BACTERIAL RESISTANCE TO ANTIBIOTICS IN VIVO

II. Population Patterns among Staphylococci

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ABSTRACT

Bliss, Eleanor A. (Bryn Mawr College, Bryn Mawr, Pa.) and Burr M. Alter. Bacterial resistance to antibiotics in vivo. II. Population patterns among staphylococci. J. Bacteriol. **84**:125–129. 1962—The general level of resistance to streptomycin of a sensitive strain of *Staphylococcus aureus* was increased by exposure to the antibiotic in vivo. Although a rise in the minimal inhibitory concentration of streptomycin in broth was seen infrequently, over half the cultures from treated mice showed a shift in the population pattern toward greater tolerance for the antibiotic. A similar change did not occur in staphylococci after exposure in vivo to penicillin or chlortetracycline. Except in the case of highly resistant mutants, the level of population tolerance for streptomycin appears to be related to the concentration of the antibiotic in the tissues of the host, since strains already possessed of a measure of resistance failed to gain in resistance when exposed to streptomycin in vivo. The mechanism of survival and outgrowth of the occasional highly resistant mutant is considered.

In the first phase of this study (Bliss and Alter, 1959), a very low incidence of drug resistance was encountered among strains of staphylococci recovered from mice which had been experimentally infected and treated with streptomycin. The degree of resistance was as likely to be high as low but, whereas two of six strains with high resistance were derived from mice which had received no streptomycin, seven strains with low resistance all came from treated mice. The criterion of resistance was an increase of fourfold or greater in the minimal inhibitory concentration (MIC) of streptomycin, as determined by the tube-dilution technique. It was recognized, however, that the end point in a fluid medium is determined by the tolerance of the few most resistant members of a culture, and that, at concentrations below this, progressively greater proportions of the population are able to grow in the presence of the drug. The present study was undertaken to test the possibility that exposure to streptomycin in vivo might result in an increase in the proportions of a bacterial population able to tolerate low concentrations of the antibiotic.

MATERIALS AND METHODS

A description of the strains and details of the technique were given earlier (Bliss and Alter, 1959). CF_1 mice were infected by the intravenous inoculation of massive doses of *Staphylococcus aureus* strain Zeut. About one-half of the mice served as controls, and the remainder were treated, usually twice a day, with 1 or 2 mg of streptomycin sulfate (Parke, Davis and Co., Detroit, Mich.) administered by the subcutaneous route. Treatments were continued for 2 weeks if the animals survived that long. Cultures were made from the cardiac blood of animals that died in the first few days, and from the kidneys of those that died or were killed later. Cultures were planted in blood broth, on blood agar plates, and on blood agar containing streptomycin (125 μg/ml). The blood agar containing streptomycin served to reveal frank resistance, and the plain agar plates provided a rough estimate of the degree of infection.

It was from the blood broth cultures that the population analyses were made. Cultures were stored in the refrigerator pending use, a delay which at times amounted to as much as 3 weeks for the controls, since it was desirable to include cultures from both treated and untreated mice in each run. Occasionally, cultures of the parent strain were also included. Samples (0.1 ml) of each culture, suitably diluted, were spread upon the surface of four blood agar plates, three containing streptomycin, 3.9, 15.6, and 62.5 μg/ml, and one drug-free. Tube sensitivities were determined at the same time. The ratios of the colony counts on the streptomycin plates to that on the

drug-free plate gave the proportions of the population resistant to the three concentrations of the antibiotic. Ordinarily, no growth was visible at 24 hr if the concentration of antibiotic exceeded $\frac{1}{16}$ of 1 MIC. In the presence of $\frac{1}{8}$ to 2 MIC, colonies appeared on the second day. After this a twofold increase in the count was commonly noted, and occasionally the difference between the second and fifth day counts was as much as 100-fold. Although the significance of such late outbursts of growth is uncertain, it seemed best to use the counts at 5 days for establishing the proportions.

RESULTS

Four groups of mice were infected with *S. aureus* strain Zeut in experiments labeled L, N, R, and S; 71 substrains were recovered, 29 from control mice and 42 from mice treated with streptomycin. The end points (MIC) in streptomycin broth of these substrains and of the parent strain are shown in Table 1. All of the control strains were sensitive to streptomycin, five strains from treated mice were slightly resistant, and one was extremely resistant. In Table 2 are given the high, low, and median proportions for the same strains on streptomycin agar. On agar with 3.9 μg/ml of streptomycin, one-half of the cultures from control mice had proportions above 3.5×10^{-6} and the highest proportion was 45×10^{-6}. In general, their proportions were lower than those for the parent strain. The median for cultures from treated mice on this concentration was 32×10^{-6}. The highest proportion (over 50% of the population) was that exhibited by the very resistant strain mentioned above, but the proportions of 18 other strains were higher than those obtained in any test with strain Zeut and 19 were higher than any control. Similar differences between strains from control and treated mice were observed on the two higher concentrations.

In short, exposure to streptomycin in vivo resulted in a rise in the general level of tolerance in at least one-half of the strains. Up to a point, the results on agar agreed with those in broth, cultures with the highest proportions having the highest broth end points and those with lowest proportions showing the greatest sensitivity in broth. Beyond this point, however, inconsistencies between the two tests were observed. All but 3 strains produced at least a few colonies on agar with 15.6 μg/ml streptomycin, and 50 (16 from controls) did so on agar containing 62.5 μg/ml of streptomycin; yet 40 strains failed to grow in broth with 15.6 μg/ml of streptomycin and only 6, all from treated mice, grew in broth containing 62.5 μg/ml of streptomycin. As reported earlier (Bliss and Alter, 1961) the difference between the results in the two media is greatly reduced when allowance is made for the smaller inoculum used in the broth tests. Analysis of the data for each strain indicated that about 50 organisms, resistant to a given concentration of streptomycin, were required to initiate growth in streptomycin broth of that concentration.

Tests were made to see whether second and third exposures to streptomycin in vivo would increase the difference between strains from treated mice and the parent strain Zeut. For experiments O and Q, the most resistant cultures from treated mice of the antecedent series were used; for T and U, median cultures were chosen. Data on tube sensitivities of the substrains which were recovered are given in Table 1, and the median proportions are shown in Table 3. The results were variable, but there was an indication, notably in experiment Q, that the level to which population resistance can be raised through the selective action of the antibiotic in vivo is limited. Support for this view was obtained by failure to change, in vivo, the resistance spectrum of *S. aureus* strain 284, whose end point in streptomycin broth is about four times higher than that of strain Zeut.

Attempts in this laboratory to increase the resistance of staphylococci to penicillin and to chlortetracycline in vivo have been unsuccessful. Substrains of strain Zeut recovered from 18 mice, which had been treated with chlortetracycline (200 μg) twice a day for 2 weeks, and from 7 mice treated for a similar period with penicillin (100 μg) were indistinguishable from their respective controls in both the proportions resistant and tube sensitivity.

DISCUSSION

McCune, Dineen, and Batten (1956) observed that staphylococci may persist for considerable periods in the kidneys of mice treated with various antibiotics, without necessarily becoming resistant. The results of the present study are in agreement with this observation, insofar as frank resistance, such as is disclosed by the tube sensitivity test, is concerned. However, exposure to streptomycin in the mouse did, in a majority

TABLE 1. *End points in streptomycin broth of Staphylococcus aureus strain Zeut and substrains recovered from mice*

Expt	Source of strains	No. of tests or cultures	Minimal inhibitory concn of streptomycin (μg/ml)								
			Not resistant				Slightly resistant				Very resistant
			7.8	15.6	31.2	62.5	125	250	500	1,000	16,000
	First series (no previous exposure)										
L, N, R, S	Zeut (parent)	91 tests	10	45	35	1					
	Control mice	29 cultures	1	20	8						
	Treated mice	42 cultures	2	17	14	3	4	1			1
	Second series (from treated mice of series 1)										
O	N 12 (parent)	3 tests					2	1			
	Control mice	9 cultures			1	3	5				
	Treated mice	10 cultures				2	7	1			
T	S 21 (parent)	1 test		1							
	Control mice	6 cultures		5	1						
	Treated mice	5 cultures		1	1	1	2				
	Third series (from treated mice of series 2)										
Q	O 17 (parent)	10 tests					4	4	2		
	Control mice	9 cultures					4	4	1		
	Treated mice	9 cultures					4	4		1	
U	T 20 (parent)	2 tests			1	1					
	Control mice	10 cultures		1	2	6	1				
	Treated mice	10 cultures			2	7	1				

of instances, affect the sensitivity spectrum of the strain as a whole. Since the change to greater overall tolerance was not seen in all the strains from treated mice, the results differ from those commonly observed after exposure in vitro. Eagle, Fleischman, and Levy (1952), who studied the effect of low concentrations of penicillin and streptomycin on staphylococci growing on agar, noted that the progeny of all colonies derived from a given concentration were able to grow on that concentration; and they (Eagle et al., 1952) and Demerec (1945) reported uniformity of tolerance within the strain. The difference between these results and the present ones is, no doubt, related to the fact that they were dealing with cultures established from single colonies, whereas the populations studied here, originating in cultures from kidney or the blood of infected animals, were less homogeneous in character.

Failure to increase resistance above a certain level, as observed in strains already possessed of enhanced tolerance, may be explained by the limited concentration of streptomycin in the blood and other tissues. A concentration of 50 to 70 μg/ml is observed in the mouse 15 min after a 1-mg dose, but this declines rapidly to 5 μg/ml or less in 2 hr. Transience of significant blood levels may also account for the persistence of sensitive organisms in the tissues of treated mice. Tests in this laboratory have shown that exposure to 75 μg/ml streptomycin in broth for as much as 3 hr is insufficient to kill all the sensitive individuals in a culture of staphylococci, and the intervals be-

TABLE 2. *Population proportions on streptomycin agar of Staphylococcus aureus strain Zeut and substrains from control and treated mice*

Source of strains	No. of tests or cultures	Streptomycin concn (μg/ml)					
		3.9		15.6		62.5	
		Proportions					
		Median	Range*	Median	Range†	Median	Range‡
Parent strain Zeut	8 tests	10×10^{-6}	4×10^{-6} to 200×10^{-6}	13×10^{-7}	5×10^{-7} to 100×10^{-7}	19×10^{-9}	$< 10^{-9}$ to 230×10^{-9}
Control mice	29 cultures	3.5×10^{-6}	$<10^{-6}$ to 45×10^{-6}	6×10^{-7}	0.1×10^{-7} to 24×10^{-7}	3×10^{-9}	$<10^{-9}$ to 200×10^{-9}
Treated mice	42 cultures	32×10^{-6}	$<10^{-6}$ to >50%	34×10^{-7}	$<10^{-7}$ to >50%	29×10^{-9}	$<10^{-9}$ to >50%

* In the treated mice, 20 cultures were higher than any control; 19 were higher than any strain Zeut.
† In the treated mice, 23 cultures were higher than any control; 18 were higher than any strain Zeut.
‡ In the treated mice, 10 cultures were higher than any control or strain Zeut

TABLE 3. *Population proportions on streptomycin agar of serial substrains of staphylococci from treated mice*

Expt	Strain	Streptomycin concn (μg/ml)					
		3.9	15.6	62.5	125	500	2,000
		Median proportions					
	Zeut	10^{-5}	13×10^{-7}	19×10^{-9}	ND*		
	Second series						
	Parent N 12	0.64	0.30	1.5×10^{-5}	9×10^{-7}	2.1×10^{-8}	ND
O	Control mice	0.59	0.07	1.7×10^{-5}	15×10^{-7}	ND	ND
O	Treated mice	0.71	0.12	5.9×10^{-5}	75×10^{-7}	ND	ND
	Third series						
	Parent O 17	ND	0.14	8.9×10^{-3}	4.9×10^{-3}	3.7×10^{-4}	4.5×10^{-5}
Q	Control mice	ND	0.04	1.0×10^{-5}	8.9×10^{-7}	9.0×10^{-8}	$<10^{-9}$
Q	Treated mice	ND	0.14	8.3×10^{-5}	9.6×10^{-6}	6.8×10^{-7}	6.0×10^{-9}
	Second series						
	Parent S 21	3.0×10^{-3}	8.2×10^{-6}	1.0×10^{-9}	ND		
T	Control mice	3.1×10^{-6}	9.8×10^{-7}	1.4×10^{-9}	ND		
T	Treated mice	0.10	7.4×10^{-6}	1.6×10^{-7}	ND		
	Third series						
	Parent T 20	0.09	8.9×10^{-6}	3.9×10^{-7}	ND		
U	Control mice	0.21	2.1×10^{-5}	1.5×10^{-6}	ND		
U	Treated mice	0.23	1.5×10^{-5}	1.6×10^{-6}	ND		

* ND = not done.

tween treatments could allow time for the organisms to recover.

At the same time, sensitive staphylococci, which have been exposed even briefly to streptomycin in vitro, tend at first to grow more slowly than is normal in drug-free media. A disability of this nature might render them less able to compete in vivo with organisms possessed of some tolerance for the antibiotic and would account for the increase in the proportions of resistant individuals seen in cultures from treated mice.

However, the case of the resistant mutant that manages, in the absence of antibiotic, to increase from 1 in a billion until it reaches 10 to 50% of the population requires a different explanation. Eagle and Magnuson (1944) reported an experience of this sort, namely, the acquisition of resistance to arsenicals by a strain of *Trypanosoma equiperdum* maintained by mouse passage in the absence of any exposure to arsenic. During the present study, two such instances were encountered; cultures in which highly resistant staphylococci apparently formed at least 10% of the population were recovered from the tissues of control mice in two separate experiments. In addition to these strains from controls, four strains with high resistance were recovered from treated mice. It is suggested that the source of the cultures in this study may provide an explanation for such occurrences. Promptly after the intravenous inoculation of a massive dose of a relatively avirulent organism, such as this strain of staphylococcus, there is a rapid reduction of the bacterial population. It is not inconceivable that a resistant mutant might be among the few survivors, resulting in a growing population that would show an unusually high proportion of resistant organisms.

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