Further Genetic Studies on the Kanamycin Resistance of *Mycobacterium Avium*.

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Genetic information concerned with the resistance to a new antibiotic, kanamycin, in microorganisms is yet scant. The author and his associates (Tsukamura *et al.*, 1959) have reported that the genetic system of kanamycin resistance in *Mycobacterium tuberculosis* consists of multiple factors that differ in their potency, and high resistance to kanamycin is produced by a single gene mutation or by polygenic system. This genetic system resembled that for streptomycin resistance in this organism (Tsukamura *et al.*, 1958) and in other bacteria previously reported by Hotchkiss (1952, 1955), Ushiba and Watanabe (1955), Hashimoto (1957), and Hsu (1957). The authors (Tsukamura and Yamamoto, 1959) have detected that the kanamycin resistance-system of *Mycobacterium avium*, a R-type mutant Jucho strain, differs from that of *M. tuberculosis*. It lacks such a system producing high resistance. The present paper concerns the genetic system of the original S-type Jucho strain of *M. avium* for kanamycin resistance. The genetic system of this organism has showed an interesting feature being different from the genetic systems of other microorganisms controlling drug resistance. It has consisted of two systems. The one is a polygenic system producing kanamycin resistance up to 1 ug/ml.; and the other is a single gene system producing almost infinitely high resistance to kanamycin that may be called as "kanamycin indifference". Unlike the case of streptomycin resistance, high kanamycin resistance has appeared to be not produced by the polygenic system.

Methods

Strain. *Mycobacterium avium*, strain Jucho, was used throughout the study. The parent strain used in the study was derived from a single colony of the stock culture. The strain represented S-type colony morphology.

Medium. Sauton medium and Sauton agar medium were used. Sauton medium consisted of 4.0 gm. of sodium glutamate, 0.5 mg. of dibasic potassium phosphate, 0.5 mg. of magnesium sulfate, 2.0 gm. of sodium citrate, 0.05 gm. of ferric ammonium citrate, 40 ml. of glycerin, and 960 ml. of distilled water. The pH was adjusted to 7.2 and the resultant medium was dispensed in 50 ml. amounts into 200 ml. Erlenmeyer

flasks and autoclaved at 115°C for thirty minutes. Sauton agar medium consisted of Sauton medium, pH 7.0, containing 3 per cent agar. The Sauton agar medium was dispensed in 20 ml. amounts to Petri dishes, 9 cm. in diameter. Kanamycin sulfate (Meiji Co.) was dissolved aseptically in distilled water and added to media immediately before use.

Calculation of viable bacterial numbers. Seven-day-old cultures in Sauton medium were homogenized by shaking with glass beads and suspended in physiological saline. The cell suspension*) was tenfold diluted. Each 0.1 ml. of different dilutions were inoculated to a series of Sauton agar media containing graded concentrations of kanamycin including control without drug. The plates inoculated were incubated at 37°C for five days and counts were made.

Determination of population structure of clones. A single whole colony was picked from Sauton agar medium and subcultured in a drug-free Sauton medium to form a large population. The clone resulted, a bacterial population derived from a single colony, was analysed for its population structure. The population structure of clones was expressed as a survival curve, i.e., a series of survival ratios on Sauton agar media containing graded concentrations of kanamycin.

Principle of the study. It appears to be a general rule in the genetics to suppose the existence of one genotype corresponding to one character. Phenotypic characters readily are observed in each individual of higher organisms. They are almost impossible to be observed in microorganisms. Characters of a cell are not observed but those of a clone are observed. The character of clones in relation to drug resistance must be the population structure of clones in relation to drug resistance. The present study will be conducted by assuming a hypothesis that one type of population structure of clones corresponds to one genotype.

Results

1. Survival curve (population structure) of the parent strain.

The survival curve of the parent strain previously not exposed to any drug was shown in Fig. 1. Most cells of the parent strain could grow on media containing up to 0.3 $\mu\text{g/ml.}$ to 0.4 $\mu\text{g/ml.}$ of kanamycin, and the curve rapidly decreased on media containing more than 0.5 $\mu\text{g/ml.}$ of kanamycin. Survivors capable of growing on media containing 0.5 $\mu\text{g/ml.}$ of kanamycin (surviving colonies resistant to 0.5 $\mu\text{g/ml.}$ of kanamycin) were found at a rate of several cells per 10^5 viable population and survivors resistant to 1 $\mu\text{g/ml.}$ of kanamycin were found at a rate of about 10^{-8} . Survivors resistant to 2 $\mu\text{g/ml.}$ of kanamycin were not found among 10^9 viable population of the parent strain. Such survivors could be detected in two of about ten experiments, in which about 10^9 viable population were utilized for each test. It has been suggested

*) The cell suspension consisted mostly of single cells and clumps of several cells. It was demonstrated that, under the conditions tested, only one cell is viable among about five cells, because the cell suspension used here gave about one fifth of viable counts given by a cell suspension prepared from a one-day-old culture, which exhibited the same optical density. It has been thus considered that each colony may be derived from a single viable cell.

that such survivors appear at a rate of about 10^{-10} in the parent strain. Survivors resistant to above $5 \mu\text{g}/\text{ml}$. could not be found by the first-step selection of the parent strain throughout the study.

2. Survival curves (population structures) of the first-step resistant clones.

Survivors grown on media containing different concentrations of kanamycin indicated by the arrows in figure 1 were picked and subcultured in Sauton medium to form a large population. The clones obtained were analysed for their population structures and the results are shown in fig. 2.

Clones derived from single colonies grown on media containing up to $0.4 \mu\text{g}/\text{ml}$.

Fig. 1. Survival curve for the parent strain of *M. avium* (Jucho) on different concentrations of kanamycin.

Fig. 2. Survival curves for first-step, second-step, and third-step resistant clones on different concentrations of kanamycin.

Survival curves of first-step, second-step, and third-step resistant clones (strains) were derived from a single colony grown on media with kanamycin concentrations indicated by the arrows, respectively. R_n indicates the survival curve of a first-step resistant clone derived from a single colony grown on medium containing $n \mu\text{g}/\text{ml}$. of kanamycin, and R_{n-m} indicates the survival curve of a second-step clone derived from a single colony grown on medium containing $m \mu\text{g}/\text{ml}$. of kanamycin. The process was repeated until third-step resistant clones were obtained.

of kanamycin showed survival curves similar to the survival curve of the parent strain. As mentioned in the principle of the study, the results suggest that these clones have the wild genotype, that is, the same genotype as the parent strain. On the other hand, clones derived from single colonies grown on media containing kanamycin more than $0.5 \mu\text{g/ml}$. represented survival curves different from the survival curve of the parent strain. The results suggest that these clones have any genotypes different from the wild genotype. In addition, they differed in their form depending upon the concentration of kanamycin, on which the colonies, from which the clones were derived, had grown. The existence of different types of population structures (survival curves) of the clones suggest that these clones have different genotypes differing in their potency.

It appeared remarkable that two clones tested each derived from a single colony grown on medium containing $2 \mu\text{g/ml}$. of kanamycin could survive on media containing much higher concentrations (more than $1,000 \mu\text{g/ml}$.) of kanamycin, whereas the clones derived from a single colony grown on medium containing $1 \mu\text{g/ml}$. of kanamycin could survive on media containing up to $1 \mu\text{g/ml}$. of kanamycin. Highly resistant survivors were not found in about 10^8 viable cells of the clones derived from the survivors on medium containing $1 \mu\text{g/ml}$. of kanamycin. The use of large inocula of these clones produced some membranous growth on medium containing $2 \mu\text{g/ml}$. of kanamycin, probably derived from pseudoresistance as indicated by Tsukamura *et al.* (1959) in *M. tuberculosis*.

3. Survival curves (population structures) of the second-step and third-step clones.

Selections of the first-step resistant clones with higher concentrations of kanamycin produced clones showing more resistant survival curves (fig. 2). However, any resistant clones were not obtained, which are more resistant than $1 \mu\text{g/ml}$. of kanamycin. Further selections of the clones resistant to $1 \mu\text{g/ml}$. remained ineffective.

4. First-step selection of highly resistant mutants.

Tests have been made in which a whole growth of Sauton medium cultures were planted into a fresh Sauton medium containing $100 \mu\text{g/ml}$. of kanamycin, but no pellicle formation was obtained in the medium. However, it has been remarkable that, when such a whole growth was planted into fresh Sauton medium containing both $100 \mu\text{g/ml}$. of kanamycin and $100 \mu\text{g/ml}$. of ammonium sulfate, a new pellicle formation occurred in the medium. This pellicle consisted of mutants resistant to more than $1,000 \mu\text{g/ml}$. of the drug. The resultant mutants could readily grow in medium containing no ammonium sulfate.

Discussion

The results obtained suggest that survivors on media containing more than $0.5 \mu\text{g/ml}$. of kanamycin are resistant mutants, since they maintained their resistance properties throughout subcultures in drug-free medium and the property of kanamycin resistance appeared to be stable genetically. They also suggest that the survival on media containing up to $0.4 \mu\text{g/ml}$. of kanamycin is produced by the existence of the

wild genotype. The existence of different survival curves (population structures) of clones tested suggests that there are different genotypes that may be produced by a polygenic system. It appears that double or multiple step mutations of the polygenic system produce more resistant clones, since further selections of the first-step resistant clones produced more resistant clones. It appears also noteworthy that kanamycin resistance levels produced by the polygenic system have the upper limit of about $1\text{ }\mu\text{g/ml.}$ of kanamycin. Therefore, it would be said that the polygenic system can produce only a narrow increase of kanamycin resistance in the organism.

Survivors resistant to $2\text{ }\mu\text{g/ml.}$ of kanamycin obtained by the first-step selection could produce clones resistant to infinitely high resistance to kanamycin. The clones appeared as if "indifferent" from kanamycin. These kanamycin-indifferent mutants were not found among 10^9 viable cells of kanamycin-resistant clones but seemed to be found at a rate less than 10^{-10} in the parent strain. The origin of the kanamycin-indifferent mutants appears to be different from kanamycin-resistant mutants produced by the polygenic system and to be derived from any single gene mutation.

Usual successive transfers of the organism in Sauton media containing increasing concentrations of kanamycin do not give the development of the kanamycin-indifferent strain. The kanamycin-indifferent mutants sometimes could be detected by inoculating massive inocula of the parent strain onto Sauton agar media containing $2\text{ }\mu\text{g/ml.}$ of kanamycin with or without $100\text{ }\mu\text{g/ml.}$ of ammonium sulfate. Sometimes it has appeared that the subculture of the organism in Sauton medium containing nucleic acids extracted from the parent strain has increased a chance to obtain such mutants. The kanamycin-indifferent mutants were isolated by inoculating a massive inoculum (a whole growth of Sauton medium cultures) into Sauton medium containing both $100\text{ }\mu\text{g/ml.}$ of kanamycin and $100\text{ }\mu\text{g/ml.}$ of ammonium sulfate per ml. of medium. However, the mutants once isolated did not require ammonium sulfate for the growth.

The author's unpublished data demonstrated that the streptomycin resistance-system of the test organism also was similar to the streptomycin resistance-systems previously reported in other bacteria (Hotchkiss, 1952, 1955; Ushiba and Watanabe, 1954, 1955; Hashimoto, 1957; Hsu, 1957; Tsukamura *et al.*, 1958). The streptomycin resistance-system consists of a single gene system producing streptomycin indifference and a multiple gene system producing both high and low resistance. The multiple gene system for streptomycin resistance can produce phenotypes extremely, as if infinitely, high resistance to streptomycin. It appears remarkable that, in the case of kanamycin, the polygenic system can produce a narrow increase of resistance levels ranging from $0.5\text{ }\mu\text{g/ml.}$ to $1\text{ }\mu\text{g/ml.}$ and cannot produce high resistance to kanamycin.

Summary

Genetic studies on the kanamycin resistance-system of *Mycobacterium avium*, original S-type Jucho strain, have been conducted by the population analysis method. The resulting conclusions are as follows:

There are multiple population structures of clones obtained by the first-step

selection of the parent strain previously not exposed to any drug. The results suggest that there are multiple genotypes producing different population structures of clones.

The wild genotype produces resistance levels to kanamycin up to 0.4 $\mu\text{g/ml}$. Resistance levels up to 1 $\mu\text{g/ml}$. of kanamycin seem to be produced by mutations of the polygenic system. High resistance levels seem to be produced by a possible single gene mutation. There is a marked difference between the streptomycin resistance-system and the kanamycin resistance-system of the test organism. The polygenic system for kanamycin resistance can produce only a limited levels of resistance (up to 1 $\mu\text{g/ml}$.), while the multiple gene system for streptomycin resistance-system can do extremely high resistance levels.

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