RESISTANCE PATTERN TO TUBERACTINOMYCIN-N AND CROSS-RESISTANCE-RELATIONSHIPS BETWEEN TUBERACTINOMYCIN-N, LIVIDOMYCIN, CAPREOMYCIN, VIOMYCIN, KANAMYCIN, AND STREPTOMYCIN RESISTANCES OF *MYCOBACTERIUM TUBERCULOSIS* (H 37 Rv)

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Introduction

Tuberactinomycin-n (TUM-N) is a new aminoglucoside-antibiotic recently produced in Japan[1,2]. TOYOHARA *et al.*[3] reported that viomycin-resistant strains and capreomycin-resistant strains of *Mycobacterium tuberculosis* were resistant to this antibiotic, and very recently, SAITO[4] and KOSEKI *et al.*[5] reported that TUM-N-resistant strains of *M. tuberculosis* were resistant to viomycin and capreomycin. These authors isolated TUM-N-resistant strains about 8-times more resistant to TUM-N than the parent H 37 Rv strain. However, pattern of resistance development has not yet been studied, as the studies by these authors remained still non-quantitative. The purpose of the present study is to deal with the pattern of resistance development to TUM-N in *M. tuberculosis* by a quantitative method and cross-resistance-relationships between TUM-N resistance and resistances to other aminoglucoside-antibiotics in this organism.

Materials and Methods

Strain. *Mycobacterium tuberculosis* strain H 37 Rv was used.

Medium. OGAWA egg medium was used. The composition of this medium is as follows: Basal solution (1% KH_2PO_4 and sodium glutamate), 100 ml; whole eggs, 200 ml; glycerol, 6 ml; 2% malachite green solution, 6 ml. The medium was poured in 8 ml quantities into tubes, 170 by 17 mm, and made as slopes by sterilization at 95°C for 60 minutes. Aminoglucoside-antibiotics were dissolved in distilled water and added to the medium before sterilization. The following agents were used: Tuberactinomycin-N sulfate (TUM-N) (Toyo Jozo Co., Shizuoka, Japan); Lividomycin sulfate (LVM) (Kowa Co., Tokyo); Capreomycin sulfate (CPM) (Eli Lilly Co., Basingstoke, England); Viomycin sulfate (VM) (Parke, Davis & Sankyo Co., Tokyo); Kanamycin sulfate (KM) (Meiji Seika Co., Tokyo); Streptomycin sulfate (SM) (Meiji Seika Co., Tokyo). The following antituberculous agents also were used: Isoniazid (INH) (Shionogi Co., Osaka); Sodium *p*-aminosalicylate (PAS) (Shionogi Co., Osaka); Ethionamide (TH) (Shionogi Co., Osaka); Amithiozone (TB) (Shionogi Co., Osaka); Ethambutol (EB) (Kaken Kagaku Co., Tokyo); Rifampicin (Lepetit, Milano, Italy). TH, TB and RFP were first dissolved in propylene glycol and added to medium.

Method of Studies of Resistance-Pattern.

The method was described previously[6]. It consists of preparing survival curves for strains isolated on various concentrations of the test agent. The test strain was cultivated in drug-free medium at 37°C for 3 weeks. The growing organisms were homogenized by shaking with glass beads, and a bacterial suspension (20 to 30 mg, moist weight/ml) was prepared. The suspension was diluted with saline (0.9% NaCl solution) in order to obtain a series of ten-folds dilutions. A 0.02 ml sample from each dilution was inoculated to a series of media containing various concentrations of an agent, using a spiral loop. The number of series of media was equal to the number of dilutions. In each series, the media consisted of a medium containing no agent and media each containing a concentration of the antibiotic. Usually, the following concentrations were used: 2, 3, 5, 10, 20, 30, 50, 100, 200, 300, 400, 500, 1,000 μg/ml. Though, in the previous study[6], ten media were used for a concentration, "one medium-one concentration"-system was used in the present study.

The tubes inoculated were stoppered with a gum

cap and incubated at 37°C for 4 to 8 weeks. The number of colonies was counted, as a rule, at 4 weeks. However, it was counted at 6 weeks at primary isolation of resistant colonies (on media inoculated with the parent strain), as the colonies grew slowly on primary isolation medium. Ratios of the number of colonies surviving at each concentration to the total viable population were calculated and, from these data, a survival curve for the parent H 37 Rv strain which was not exposed previously to any agent was prepared (survival curve P). At the next experiment, single colonies were picked up and cultivated in drug-free media for 3 weeks. Strains, each derived from single colony, were named "first step-strains" and were designated as, for example, TUM-N 50 a R. This is a strain derived from single colony grown on medium containing 50 μg/ml TUM-N. If three strains were obtained on the same concentration, these were named as a, b, and c. Survival curves of the first step-strains were prepared as described in the above.

The second step-strains were designated as, for example, TUM-N 50 b-200* R. This indicates a strain derived from single colony isolated from the first step-strain TUM-N 50 b R by inoculating it on a medium containing 200 μg/ml TUM-N, and a symbol* shows that the colony grown on the medium containing 200 μg/ml TUM-N was a small or minute one.

Method of Measurement of Resistance-Level of Strains.

Resistance-level of a strain to an agent was measured by the "actual count" method[7~9]. The resistance-level was expressed as the highest concentration of an antibiotic, on which a small inoculum consisting of 20 to 50 (if this is not obtained, 10 to 100) viable organisms could grow after incubation at 37°C for 4 weeks. The results were shown by this method, unless specially noticed. The data concerning with TUM-N were read at both 4 and 6 weeks.

The concentrations of aminoglucoside-antibiotics used and the methods for preparation of bacterial suspensions and for inoculation were similar to those used in preparation of survival curves.

Resistance-levels to antituberculous agents other than the aminoglucoside-antibiotics also were measured by the same method. The concentrations used were as follows : INH- 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 5, 10 μg/ml ; PAS- 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 5, 10 μg/ml ; TH- 0, 5, 10, 20, 30, 50, 100 μg/ml ; RFP- 0, 5, 10, 20, 30, 50, 100 μg/ml ; EB, 0, 1, 2, 3, 5, 10, 20, 40 μg/ml ; TB- 0, 5, 10, 20, 50, 100 μg/ml.

Results and Discussion

Resistance Pattern of *M. tuberculosis* H 37 Rv to Tuberactinomycin-N.

There were two phenotypes of resistance to TUM-N ; one showing resistance-level 50 to 200 μg/ml and another showing resistance-level more than 1,000 μg/ml. Resistance-levels belonging to 50 to 200 μg/ml were considered to be a phenotype, as the levels became equally 200 μg/ml at 6 weeks and variation between 50 to 200 μg/ml resistance-levels were considered to be due to experimental variation (Table 1).

The results are summarized in Figure 1. There are three types of survival curves ; the first type of the parent strain (P) ; the second type of low resistance (R 1) ; the third type of high resistance (R 2).

Previously SAITO[4] and KOSEKI *et al.*[5] reported that the resistance-level of their TUM-N-resistant strains of *M. tuberculosis* H 37 Rv was about 8-times higher than that of the parent strain. It was found in the present study that there was another type, high resistance to this antibiotic. The resistance-level of this type was as high as more than 100-to 200-times of that of the parent strain. It was considered that there are two phenotypes of TUM-N resistance in *M. tuberculosis* H 37 Rv.

Fig. 1 Survival curves for strains of *M. tuberculosis* H 37 Rv isolated on various concentrations of TUM-N

P : Survival curve for parent strain, TUM-N 5 R, and TUM-N 30* b, c R.

R 1 : Survival curve for TUM-N 30 a R, TUM-N 50 R, TUM-N 100 R, TUM-N 25* b-50 R, TUM-N 25* b-200* R, and TUM-N 25* c-200* R.

R 2 : Survival curve for TUM-N 200 R, TUM-N 200 a-1000 R, and TUM-N 200 b-500 R.

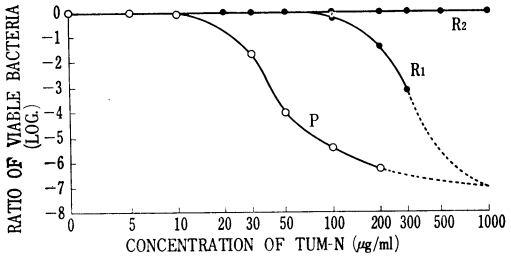


Table 1 Resistance-levels to various aminoglucoside-antibiotics of TUM-N-resistant strains of *M. tuberculosis* strain H37Rv

Strain	Resistance level (μg/ml) to :						
	TUM-N §	TUM-N §	LVM	CPM	VM	KM	SM
Parent	5	10	30	10	10	10	5
TUM-N 10a R	5	10	30	10	10	10	5
TUM-N 10b R	10	10	30	10	10	10	5
TUM-N 10c R	10	10	30	10	10	10	10
TUM-N 30a R	50	200	30	200	200	20	5
TUM-N 30b*R	10	10	30	10	10	10	5
TUM-N 30c*R	10	10	30	10	10	10	10
TUM-N 50a R	50	200	30	200	200	20	5
TUM-N 50b R	50	200	30	200	300	20	10
TUM-N 50c R	50	200	30	200	200	20	10
TUM-N 100a R	200	200	50	200	300	30	10
TUM-N 100b R	200	200	50	200	300	20	10
TUM-N 100c R	50	200	30	200	200	20	10
TUM-N 200a R	>1000	>1000	>1000	>1000	>1000	>1000	3
TUM-N 200b R	>1000	>1000	>1000	>1000	>1000	>1000	10
TUM-N 200c R	>1000	>1000	>1000	>1000	>1000	>1000	5
TUM-N 25a-100 R	200	200	50	200	200	20	10
TUM-N 25b*-50 R	100	200	30	100	100	10	5
TUM-N 25b*-200*R	100	200	30	200	200	10	5
TUM-N 25c*-200*R	200	200	20	200	200	10	5
TUM-N 200a-1000 R	>1000	>1000	>1000	>1000	>1000	>1000	5
TUM-N 200b-500 R	>1000	>1000	>1000	>1000	>1000	>1000	10

§ Left : Read at 4 weeks; Right : Read at 6 weeks. * Strain originated from a small colony.

Table 2 Resistance-levels to various antituberculous agents of TUM-N-resistant strains of *M. tuberculosis* H37Rv

Agent	Resistance level (μg/ml)
Isoniazid (INH)	<0.1
Sodium p-aminosalicylate (PAS)	<0.1
Ethionamide (TH)	5 to 10
Rifampicin (RFP)	5 to 10
Ethambutol (EB)	1
Amithizone (TB)	<5

The strains used for tests are shown in Table 1.

TUM-N-resistant strains showed the same resistance-level as the parent strains.

Cross-Resistance-Relationships between TUM-N Resistance and Resistances to Other Antituberculous Agents in *Mycobacterium tuberculosis* H 37 Rv.

All TUM-N-resistant strains shown in Table 1 were as susceptible as the parent H 37 Rv strain to antituberculous agents other than aminoglucoside-antibiotics (Table 2). Resistance-levels to various aminoglucoside-antibiotics of the strains are shown in Table 1.

Resistance-levels to TUM-N of the strains resistant to other aminoglucoside-antibiotics are shown in Table 3.

From the results shown in Table 2 and Table 4, it is evident that there is no cross-resistance relationship between resistances to aminoglucoside-antibiotics and resistances to INH, PAS, RFP, TH, EB and TB.

From the results shown in Table 1 and Table 3, cross-resistance-relationships between resistances to aminoglucoside-antibiotics are as follows :

(1) SM-resistant strains, both high and low, are susceptible to all others, LVM, CPM, VM, KM, and TUM-N ;

(2) KM-lowly-resistant strains are resistant to only KM and are susceptible to all others, TUM-N, LVM, CPM, VM, and SM ; KM-highly-resistant strains are resistant to KM, LVM and CPM and susceptible to TUM-N, VM, and SM ; one-way-cross-resistance from KM-high resistance to CPM resistance is not marked in strains obtained by one step-selection, but it is significant in strains ob-

Table 3 Resistance-levels to various aminoglucoside -antibiotics of strains resistant to LVM, CPM, VM, KM and SM isolated from *M. tuberculosis* H37Rv

Strain	Resistance level (μg/ml)					
	TUM-N	LVM	CPM	VM	KM	SM
LVM 400 R	10	500	200	100	200	5
CPM 100 R	200	500	200	100	10	5
VM 100 R	200	30	100	100	10	5
KM 100 R	10	30	10	10	200	10
KM 500 R	10	500	30	10	>1000	10
KM 100-500 R	10	500	200	10	>1000	10
SM 20 R	5	30	10	10	10	20
SM 1000 R	5	30	10	10	10	>1000
SM 100-1000 R	5	30	10	10	10	>1000

Table 4 Resistance-levels to aminoglucoside-antibiotics of strains resistant to various antituberculous agents isolated from *M. tuberculosis* H37Rv

Strain	Resistance level (μg/ml)					
	TUM-N	LVM	CPM	VM	KM	SM
Parent	10	30	10	10	10	5
INH 10-100 R	20	30	10	10	10	5
PAS 1-100-1000 R	10	30	10	10	10	2
TH 100 R	10	30	10	10	10	5
EB 10 R	10	30	10	10	10	5
RFP 100 R	10	20	10	10	10	5
TB 10 R	10	30	10	10	10	5

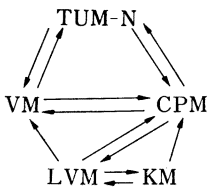
tained by multi-step-selection[10] (Table 3) ;

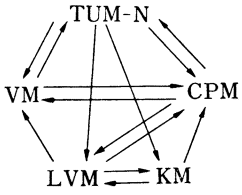
(3) VM-resistant strains are resistant to VM, TUM-N and CPM and are susceptible to others, LVM, KM and SM ;

(4) CPM-resistant strains are resistant to CPM, TUM-N, LVM, and VM, but are susceptible to KM and SM ;

Fig. 2 Cross-resistance-relationships between TUM-N-, LVM-, CPM-, VM-, and KM-resistances of *M. tuberculosis* H 37 Rv

Arrow A to B indicates that strains resistant to agent A are resistant to agent B. In figure, KM indicates KM-highly-resistant strains. Upper : TUM-N -lowly resistant strains. Lower : TUM-N-highly resistant strains.





(5) LVM-resistant strains are resistant to LVM, CPM, KM, and VM, but are susceptible to TUM-N and SM ;

(6) TUM-N-lowly-resistant strains are resistant to TUM-N, VM and CPM, but susceptible to others, LVM, KM and SM ; TUM-N-highly-resistant strains are resistant to TUM-N, LVM, CPM, VM, and KM, and are susceptible to only SM.

Cross-resistance-relationships between LVM, KM, CPM and VM were reported previously. These were confirmed in this study, though one way-cross-resistance relationship between VM and KM resistances were considered to be strain-specific[10~15].

Cross-resistance-relationships between resistances to TUM-N, LVM, CPM, VM and KM are summarized in Fig. 2 and 3.

Both highly- and lowly-resistant strains were obtained by one step-selection with TUM-N. Therefore, pattern of resistance development of this organism to TUM-N is considered to be a facul-

Fig. 3 Cross-resistance-relationships between LVM-, CPM-, VM-, and KM-resistances of *M. tuberculosis* H 37 Rv.

Arrow from A to B indicates that strains resistant to agent A are resistant to agent B. In figure, KM indicate KM-lowly-resistant strains.

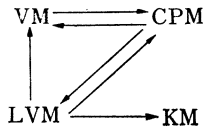


Table 5 Comparison of resistance patterns of *M. tuberculosis* H37Rv to various aminoglucoside -antibiotics

Antibiotic	Number of Phenotypes for resistance	Resistance-level (s) (μg/ml)	Upper limit of resistance expressed as "Resistance Ratio"	Pattern of resistance development
SM	several	20; --- >1000	>200	facultative single-step
KM	2	-200; >1000	>100	facultative single-step
CPM	1	200	20	single-step
VM	1	200	20	single-step
LVM	1	500	16	single-step
TUM-N	2	200; >1000	>100	facultative single-step

tative single step-pattern. There are two phenotypes in TUM-N resistance of *M. tuberculosis* H 37 Rv. The resistance-level of TUM-N resistance is more than 1,000 μg/ml. Thus, pattern of resistance to TUM-N of *M. tuberculosis* H 37 Rv is summarized in Table 5. In table, the patterns in resistances to other aminoglucoside-antibiotics[6,16~19] are shown in Table 5 for comparison. KM resistance was reported previously to have multi phenotypes[6], but it has been shown recently to have two phenotypes, low resistance and high resistance (unpublished data).

It is remarkable that TUM-N-highly resistant organisms are multi resistant and the resistance-levels of the organisms to CPM, LVM and VM are higher than the levels obtained by selection with LVM, VM or CPM. As these organisms are found in the parent strain at a rate of 10^{-6} to 10^{-7}, they are considered to be mutants (the characters of multi resistances are stable through transfers on drug-free medium). If these organisms had been present in the parent strain showing their phenotype, they should have been obtained by other antibiotics, for example, LVM, KM, CPM or VM. However, this could not occur. The organisms were isolated only by selection with TUM-N. Therefore, it is considered that these organisms have been present in the parent strain before exposure to TUM-N without showing their phenotype and the phenotype of multi resistances have been developed by contact with TUM-N. The phenotype is considered to have been induced by TUM-N. TUM-N is considered to have acted as an inducing agent as well as a selective agent.

Summary

Mycobacterium tuberculosis strain H 37 Rv has two phenotypes of TUM-N resistance ; one low resistance-type and another high resistance-type. The former is resistant to 200 μg/ml level of TUM-N, CPM and VM. The latter is resistant to more than 1,000 μg/ml level of TUM-N, CPM, VM, LVM and KM. Both types are susceptible to other antituberculous agents.

VM-resistant strains of the organism are resistant to VM, CPM and TUM-N. KM-highly resistant strains are resistant to KM, LVM and CPM, and KM-lowly resistant strains are resistant to only KM. CPM-resistant strains are resistant to CPM, VM, TUM-N and LVM. LVM-resistant strains are resistant to LVM, VM, CPM and KM. SM-resistant strains are susceptible to other agents.

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