final readings until the 4th or 5th day.

Tests in vivo. Male CF1 mice, weighing 18 to 23 g, were used. They were inoculated by the intravenous route with massive doses of the test organism. The culture, grown for 18 hr at 37 C on the surface of a blood agar plate, was suspended in 2 to 4 ml of broth; 0.1 ml of a 1:10 broth dilution of the suspension was injected in a tail vein. The dosage, as determined by colony count, ranged from 0.5 to 2.5 billion organisms. The bacteremia so produced may persist for 3 or even 5 days. If the animals survive this period, abscesses are formed, especially in the kidneys, and staphylococci can be recovered after long intervals. Mice infected with 284 usually die in 2 or 3 days if not treated. Infected with Zeut, untreated mice live longer and occasionally appear to recover, but even in such apparently healthy animals there is usually evidence of kidney damage (although abscesses are rare) and staphylococci are present. Antibiotics were given by subcutaneous injection. The usual schedule was 0.1 mg of penicillin or 1.0 mg of streptomycin, first administered shortly after the infecting dose and repeated twice daily for 2 weeks. Evidence
of renal damage is less constant and a smaller proportion of cultures is positive for staphyloccoci in mice that have received therapy. The cultures were made from heart blood of mice that died within 3 days and from the kidney tissue of those that died later. Their subsequent handling is described below.

RESULTS

In the preliminary experiments, series P-1 and P-2, 2 colonies were picked from each plate which had been incubated with tissue from a mouse, and were transferred to a single tube of blood broth. These broth cultures were tested for sensitivity to streptomycin and penicillin. In series P-1, strain Zeut was used. Eighty infected mice were treated with streptomycin and from them 59 cultures of *S. aureus* were recovered. One of the cultures proved to be highly resistant (1000-fold) to streptomycin. Four exhibited a low (4- to 16-fold) resistance. Seventy-nine mice either received no antibiotic or were treated with penicillin. Seventy cultures were recovered, none of which had any resistance to streptomycin. No change in sensitivity to penicillin was noted in any of the cultures.

In series P-2 the penicillinase producing strain 284 was used. Forty-five cultures recovered from 38 mice treated with streptomycin were all sensitive to this antibiotic. But of 37 cultures from untreated mice, 1 possessed a 500-fold resistance to streptomycin. All 82 cultures were as resistant as the parent strain to penicillin.

Recovery of a highly resistant strain from material that had never been exposed to streptomycin not only threw doubt on the role of the drug in the origin of the first strain but raised a question about the technique. The practice of picking two colonies had been adopted to allow for the possibility that the cultures from mice might not be homogeneous—that they might be mixtures of resistant and sensitive organisms. It now seemed that in selecting only two colonies other positive cultures might be missed, since, if resistance occurred spontaneously and without the selective aid of the drug, it might be manifest in only a small proportion of the population. Accordingly, blood broth, as well as blood agar, cultures were now made with bits of mouse tissue and the number of colonies subjected to further analysis was increased to 10, on the average.

Later, because of doubt as to whether resistance noted in secondary cultures had arisen in vivo or during primary culture, streptomycin agar was added to the primary culture media.

In the next 4 experiments, 94 mice were infected with strain 284; 60 received no antibiotic. Again, a highly resistant strain was recovered from 1 of the cultures derived from a control mouse. Two of 10 colonies from the drug-free plate inoculated with material from this mouse showed 500- to 1000-fold resistance. A highly resistant strain was also recovered from 1 of the 44 mice treated with streptomycin—1 colony of 10 isolated from its plate showing 1000-fold resistance. Two other treated mice were the source of 2 strains with weak resistance. These mice were part of a group of 20 which were given an initial dose of 10 mg streptomycin; 6 of the mice died within 1 hr and subsequent therapy was therefore reduced to 2 mg twice a day. Only 6 positive cultures were obtained at

<table>
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<th>Strain</th>
<th>Streptomycin Treated Mice</th>
<th>Untreated Mice*</th>
<th>Total Mice</th>
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<tr>
<td></td>
<td>Mice Cultures Streptomycin resistance</td>
<td>Mice Cultures Streptomycin resistance</td>
<td>Mice Streptomycin resistance</td>
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<tr>
<td></td>
<td>High Low</td>
<td>High Low</td>
<td>High Low</td>
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<td>Zeut...</td>
<td>144 117 3 5</td>
<td>144 135 0 0</td>
<td>288 3 1.0 5 1.7</td>
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<td>284....</td>
<td>82 78 1 2</td>
<td>86 77 2 0</td>
<td>168 3 1.8 2 1.2</td>
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<tr>
<td>Total...</td>
<td>226 195 4 7</td>
<td>230 212 2 0</td>
<td>456 6 1.3 7 1.5</td>
</tr>
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* Fifteen mice treated with penicillin included.
autopsy and the fact that 2 of them showed some resistance, whereas all 10 cultures from 10 mice that received 2 mg from the start were fully sensitive, suggests that the heavier dosage may have had an effect.

Strain Zeut was used for the next 5 experiments. Half the 129 mice were treated; from them, 58 cultures were obtained of which 2 were highly resistant. In the case of 1 of these, 9 of 10 colonies picked from the drug-free agar showed 500- to 1000-fold resistance; in the other instance, 6 of 10 colonies were resistant. Both grew heavily on the primary streptomycin (125 μg/ml) plate. A third culture from a treated mouse had a low (16-fold) resistance. None of the 65 cultures obtained from the untreated mice showed any resistance.

The data are summarized in table 1. From a total of 456 mice, 288 infected with staphylococcus strain Zeut and 168 with strain 284, 6 substrains with high resistance to streptomycin and 7 with low resistance were recovered. Two of the highly resistant strains came from mice that had received no streptomycin; both of these were variants of strain 284. The other 4 strains with high resistance and all 7 with low resistance originated in cultures from treated mice. In short, resistance was encountered only occasionally and when it occurred it was as likely to be high as low in degree.

**DISCUSSION**

As compared with the great number of studies in *vivo*, there are relatively few reports of attempts to induce resistance to antibacterial agents, experimentally, *vivo*. That it is possible to do so has been demonstrated from time to time. In several of the previous studies the method, successive transfer in mice treated with increasing doses of the therapeutic agent, resembled that employed in *vivo*. By this means Miller and Bohnhoff (1948) were able to obtain a considerable degree of penicillin resistance in meningococci. In the present study, the purpose was to simulate the conditions under which bacterial resistance to a drug develops in a patient. Here the microorganism is exposed, not to increasing concentrations of the drug, but to repeated assaults with the same dose over a long period of time. This was the procedure used by Youmans *et al.* (1949), Williston and Youmans (1950), and Wolinsky and Steenken (1953) in their studies of streptomycin resistance in tubercle bacilli recovered from mice and other animals which had been treated with this antibiotic. In the first two studies mentioned the investigators noted a high incidence of resistance in cultures from heavily treated mice, although in most instances the degree of resistance was low. Wolinsky and Steenken, on the other hand, were struck by the infrequency with which resistance developed and commented on the difference between their experimental results and the effect on the bacilli of streptomycin therapy in man. Thus, their results were similar to those obtained here and it is pertinent to note their suggestion that something in addition to spontaneous mutation and selection—possibly host factors—must play a part in the emergence of resistance in *vivo*.

With regard to the results of the present study, it is evident that although exposure to streptomycin may have been responsible, through selective action, for the strains that showed a low degree of resistance, it had little bearing on either the origin or the outgrowth of the 6 highly resistant strains. Not only had 2 of these strains received no exposure to the drug but for none of them was the exposure commensurate with the degree of resistance attained. According to our own observations and those of Zubrod (1948), the highest blood level in mice, following the dosage used in the present study, is only 50 to 70 μg/ml streptomycin and this dwindles in 2 hr to 5 μg/ml or less. Granted that the high resistance originated as a spontaneous mutation of some sort, the mechanism by which the mutants survived and occasionally outgrew the sensitive members of the population is obscure.

**SUMMARY**

Streptomycin resistance occurred only rarely in the course of the study. Of 456 mice infected with one or the other of two strains of *Staphylococcus aureus*, 6 yielded substrains with high resistance and 7 yielded substrains with low resistance. About half the mice received no treatment with streptomycin and from them 2 of the very resistant strains were derived. From this observation it is concluded that high resistance is a result of spontaneous mutation. All of the strains with low resistance came from the treated series and their survival may be attributable to the presence of low concentrations of streptomycin in the tissues of the host.
REFERENCES


