A MECHANISM FOR THE DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN AND PENICILLIN[1]

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Demerec (1945) and Luria (1946), in studying the resistance of staphylococci to penicillin, found that for a given inoculum (approximately 300,000,000 bacteria) there was a variation in resistance of approximately tenfold for all of their strains; e.g., if the inoculum required 0.1 unit of penicillin for complete inhibition, many of the bacteria in the inoculum would be inhibited by as little as 0.01 unit. The titer of 0.1 unit represented the resistance of a relatively small number of bacteria in the inoculum. Demerec indicated that the development of penicillin resistance resulted from the selection of the most resistant bacteria in concentrations of penicillin not completely inhibiting growth. By continued subcultures in higher penicillin concentrations resistance gradually developed through a series of small increments.

We found that, in the case of streptomycin (Klein and Kimmelman, 1946) and penicillin, if one examined only several million bacteria, there was a similar relatively small range of variation in resistance. However, by examining very large numbers of bacteria not previously exposed to streptomycin, namely, several billion, it was possible to isolate from all of six strains of the shigellae studied (standard inocula of which were inhibited by 3 to 7 units of streptomycin) variants resistant to more than 1,000 units of streptomycin. The presence of these highly resistant variants (approximately one resistant bacterium to one billion susceptible cells) was indicated to be the critical factor for the very rapid development of streptomycin resistance.

Clinically and *in vitro* (Graessle and Frost, 1946) bacteria become resistant to penicillin at a far slower rate than to streptomycin. If the few highly resistant variants are the factors determining the very rapid development of streptomycin resistance, then it follows that highly resistant variants should regularly be found present in strains showing a rapid rate of development in their streptomycin resistance. Highly resistant penicillin variants should be absent or far less frequent in strains showing a slow rate in their development of penicillin resistance.

In the present work we have tested several billion bacteria in each member of a group of strains for the presence of highly resistant variants against penicillin and streptomycin. We have found that while highly resistant variants against streptomycin were present in all the strains tested, no highly resistant variants were found against penicillin. These results were reflected by the rates at which the strains became resistant *in vitro* to the two chemotherapeutic agents.

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EXPERIMENTAL

The methods used in the assay procedures for resistant variants and the development of resistant strains are essentially similar to the procedures previously used (Klein and Kimmelman, 1946). The various penicillin and streptomycin dilutions were prepared in a final volume of 5 ml of extract broth, pH 7.3.[2]

Incidence of variants resistant to penicillin and streptomycin. In our assays very large inocula were used in order to permit the examination of many bacteria for the very few resistant variants present. The initial titers of the test strains

TABLE 1

*Incidence of variants resistant to penicillin**

TEST BACTERIA	INITIAL TITER; UNITS OF PENICILLIN PER ML INHIBITING GROWTH	NUMBER OF POSITIVE CULTURES (RESISTANT VARIANTS) IN 100 TUBES	TEST CONC. OF PENICILLIN; UNITS PER ML	FOLD INCREASE OF TEST PENICILLIN CONCENTRATION OVER INITIAL TITER
Staphylococcus aureus no. 726	0.1	0	2.0	20
Staphylococcus aureus no. 726	0.1	0	7.5	75
Staphylococcus aureus no. 726	0.1	0	7.5	75
Staphylococcus aureus no. 4A	0.5	0	2.5	5
Staphylococcus aureus no. 5A	0.1	0	2.0	20
Staphylococcus albus no. 7	0.1	0	2.5	25
Staphylococcus albus no. 7	0.1	0	7.5	75
Staphylococcus albus no. 7	0.1	0	7.5	75
Staphylococcus aureus no. 725	0.1	0†	1.0	10
Staphylococcus aureus no. 725	0.1	0	2.5	25
Staphylococcus aureus no. 725	0.1	0	4.0	40
Staphylococcus aureus no. 725	0.1	0	7.5	75
Staphylococcus aureus no. 723	0.1	0	1.5	15
Staphylococcus aureus no. 723	0.1	0	2.5	25
Staphylococcus aureus no. 723	0.1	0	7.5	75
Streptococcus viridans	6.0	0	600	100
Streptococcus viridans	6.0	0	250	41
Staphylococcus aureus no. 1A	0.05	0	0.5	10

* Inoculum used in determining initial titer was 0.4 ml of a 20- to 24-hour broth culture (approximately 80,000,000 bacteria). Number of resistant variants in each assay was determined by seeding 0.4 ml of culture into each of 100 broth tubes containing 5 ml of indicated test concentration of penicillin.

† Growth was observed after 72 hours in one tube of this assay.

listed in tables 1 and 2 were determined by using as our standard inoculum 0.4 ml of a 20- to 24-hour broth culture. As we have previously shown in the case of the shigellae (Klein and Kimmelman, 1946), though a given concentration of streptomycin may inhibit the growth of a single test inoculum, one cannot conclude that this specific strain cannot give rise to variants resistant to greater streptomycin concentrations. These highly resistant variants can only be found by examining very large numbers of bacteria.

[2] We wish to thank Dr. Chester S. Keefer, Chairman of the Committee on Chemotherapeutic Agents of the National Research Council, for our supply of streptomycin.

We tested our strains for variants resistant to penicillin and streptomycin by inoculating 100 of the standard 0.4-ml inocula into concentrations of penicillin or streptomycin many times greater than the concentration found inhibitory in the initial assays. For example, it was found that the 0.4-ml inoculum of *Staphylococcus aureus* no. 725 (table 1) was inhibited by 0.1 unit of penicillin. Four series of one hundred 0.4-ml samples were tested for variants resistant to 1, 2.5, 4, and 7.5 units of penicillin, which represented a 10-, 25-, 40-, and 75-fold increase, respectively, over the initial titer. For a given assay, if growth occurred in a single tube after 48 hours' incubation at 37 C, it indicated that one of the one hundred 0.4-ml inocula contained a bacterium resistant to the high test concentration of the chemotherapeutic agent. The results are summarized in tables 1 and 2.

Only four of the strains were tested against both of the agents, since they were the only strains sufficiently susceptible to both agents to permit an assay for

TABLE 2

Incidence of variants resistant to streptomycin

TEST BACTERIA	INITIAL TITER; UNITS OF STREPTOMYCIN PER ML INHIBITING GROWTH	NUMBER OF POSITIVE CULTURES (RESISTANT VARIANTS) IN 100 TUBES	TEST CONC. OF STREPTOMYCIN; UNITS PER ML	FOLD INCREASE OF TEST STREPTOMYCIN CONCENTRATION OVER INITIAL TITER
Escherichia coli	12	18	1,000	84
Proteus vulgaris	64	1	1,000	15
Staphylococcus albus no. 7	4	9	100	25
Staphylococcus albus no. 7	4	2	1,000	250
Staphylococcus aureus no. 4A	16	25	1,000	62
Staphylococcus aureus no. 5A	10	1	1,000	100
Staphylococcus aureus no. 726	16	1	1,250	80

resistant variants. For example, *Escherichia coli* and *Proteus vulgaris* were assayed only against streptomycin for resistant variants, since both strains grew in more than 50 units of penicillin per ml in the initial assay. As shown in tables 1 and 2, the incidence of resistant variants to the two agents differed markedly. On the assumption that growth in each tube represents the presence of one resistant variant, 57 variants resistant to high concentrations of streptomycin were found after seven assays of 100 tubes each. No highly resistant penicillin variants were found in eighteen 100-tube assays.

The rate of development of resistance to penicillin and streptomycin. Since highly resistant variants were present in the streptomycin assays but not in the penicillin assays, we studied the rate at which the four test strains (table 3) became resistant *in vitro* in order to determine the correlation between the incidence of resistant variants and the rate of development of resistance. Using an inoculum of 0.1 ml of a 20- to 24-hour broth culture, 0.1 ml was subcultured from the last tube showing growth in the penicillin and streptomycin assays to higher concentrations of the respective agents. The fold increase in resistance

is indicated after 3 subcultures in the case of streptomycin and after 3 and 6 subcultures in the case of penicillin. The results show that the four strains became resistant to streptomycin at a much more rapid rate than to penicillin. After only 3 subcultures all of the strains grew in 5,000 units of streptomycin per ml, the highest test concentration. The penicillin strains showed only a slight increase in resistance after 3 subcultures, and even after 6 subcultures the penicillin strains (excepting *S. aureus* no. 5a) did not reach the same level of resistance as was obtained with streptomycin.

We also attempted to study the presence of highly resistant variants in sulfadiazine. However, our standard inoculum of 0.4 ml grew in a saturated solution of sodium sulfadiazine in a casein hydrolyzate medium, and even a 100-fold reduction in the size of the inoculum still gave us growth in this sulfonamide concentration (1:500). Since it was not feasible under the conditions of our assay to

TABLE 3

Rate of development of resistance to penicillin and streptomycin

TEST BACTERIA	FOLD INCREASE IN RESISTANCE			
	After 3 subcultures		After 6 subcultures	
	Penicillin	Streptomycin	Penicillin	Streptomycin*
Staphylococcus albus no. 7	2	1,250	10	—
Staphylococcus aureus no. 4A	10	315	100	—
Staphylococcus aureus no. 5A	50	500	600	—
Staphylococcus aureus no. 726	2	315	100	—

* After 3 subcultures all of the strains grew in 5,000 units of streptomycin per ml, the highest concentration tested.

examine as large numbers of bacteria as had been tested with penicillin and streptomycin, no extended studies for resistant variants were done with the sulfonamides.

DISCUSSION

Our results indicate clearly that though variants showing a high degree of resistance can be found in cultures assayed against streptomycin, highly resistant variants are not found against penicillin. These results indicate that the following factors are involved in the rate of development of drug resistance.

If one inoculates bacteria into partially inhibitory concentrations of, say, 4 units of streptomycin, many of the bacteria will be killed; and as the most resistant bacteria in the inoculum multiply, they will show a distribution pattern in their resistance. Since the bacteria are now growing in 4 units of streptomycin, variation of the bacteria will occur in the direction of resistance greater than 4 units, and the degree of increased resistance will be determined by the extent of variation in resistance of the new bacteria. If the bacteria give rise to cells in which the range of resistance is very great, e.g., bacteria growing in 4 units of streptomycin giving rise to variants resistant to 100 or 1,000 units of strepto-

mycin, then the rate of development of resistance will be very rapid. This is the probable pattern of development in the case of streptomycin.

In the case of penicillin, however, there is not a broad range of penicillin-resistant bacteria, and highly resistant variants do not occur. Therefore, if bacteria are growing in partially inhibitory concentrations of, say, 0.1 unit of penicillin, the distribution of resistance in bacteria growing in this concentration will always be relatively close to 0.1 unit, and the rate of development of resistance will be slow.

Whether or not the selection of resistant variants is the only factor involved in the development of resistance is difficult to determine. Though we have previously found (Klein and Kimmelman, 1946) that the shigellae when in the resting stage show no adaptation to streptomycin after prolonged exposure, it is possible that a change in resistance can occur when streptomycin acts on susceptible dividing bacteria. The problem is difficult because, if one works with dividing cells, it would be hard to determine whether the increased resistance results from the presence of resistant variants or the interaction between susceptible dividing bacteria and streptomycin.

SUMMARY

A total of 57 variants resistant to high concentrations of streptomycin were isolated from all of six strains tested. No variants possessing a high degree of resistance to penicillin were found in a total of eight strains. The incidence of resistant variants against the two chemotherapeutic agents was correlated with the rate at which resistance developed *in vitro*.

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