Loss of Hextuple Resistance to Aminoglycoside Antibiotics in *Mycobacterium tuberculosis* (H37Rv) by Mutation to Isoniazid Resistance and by Incubation at High Temperature

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INTRODUCTION

Tsukamura & Mizuno (1975) reported that various types of multi-resistant mutants could be isolated by single-step selection from the H37Rv strain of *Mycobacterium tuberculosis*, which had not been exposed previously to any antibiotic. Hextuply-resistant mutants (6R mutants), which are resistant to high concentrations of six aminoglycoside antibiotics, emviomycin (tuberactinomycin N), viomycin, capreomycin, kanamycin, lividomycin and paromomycin, were thought to be produced by a single mutation. This paper describes studies carried out on the 6R mutants, which lose their hextuple resistance on mutation to isoniazid resistance and on incubation at 45 °C.

METHODS

Strain. All mutants were isolated from the H37Rv strain of *Mycobacterium tuberculosis*. They were all derived from single colonies, and all resistant mutants were isolated by single-step selection, unless otherwise stated. Three 6R mutants (05077, 05078 and 05079) were isolated from media containing paromomycin (1 mg ml⁻¹). Isoniazid-resistant mutants were obtained from them by selection on medium containing isoniazid (10 µg ml⁻¹). A 4R mutant (05083) was isolated by selection with paromomycin (1 mg ml⁻¹). Mutant 05009 was isolated by two-step selection, first with 10 µg isoniazid ml⁻¹ and then with 100 µg isoniazid ml⁻¹; it was resistant to 100 µg isoniazid ml⁻¹. Mutant 05021 was isolated using 10 µg isoniazid ml⁻¹ and was resistant to 10 µg isoniazid ml⁻¹.

Measurement of resistance levels. The antibiotics and methods were mainly those used previously (Tsukamura & Mizuno, 1975), except for a simplified method for measuring resistance. One loopful (about 0.2 mg moist weight) of the test strain was inoculated on to Ogawa egg medium slants containing various concentrations of the antibiotics and incubated at 37 °C for 3 weeks. The resistance levels were taken to be the highest antibiotic concentration at which the test organism showed the same growth as on control medium without the drug. The method gave almost the same results as the quantitative method used previously.

Emviomycin (previously called tuberactinomycin N), viomycin, capreomycin, kanamycin, lividomycin, and paromomycin were used at concentrations of 0, 100, 200, 500 and 1000 µg ml⁻¹. The concentrations of isoniazid (Shionogi Co., Osaka, Japan) were 0, 0.1, 1, 10 and 100 µg ml⁻¹.

Incubation at 45 °C. Two types of experiments were carried out. (1) The test strain was inoculated on to Ogawa egg medium slants and incubated at 37 °C for 3 weeks before trans-

ferring the slants to an incubator at 45 °C. Immediately, and after 1, 2 and 3 days incubation, a bacterial suspension (10 mg wet wt ml⁻¹) was prepared and inoculated with a spiral loop (0.02 ml) on to media containing various concentrations of antibiotics to determine resistance levels.

(2) A bacterial suspension (1 ml; 10 mg wet wt ml⁻¹) was added to modified Sauton medium (5 ml) and the mixture was incubated at 45 °C. Immediately, and after 3, 6, 24 and 48 h, it was inoculated with the spiral loop on to the media containing various concentrations of antibiotics. The modified Sauton medium contained: sodium glutamate, 4.0 g; glycerol, 30 ml; KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.5 g; ferric ammonium citrate, 0.05 g; citric acid, 2.0 g; purified agar, 20.0 g; distilled water, 970 ml. The pH was adjusted to 7.0 by adding 10 % (w/v) KOH solution and the medium was sterilized by autoclaving at 120 °C for 20 min.

Designation of phenotypes. The phenotypes were defined according to the numbers of antibiotics to which they were resistant (see Tsukamura & Mizuno, 1975). Four subtypes of the 6R phenotype had previously been distinguished, 6R, 6R', 6R'' and 6R''', the last three being considered modifications of the 6R phenotype. However, all the 6R strains resembled the 6R'' phenotype after several transfers on medium containing no drug and in the present study were all designated as 6R.

RESULTS

Effect of mutation to isoniazid resistance

The three 6R mutants, 05077, 05078 and 05079, were inoculated on to media containing isoniazid (10 µg ml⁻¹). Isoniazid-resistant mutants occurred at a frequency of 1 to 2 in 10⁶ of the total viable population. Single colonies were subcultured on medium without any drug and tested for their phenotype. Out of 35 isoniazid-resistant mutants tested, 29 (83 %) had lost their hexuple resistance and the remaining six (17 %) maintained their 6R phenotype.

Attempts were made to isolate back mutations to the 6R phenotype from these isoniazid-resistant hexuply-sensitive mutants. Out of 12 colonies of a revertant (05077-INH R) that survived on medium containing paromomycin (1 mg ml⁻¹), two were 6R mutants and the remaining 10 were 4R mutants. Out of 30 colonies of another revertant strain (05079-INH R) that survived on the same medium, none were 6R mutants; all 30 colonies had the 4R phenotype. The numbers of surviving colonies on the medium containing paromomycin (1 mg ml⁻¹) were 183 and 72 out of 10⁹ viable bacteria for strains 05077 and 05079, respectively.

With the parent H37RV strain, the number of surviving colonies on medium containing paromomycin (1 mg ml⁻¹) was 145 out of 10⁹ viable bacteria; of 56 such colonies, 33 were of the 6R phenotype, 14 the 4R phenotype, and nine the 3Ra phenotype (Tsukamura & Mizuno, 1975). Thus, the frequency of the 6R mutants in the revertants appeared to be less than that of the parent H37RV strain.

For comparison, isoniazid-resistant mutants were selected from the 4R strain (05083). Isoniazid resistance occurred at a frequency of 9×10^{-6} . Of 15 mutants derived from single colonies surviving on medium containing isoniazid (10 µg ml⁻¹), all retained their quadruple resistance.

The loss of hexuple resistance that occurred in 6R strains on mutation to isoniazid resistance suggested that isoniazid-resistant strains would have diminished capacity to develop hexuple resistance. To test this possibility, mutants of the 6R type were selected from two isoniazid-resistant mutants (05009 and 05021) by their resistance to lividomycin or paromomycin (1 mg ml⁻¹). However, 6R mutants were produced from isoniazid-resistant strains as readily as from the parent H37RV strain (Table 1).

Table 1. *Phenotype of aminoglycoside-resistant strains isolated from isoniazid-resistant strains by single-step selection with lividomycin (1 mg ml⁻¹) or paromomycin (1 mg ml⁻¹)*

Strain	Antibiotic used for selection	No. of surviving colonies*	No. of resistant strains identified of each phenotype:			No. of strains tested
			6R-INH R	4R-INH R	3Ra-INH R	
05009	Lividomycin	30	16	3	1	20
	Paromomycin	88	15	32	3	50
05021	Lividomycin	53	12	1	2	15
	Paromomycin	171	11	38	1	50
H37RV	Lividomycin	21	3	9	0	12
	Paromomycin	145	33	14	9	56

* Out of 10⁸ viable parent bacteria.

Loss of phenotype of hexuple resistance by incubation at high temperature

Three-week-old cultures of the three 6R mutants were incubated at 45 °C. At intervals, the mutants were inoculated on media containing various concentrations of the six antibiotics. They appeared to lose their resistance after 2 to 3 days (method 1) or 24 to 48 h (method 2). Ten colonies were isolated from each strain and were shown to be susceptible to all six antibiotics. These sensitive colonies had a white, smooth appearance unlike the rough original H37RV strain. Like the latter, they were acid-fast, reduced nitrate and produced niacin.

The same experiment was carried out with the 4R strain, but no loss of quadruple resistance occurred.

To exclude the possibility that spontaneous antibiotic-sensitive revertants which had been derived from the 6R mutants were more resistant to heating than their parents, the resistance to heating of the original H37RV strain was compared with that of the multi-resistant strains. The heat resistance of the strains was similar; the numbers of viable organisms were reduced by about 10³ after 3 days incubation. It was concluded that these antibiotic-sensitive mutants were not the result of a selective mechanism but of a conversion of hexuply-resistant mutants to susceptible organisms by heat. The change seemed to occur under the condition in which the number of viable organisms of the 6R strains had decreased to 0.1 %.

Three 6R strains were incubated in 0.067 M-phosphate buffer (pH 7.1) containing 0.01, 0.1 and 1 % (w/v) acrinol (6,9-diamino-2-ethoxy-acridine lactate hydrate) for 3 to 72 h, but no loss of the 6R phenotype occurred. The rates of occurrence of doubly-resistant mutants were estimated in mixed cultures of a 6R strain with a streptomycin-resistant strain, a rifampicin-resistant strain or an isoniazid-resistant strain, but no significant increase of the prevalence of doubly-resistant mutants (kanamycin-streptomycin, kanamycin-rifampicin, or kanamycin-isoniazid) was observed.

DISCUSSION

In this study, we have shown that 6R mutants lost their hexuple resistance after mutation to isoniazid resistance. The mutation to isoniazid resistance in *M. tuberculosis* is known to be accompanied by modification of other characters, for example, decrease in the uptake of isoniazid (Barclay, Ebert & Koch-Weser, 1953), attenuation of virulence for guinea pigs (Peizer, Widelock & Klein, 1953; Middlebrook & Cohn, 1953; Mitchison, 1954), decrease

in catalase activity (Middlebrook, 1954), decrease in peroxidase activity (Tirunarayanan & Vischer, 1957), increased susceptibility in nitrofurans (Beutner, Doyle & Evander, 1963), and increased susceptibility to *p*-aminophenol (Tsukamura & Tsukamura, 1964). Furthermore, the phenotype of isoniazid resistance appears promptly without phenomic lag, unlike the phenotype of streptomycin resistance (Tsukamura, 1962). All these findings suggest that the mutation to isoniazid resistance involves a deletion in the genome and occurs without re-organization or synthesis of enzymes, and that the deletion is accompanied by deletion of other genes.

Jones & David (1972) found that one type of streptomycin resistance in *M. smegmatis* was apparently carried by a plasmid. It is possible that hexuple resistance in *M. tuberculosis* is conferred by a plasmid which is lost during mutation to isoniazid resistance, and is eliminated by heating, as are some plasmids of *Staphylococcus aureus* (Lacey, 1975). Unlike streptomycin resistance in *M. smegmatis*, however, hexuple resistance is not 'cured' by acrinol.

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