

FACTORS AFFECTING VIRULENCE OF DRUG-RESISTANT BACTERIA

Animal Experiments with Isoniazid-Resistant Tubercle Bacilli

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Introduction

It has been known for a long time that tubercle bacilli resistant to isoniazid are attenuated in virulence for guinea pigs¹⁻⁷). In addition to this, the attenuation of virulence was also reported with tubercle bacilli resistant to rifampicin^{8,9}).

The virulence of tubercle bacilli highly resistant to isoniazid for human subjects is also considered to be attenuated because of infrequent occurrence of infection with these bacilli¹⁰).

The attenuation of virulence of rifampicin-resistant bacteria was reported not only on tubercle bacilli, but also on *Staphylococcus* or *S. typhimurium*¹¹), indicating that the attenuation of virulence in drug-resistant bacteria is not such a phenomenon as to be observed only in tubercle bacilli.

Even in isoniazid-resistant tubercle bacilli, however, the attenuation of virulence was not always observed in all the resistant bacilli. Although highly resistant bacilli tend to be attenuated, highly virulent bacilli can be found among those which are highly resistant and it is also possible to detect remarkable attenuation among with low degree of resistance.

These facts suggest that the virulence of drug-resistant bacilli are determined not only by the degree of the drug-resistance, but also by other factors concerned.

This paper reports the results from our animal experiments on the factors affecting virulence of isoniazid-resistant tubercle bacilli.

Methods

The experiments consist of 2 parts.

1. Methods of Experiment 1

Tubercle bacilli were inoculated into guinea pigs after 3 transfers on solid egg media containing various concentrations of isoniazid. Tubercle bacilli used were *Mycobacterium tuberculosis* var. *hominis*, strain Frankfurt, and the medium used was OKA-KATAKURA's solid egg medium. The isoniazid concentrations in the media were 0.1, 0.3, and 1 μ g/ml, including one without isoniazid.

The intervals of the cultivations were 4, 8, and 4 weeks for three transfers, respectively. For the inoculation, tubercle bacilli were suspended in saline at the concentration of 20 mg/ml, of which 0.1 ml was spread on the surface of the media. Tubercle bacilli, grown for 4 weeks on the third medium, were suspended in saline at the concentration of 0.2 mg/ml, of which 0.5 ml was inoculated subcutaneously in the femoral region of guinea pigs. And the dilution of this suspension was spread on the surface of the media. After 4 weeks of cultivation viable units were counted. The population of isoniazid-resistant bacteria was also counted on the isoniazid-containing media.

Eighteen weeks after the infection, the animals were sacrificed and each organ of the animal was macroscopically examined to detect pathological changes. And a portion of the organ was ground and suspended in 2% sulfuric acid at the concentration of 100 mg/ml, of which 0.1 ml was spread on the media. After 4 weeks of cultivation viable units were counted. The population of isoniazid-resistant bacteria was also counted on the isoniazid-containing media.

In cases of bacteria with marked attenuation, it was impossible to isolate viable bacteria from the organs 12 weeks or more after the infection. Accordingly, tubercle bacilli in organs of some animals were tested between 4 to 5 weeks after the infection for the susceptibility to isoniazid by the method described above.

2. Methods of Experiment 2

The methods employed were essentially the same as Experiment 1, except that the tubercle bacilli used were isolated from the animals in Experiment 1, and had a low degree of resistance to isoniazid but possessed fairly high virulence. Isoniazid concentrations in the media were 0.3, 1, 3, and 10 $\mu\text{g/ml}$, including one without isoniazid, and intervals at which subcultures were performed were 4 weeks in all the three transfers. The macroscopic examination of the organs and quantitative culture from the organs were conducted 12 weeks after the infection.

Results

1. Results from Experiment 1

Table 1 summarizes the results of isoniazid-susceptibility test of tubercle bacilli prepared for inoculation into guinea pigs after three subcultures of isoniazid-susceptible strain on the isoniazid-containing media. Tubercle bacilli subcultured on the medium containing low concentration (0.1 $\mu\text{g/ml}$) of isoniazid had a low degree of resistance to isoniazid only up to 1 $\mu\text{g/ml}$, while the tubercle bacilli from the medium containing 0.3 or 1 $\mu\text{g/ml}$ of isoniazid were highly resistant up to 30 $\mu\text{g/ml}$

of isoniazid.

The results from quantitative culture of animal organs obtained 18 weeks after the infection are shown in Table 2. A large number of colonies of tubercle bacilli were detected from the organs of the animals into which tubercle bacilli after subcultures on a low isoniazid (0.1 $\mu\text{g/ml}$) medium were inoculated, while growth of tubercle bacilli was scarcely observed when tubercle bacilli subcultured on relatively high isoniazid (0.3~1 $\mu\text{g/ml}$) media were inoculated into the animals.

Moreover, no bacterial growth was observed from the organs of the animals when the tubercle bacilli for infection had been first subcultured on a medium with isoniazid at relatively high concentration (1 $\mu\text{g/ml}$) followed by two subcultures at relatively low isoniazid concentration (0.1 $\mu\text{g/ml}$). Degree of gross tuberculous changes in the organs agreed essentially with the results of quantitative culture described above.

The isoniazid-susceptibility test with ground portions of organs revealed (Table 3) that tubercle bacilli grown *in vivo* after transfers *in vitro* on a low isoniazid (0.1 $\mu\text{g/ml}$) medium were completely resistant to isoniazid at 0.3 $\mu\text{g/ml}$ and organisms resistant up to 3 $\mu\text{g/ml}$ of isoniazid were also detected.

As no bacterial growth was detected from the organs 18 weeks after the infection when the tubercle bacilli had been subcultured on a medium with higher than 0.3 $\mu\text{g/ml}$ of isoniazid and inoculated to the animals, the results of susceptibility

Table 1 Isoniazid-susceptibility of tubercle bacilli (prepared for the inoculation into guinea pigs) obtained by subcultures on the media containing isoniazid—Parent strain was susceptible to isoniazid.

Concentrations of isoniazid contained in solid egg media ($\mu\text{g/ml}$)	Subcultures (Concentration of isoniazid in $\mu\text{g/ml}$)				
	0→0→0	0.1→0.1→0.1	0.3→0.3→0.3	1→0.1→0.1	1→1→1
100	0	0	9	0	0
30	0	0	5	0	3
10	0	0	1	0	C
3	0	0	3	0	3
1	0	6	3	4	1
0.3	0	35	3	28	13
0.1	0	35	21	48	25
0.05	0	35	10	80	32
0	47	30	14	61	36

The numbers represent viable units in 1/50,000 mg of tubercle bacilli.

C : Contamination

Table 2 Viable units contained in 10mg of each organ of guinea pigs, 10 weeks after infection with tubercle bacilli subcultured on the media containing isoniazid—Parent strain was susceptible to isoniazid.

Subcultures (Concentration of isoniazid contained in the media for subcultures in $\mu\text{g/ml}$)	Guinea pigs	Lungs	Liver	Spleen	Lymph nodes
1→1→1	J-1	0	0	0	0
	J-2	0	0	0	0
	L-1	0	0	0	0
	N-1	0	0	0	0
1→0.1→0.1	L-4	0	0	0	0
	N-2	0	0	0	0
	O-1	0	0	0	0
0.3→0.3→0.3	F-1	0	0	0	0
	G-1	0	0	0	1
	H-2	0	0	0	0
	L-5	0	0	0	0
0.1→0.1→0.1	F-2	23	145	+	180
	G-2	0	0	0	371
	H-3	4	0	+	310
	N-3	0	0	0	118
0→0→0	F-4	30	92	461	337
	O-2	28	46	260	519

+ : over 700 colonies.

Table 3 Isoniazid susceptibility test with emulsified and diluted organs of guinea pigs infected with tubercle bacilli subcultured on the media containing isoniazid—Parent strain was susceptible to isoniazid.

	Guinea pigs	Subcultures (Concentrations of isoniazid in $\mu\text{g/ml}$)						
		0→0→0		0.1→0.1→0.1		0.3→0.3→0.3	1→0.1→0.1	1→1→1
		F-4*	O-2*	F-2*	H-3*	L-2**	H-1**	L-3**
Concentrations of isoniazid contained in media for susceptibility test ($\mu\text{g/ml}$)	100	0	0	0	0	0	0	0
	30	0	0	0	0	1	0	20
	10	0	0	0	0	2	0	14
	3	0	0	0	113	5	0	14
	1	0	0	293	242	46	16	13
	0.3	0	0	+	+	98	10	16
	0.1	0	0	+	+	92	17	17
	0.05	0	0	+	+	89	19	15
	0	461	260	+	+	69	17	15

The numbers in the table represent viable units contained in 10mg of each organ.

+ : over 700 colonies.

* : Organs tested are spleen. 18 weeks after the infection.

** : Organs tested are local lymph nodes. 4 weeks after the infection.

test with organs 4 weeks after the infection were given in the table. When isoniazid-resistant variants had been obtained either by three subcultures on media containing isoniazid at 0.3 $\mu\text{g/ml}$ or by subcultures on medium containing 1 $\mu\text{g/ml}$ of isoniazid for the first subculture followed by 2 subcultures on media containing 0.1 $\mu\text{g/ml}$ of isoniazid and inoculated into guinea pigs, tubercle bacilli in the organs of the animals were completely resistant to 0.3 to 1 $\mu\text{g/ml}$ of isoniazid. Therefore, although isoniazid-resistance of these

tubercle bacilli is not so high, they could not continue the *in vivo* growth for 18 weeks.

Tubercle bacilli in organs of animals were completely resistant to 30 $\mu\text{g/ml}$ of isoniazid, when isoniazid-resistant variants had been obtained by three subcultures on media containing 1 $\mu\text{g/ml}$ of isoniazid and were inoculated into guinea pigs.

2. Results from Experiment 2

In Experiment 2, virulent tubercle bacilli with low resistance to isoniazid isolated from the organ of the guinea pig in Experiment 1 were subcultu-

Table 4 Isoniazid susceptibility of tubercle bacilli (prepared for the inoculation into guinea pigs) obtained by subcultures of bacilli on the media containing isoniazid—Parent strain had low degree of resistance to isoniazid.

Concentrations of isoniazid contained in solid egg media ($\mu\text{g/ml}$)	Subcultures (Concentrations of isoniazid in $\mu\text{g/ml}$)				
	0→0→0	0.3→0.3→0.3	1→1→1	3→3→3	10→10→10
100	0	0	0	0	0
30	0	0	0	4	6
10	0	0	0	28	15
3	0	0	0	137	31
1	6	24	5	225	79
0.3	131	202	185	279	65
0.1	125	199	147	246	66
0.05	127	183	157	245	55
0	73	204	191	218	71

The numbers represent viable units in 1/50,000 mg of tubercle bacilli.

Table 5 Viable units contained in 10mg of each organ of guinea pigs, 12 weeks after infection with tubercle bacilli subcultured on the media containing isoniazid—Parent strain had low degree of resistance to isoniazid.

Subcultures (Concentrations of isoniazid contained in the media for subcultures in $\mu\text{g/ml}$)	Guinea pigs	Lungs	Liver	Spleen	Lymph nodes
10→10→10	V-3	0	0	0	0
	V-4	0	0	0	0
3→3→3	U-3	4	6	186	148
	U-4	0	5	5	298
	U-5	72	27	+	+
1→1→1	T-3	0	3	4	273
	T-4	1	2	202	145
	T-5	1	4	19	+
0.3→0.3→0.3	S-3	1	2	172	137
	S-4	0	3	193	67
0→0→0	Q-3	2	7	116	147
	Q-4	0	1	258	230
	Q-5	3	0	+	193

+ : over 700 colonies.

Table 6 Isoniazid susceptibility test with emulsified and diluted organs of guinea pigs infected with tubercle bacilli subcultured on the media containing isoniazid—
Parent strain had low degree resistance to isoniazid.

		Subcultures (Concentrations of isoniazid in $\mu\text{g/ml}$)								
		0→0→0		0.3→0.3→0.3		1→1→1		3→3→3		10→10→10
Concentration of isoniazid contained in media for susceptibility test ($\mu\text{g/ml}$)	Guinea pigs	Q-3*	Q-4*	S-3*	S-4*	T-4*	T-5*	U-3*	U-5*	V-2**
	100	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	6	2
	10	0	0	0	0	0	0	22	131	2
	3	10	29	3	2	7	1	63	259	3
	1	96	99	64	100	66	11	176	+	3
	0.3	153	170	163	236	153	20	188	+	2
	0.1	175	232	117	194	185	28	179	+	3
0	116	258	172	193	202	19	186	+	3	

The numbers represent viable units in 10 mg of each spleen.

+ : over 700 colonies.

* : 12 weeks after infection.

** : 4 weeks after infection.

red on isoniazid-containing media and inoculated into guinea pigs. Results of isoniazid susceptibility test of tubercle bacilli prepared for inoculation into guinea pigs were summarized in Table 4. Isoniazid-resistant variants obtained by three subcultures on the media containing at 0.3 or 1 $\mu\text{g/ml}$ were incompletely resistant to 1 $\mu\text{g/ml}$ of isoniazid and completely resistant to 0.3 $\mu\text{g/ml}$ of isoniazid as in the case of control bacteria which were subcultured on the isoniazid-free medium. On the other hand, tubercle bacilli subcultured on the media containing isoniazid at 3 $\mu\text{g/ml}$ or higher were incompletely resistant to 30 $\mu\text{g/ml}$ of isoniazid and completely resistant to 1 $\mu\text{g/ml}$ of isoniazid.

The results of quantitative culture with ground portions of organs of guinea pigs dissected 12 weeks after the infection were shown in Table 5. A large number of bacterial growth was observed from the organs of the animals, into which tubercle bacilli after three subcultures on the media containing isoniazid at 3 $\mu\text{g/ml}$ or less had been inoculated, while no bacterial growth was observed from the organs of animals when bacteria after three subcultures on medium containing 10 $\mu\text{g/ml}$ of isoniazid had been inoculated into the animals. The degree of gross tuberculous changes in organs essentially agreed with the results of quantitative culture described above.

The isoniazid-susceptibility of tubercle bacilli in organs of guinea pigs was shown in Table 6.

Tubercle bacilli resistant up to 3 $\mu\text{g/ml}$ of isoniazid were isolated from organs of animals into which isoniazid-resistant variants obtained by three subcultures on the media containing isoniazid at 1 $\mu\text{g/ml}$ or less had been inoculated, while tubercle bacilli resistant to 10 to 30 $\mu\text{g/ml}$ of isoniazid were isolated when isoniazid-resistant variant after three subcultures on medium containing isoniazid at 3 $\mu\text{g/ml}$ had been inoculated into the animals.

Although a direct method for estimation of susceptibility to isoniazid with organs tends to give higher values for resistance¹²⁾, these results suggest that tubercle bacilli resistant to 3 to 10 $\mu\text{g/ml}$ of isoniazid may grow in guinea pig organs.

Discussion

In Experiment 1, after three subcultures of isoniazid-susceptible tubercle bacilli on the media containing isoniazid at various concentration, it was possible to isolate variants having low degree of resistance to isoniazid (0.3~1 $\mu\text{g/ml}$) with either scarcely or markedly attenuated virulence. Namely, subcultures on the media with isoniazid at lower concentration yielded virulent tubercle bacilli having low degree of resistance to isoniazid, while subcultures on the media with isoniazid at relatively high concentrations yielded tubercle bacilli which had low degree of resistance to isoniazid and were markedly attenuated.

When the first subculture in 3 subcultures was carried out on the medium with isoniazid at rela-

tively high concentration, tubercle bacilli isolated had low degree of resistance to isoniazid and were markedly attenuated, even if the following 2 subcultures were on the media with isoniazid at relatively low concentrations.

In Experiment 2, the virulent tubercle bacilli having low resistance to isoniazid obtained in Experiment 1, were subcultured on the media containing isoniazid at relatively high concentrations. The procedures resulted in the production of both the highly resistant (3~10 $\mu\text{g/ml}$) and virulent variants and more highly resistant and scarcely virulent variant. Subcultures on the medium containing isoniazid at a moderate (3 $\mu\text{g/ml}$) concentration yielded the virulent while subcultures on the medium containing isoniazid at a higher concentration yielded the attenuated variant with high degree of resistance to the drug.

These results suggest that virulent tubercle bacilli having resistance to isoniazid may be yielded when the initial isoniazid concentration is low in the growth media.

From the clinical point of view, chances may not be few for tubercle bacilli in lesions to be exposed to isoniazid at the low concentration. This may be the case, for example, when isoniazid is given at a low dose, when lesion is hard to reach for isoniazid, or when isoniazid is given to a patient whose isoniazid-inactivating activity is high. It should also be noted that, different from an *in vitro* experiment, isoniazid concentration in the lesion is not constant and may drop down to zero during the interim period between medications. Under such conditions, tubercle bacilli may have mild contacts with isoniazid and variant having low degree of resistance to the drug without a marked attenuation of virulence may be yielded. Once virulent variant having low degree of resistance is grown, it may be followed by the growth of virulent bacilli having high degree of resistance as observed in Experiment 2.

There is a paper reporting that isoniazid-resistant tubercle bacilli isolated from patients receiving large doses of isoniazid at the initiation of the chemotherapy, tend to be negative in catalase reaction¹³. Attenuation of virulence of isoniazid-resistant tubercle bacilli is not always coupled with negative catalase reaction. Attenuation of virulence was, however, frequently observed in isoniazid-resistant tubercle bacilli isolated from

patients receiving large doses of the drug at the initiation of the chemotherapy¹⁴. Exceptional cases are of course inevitable, since isoniazid concentration in each lesion is not always proportional to the doses of isoniazid.

As described above, our experimental results suggest that isoniazid concentration in the environment, in which tubercle bacilli grow, at the initiation of the isoniazid administration affects the virulence of isoniazid-resistant variants.

Summary

1. After subcultures of isoniazid-susceptible tubercle bacilli on the media with isoniazid at various concentrations it was possible to isolate variants having low degree of resistance to the drug with and without attenuation of virulence. The latter was isolated by subcultures on the media containing isoniazid at low concentration, while the former was isolated by subcultures on the media containing isoniazid at relatively high concentrations.

2. When the virulent tubercle bacilli having low degree of resistance to isoniazid were subcultured on a media with isoniazid at moderate concentration, the variant having relatively high resistance to the drug was isolated without marked attenuation of virulence.

3. It was proposed that isoniazid concentration in the environment, in which tubercle bacilli grow, at the initiation of the isoniazid administration affects the virulence of isoniazid-resistant variants.

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