TUBERCLE BACILLI RESISTANT TO ISONIAZID

VIRULENCE AND RESPONSE TO TREATMENT WITH ISONIAZID IN GUINEA-PIGS

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Barnett, Bushby, and Mitchison (1953a, 1953b) found among strains of tubercle bacilli an apparent relation between their degree of resistance to isoniazid and their virulence in guinea-pigs and mice. Strains with a high degree of resistance were of low virulence, whereas those with only a slight increase in resistance were fully virulent. However, only three out of the eight strains employed were recently isolated from human lesions. Furthermore, even with these three strains the virulence of sensitive cultures isolated from the same patients before treatment with isoniazid was not tested. To meet these objections the virulence of eight pairs of freshly isolated sensitive and resistant strains has been investigated in guinea-pigs. The response to treatment at a level comparable to that used in man was also found by treating half of the animals with 4 mg. of isoniazid per kg. per day, given by subcutaneous injection.

Methods

Strains.—The eight pairs of sensitive and resistant strains were obtained from the sputum of eight patients in the Medical Research Council (1952) trials of isoniazid. The sensitive strains, obtained before treatment with isoniazid, had been subcultured not more than three times over a period of up to six months before preparation of the inoculum. The resistant strains, obtained during or up to three months after treatment with isoniazid, were isolated on isoniazid-free Löwenstein slopes and were then subcultured twice on slopes containing isoniazid in concentrations shown in Table II. In this way admixture of organisms with a lower degree of resistance was eliminated.

The inoculum for infecting the guinea-pigs was obtained by suspending the growth from 12- to 17-day-old Löwenstein cultures in Tween–albumin medium. Quantitative sensitivity tests on Löwenstein medium and viable counts on oleic-acid–albumin agar were set up from all the suspensions as described by Barnett *et al.* (1953b).

Guinea-pig Experiments.—An injection of 0.1 mg. (moist weight) of each strain was given into the right thigh of six guinea-pigs. One subgroup of three of these animals received no treatment and the other three were given 2 mg. of isoniazid per kg. subcutaneously at both 10 a.m. and 5 p.m. daily, except on Saturdays, when only one dose was given, and on Sundays, when none was given. Treatment was begun immediately after infection and was continued till the animals either died or were killed. Pairs of guinea-pigs, consisting of one treated and one untreated animal, were killed after 6, 10, and 14 weeks. If any animal died spontaneously, none was killed in that subgroup at the next appointed time. At the necropsy a histological examination was made of the spleen, liver, and lungs of all animals in which there was little or no macroscopic tuberculosis, and of some of the remaining animals with visible disease. The degree of tuberculosis was scored out of a possible total of 100 as described by Barnett *et al.* (1953b). Portions of the spleens from animals infected with the resistant strains and from treated animals infected with the sensitive strains were cultured on Löwenstein slopes, and a sensitivity test to isoniazid was carried out by the Medical Research Council (1953) method on any growth of tubercle bacilli that appeared. Serum isoniazid concentrations were estimated by Dr. S. W. A. Kuper, of the Brompton Hospital, using the method of Cuthbertson, Ireland, Woolf, and Kuper (1954).

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Results

Sensitive Strains.—All the untreated guinea-pigs infected with the sensitive strains developed widespread tuberculosis, which was often fatal within nine weeks, as shown in Table I. The average score for the amount of tuberculosis varied from 53 to 89. Among the guinea-pigs infected with the sensitive strains and treated with isoniazid there were 18 out of 24 with no macroscopic or histological evidence of tuberculosis. In the remaining six animals there was some tuberculous involvement at the site of inoculation or in the inguinal glands. The average scores, shown in Table I, varied from 0 to 3.3. These findings demonstrate the protective ability of isoniazid in the dosage used.

TABLE I.—*Results in Guinea-pigs Infected with Eight Sensitive Strains*

Strain	Viable Units Injected Oleic-acid–albumin agar	Untreated Guinea-pigs Dying from Tuberculosis		Average Amount of Tuberculosis out of 100	
		No. out of 3	Days till Death	Untreated	Treated
O'T ..	$2{\cdot}4\times10^{6}$	2	57, 62	73	0
McF ..	$4{\cdot}3\times10^{6}$	3	39, 46, 57	81	1·3
L ..	$4{\cdot}2\times10^{6}$	2	55, 60	89	3·3
McK ..	$2{\cdot}7\times10^{5}$	0	—	60	0
H ..	$7{\cdot}9\times10^{6}$	1	86	53	1·3
Ri ..	$6{\cdot}6\times10^{6}$	2	49, 49	65	2·7
Ru ..	$4{\cdot}9\times10^{6}$	1	50	79	0
Fy ..	$3{\cdot}8\times10^{6}$	2	60, 62	76	2·7

Resistant Strains.—The results of the quantitative sensitivity tests on all eight of the resistant strains, together with one typical sensitive strain, are shown in Table II. The amount of tuberculosis in the guinea-pigs infected with the resistant strains is shown in Table III. In animals with

TABLE II.—*Results of the Quantitative Sensitivity Tests. Log_{10} Viable Units Per mg. of Bacilli*

Strain	Isoniazid Concentration μg./ml.							
	0	0·2	1	5	10	50	200	500
O'T sensitive ..	7·3	<2						
O'T resistant ..	7·2	7·2	<2					
McF	7·1	7·1	7·1	<2				
L	7·6	7·6	7·6	<2				
McK	5·9	5·9	5·5	3·8	3·2	<2		
H	6·9	6·9	6·9	6·9	6·9	3·1	<2	
Ri	6·0	7·0	7·0	7·0	7·0	3·6	<2	
Ru	7·2	7·2	7·2	7·2	7·2	6·6	3·2	<2
Fy	7·4	7·4	7·4	7·4	7·4	7·4	4·3	<2

TABLE III.—*Results in Guinea-pigs Infected with Eight Resistant Strains*

Strain	Minimal Inhibitory Concentration of Isoniazid (μg./ml.)	Isoniazid Concentration during Sub-culture (μg./ml.)	Viable Units Injected Oleic-acid–albumin agar	Average Amount of Tuberculosis out of 100	
				Untreated	Treated
O'T ..	1	0·2	$5{\cdot}1\times10^{6}$	78*	30
McF ..	5	0·2	$6{\cdot}8\times10^{6}$	58	64†
L	5	0·2	$7{\cdot}7\times10^{6}$	22	30
McK ..	5	0·2	$3{\cdot}3\times10^{5}$	11	14
H	50	10	$5{\cdot}0\times10^{6}$	13	22
Ri	50	10	$4{\cdot}8\times10^{6}$	11	12
Ru	200	50	$3{\cdot}7\times10^{5}$	10	6
Fy	500	50	$2{\cdot}5\times10^{6}$	15	20

* Two animals died from tuberculosis on the 64th and 77th days.
† One animal died from tuberculosis on the 83rd day.

scores of up to 20 there was a caseous abscess at the site of inoculation and a variable amount of caseation in the draining lymph nodes. The liver, spleen, and lungs, though macroscopically normal, often contained small tubercles or collections of histiocytes and lymphocytes. A score of 20 to 50 indicated macroscopic disease in the spleen, liver, and lungs, though less than would be found in animals infected with a fully virulent strain. Widespread generalized tuberculosis was present in animals with a score of over 50.

Virulence and Degree of Resistance.—As can be seen from Table III, an increase of resistance to isoniazid was associated with a decrease in the virulence of the strains. The scores of the amount of tuberculosis in untreated animals ranged from 78, caused by the strain with the lowest degree of resistance, to 10 to 15 caused by the four strains with the highest degrees of resistance. This relation can best be shown by including with the present results those obtained by Barnett *et al.* (1953a, 1953b), using other resistant strains, and also the results on a further two highly resistant strains (Table IV). One of these was obtained

TABLE IV.—*Relation Between the Virulence and the Degree of Isoniazid Resistance of 17 Strains of Tubercle Bacilli*

Virulence	Degree of Isoniazid Resistance			
	Growth on 0·2 μg./ml. Not on 1	Growth on 1 μg./ml. Not on 5	Growth on 10 μg./ml. Not on 50	Growth on 50 μg./ml.
High or just subnormal	3	2	0	0
Intermediate	0	1	1	0
Low	0	1	2	7*

* Three of these strains were variants of the laboratory strain H37Rv.

from a patient under treatment with isoniazid and the other was a resistant variant of the H37Rv strain obtained from Professor R. Knox, of Guy's Hospital, and used by Goulding and Robson (1952). Both were injected into two untreated guinea-pigs, which were killed after 10 weeks. In spite of differences in the time between infection and killing, all these strains could be classified into three categories of virulence : high or just subnormal (scores of above 50), intermediate (scores from 20 to 50), and low (scores below 20). A well-marked correlation between the degree of resistance to isoniazid and loss of virulence is shown.

Stimulation of Virulence by Treatment.—The individual scores of guinea-pigs infected with the six strains of the present series which were of relatively low virulence are shown in Table V. In untreated animals the disease tended

TABLE V.—*Amount of Tuberculosis in Guinea-pigs Infected with Six Resistant Strains of Low Virulence*

Strains	Amount of Tuberculosis out of 100							
	Untreated Animals Killed at				Treated Animals Killed at			
	6 Weeks	10 Weeks	14 Weeks	Average	6 Weeks	10 Weeks	14 Weeks	Average
McK ..	12−	10−	10−	11	16±	17±	10−	14
Ri ..	16±	10−	8−	11	17±	11−	8−	12
Ru ..	12±	10±	8−	10	10±	0−	8−	6
L ..	33+	17±	16−	22	15±	17±	59+	30
H ..	18±	10−	12−	13	31+	15±	20+	22
Fy ..	18±	15±	13±	15	20±	12−	29+	20
Average	18	12	11		18	12	22	

+: Macroscopic tuberculosis in spleen, liver, or lungs.
±: Histological but not macroscopic tuberculosis in spleen, liver, or lungs.
−: Normal spleen, liver, and lungs.

to heal, as demonstrated by the fall in the average scores and the decrease in the proportion of animals showing lesions in the spleen, liver, or lungs during the period from 6 to 14 weeks after infection. The average scores of treated guinea-pigs killed at 6 and 10 weeks were the same as those of similar untreated animals. However, among animals killed at 14 weeks the score in those that were treated was 22, whereas in those that were not treated it was 11. This higher average in the treated animals was due to those infected with strains L, H, and Fy. It can be seen that these three strains were slightly more virulent in the untreated animals than were the remaining strains. Thus there is a suggestion that, among resistant strains of low virulence those which were the more virulent appeared to have had their virulence increased by treatment with isoniazid between the 10th and 14th weeks after infection.

Response to Treatment.—Treatment with isoniazid afforded partial protection to the animals infected with strain O'T, which grew on 0.2 μg. per ml. but not on 1 μg. per ml. ; their score was 30 (Table III). The scores in the treated animals infected with three strains, McF, L, and McK, capable of growth on 1 μg. per ml. but not on 5 μg. per ml., were in each case higher than in the untreated animals. Whether or not the strain can grow on 1 μg. per ml. therefore seems to determine whether the infection will respond *in vivo* to treatment at 4 mg. per kg. per day.

Viable Counts.—Barnett *et al.* (1953b) found that two highly resistant strains gave much lower viable counts on oleic-acid–albumin agar than on Löwenstein medium. These differences were thought to be due to abnormal nutritional requirements of the strains. In the present investigation (Tables II and III) the counts on oleic-acid–albumin agar were equal to or slightly greater than the counts on Löwenstein medium except with the resistant strain Ru, which yielded counts of 3.7×10^6 and 1.5×10^7 respectively per mg. of bacilli.

Spleen Cultures.—Out of 21 spleens from treated guinea-pigs infected with sensitive strains, 2 yielded growth of tubercle bacilli. One of these strains was sensitive to isoniazid. The other grew on 0.2 μg. per ml. but was inhibited by 1 μg. per ml. None of the 19 cultures recovered from animals infected with the resistant strains showed any alteration in the degree of resistance from the strain injected.

Serum Isoniazid Concentrations.—The serum isoniazid concentration of guinea-pigs injected with doses of 2 mg. per kg. are shown in Table VI. For comparison mean

TABLE VI.—*Serum Isoniazid Concentrations in Patients and Guinea-pigs*

Time After Dose	Average Serum Isoniazid, μg./ml.		
	Patients; Oral	Guinea-pigs; Subcutaneous	
	1·4–2·6 mg./kg.	2 mg./kg.	10 mg./kg.
½ hour	0·9	2·3	9·9
1 „	1·2	1·7	5·4
2 hours	0·8	0·7	—
4–17 „	—	<0·5	<0·5
No. of patients or animals on which each average based	10	3	2

values for isoniazid concentrations, performed by the same method, in sera from 16 patients receiving 1.4 to 2.6 mg. per kg. by mouth were taken from the figures of Cuthbertson *et al.* (1954). If allowance is made for differences in the speed of absorption due to the different routes of administration it will be seen that the values obtained in man and in guinea-pigs were very similar. The serum concentrations in guinea-pigs injected with doses of 10 mg. per kg. were substantially higher.

Discussion

The low virulence to guinea-pigs of strains of tubercle bacilli resistant to isoniazid has also been reported by Barry, Conalty, and Gaffney (1953), although they did not state the degree of resistance of their strains isolated from patients. Since these strains also lack virulence in mice

(Barnett *et al.*, 1953b) and in rabbits (Domagk, quoted by Klee, 1953) it would seem probable that they would be of low virulence in man. However, patients in the Medical Research Council (1952) trials who developed resistant strains (growth on Löwenstein slopes containing 1 μg. of isoniazid per ml.) benefited less from treatment with isoniazid than did those whose strains remained sensitive.

There seem to be three possible explanations for the apparent contradiction. In the first place, even the most highly resistant strains set up large abscesses at the site of inoculation and usually minimal regressive lesions in the spleen, liver, or lungs. Thus these strains are not completely avirulent. Secondly, when strains from patients appear highly resistant in sensitivity tests there may be bacilli with a lower degree of resistance, and therefore higher virulence, in areas of the lungs containing progressive tuberculous lesions. Thirdly, the virulence of resistant strains may be increased by treatment with isoniazid; this may have occurred in the guinea-pigs infected with strains L, H, and Fy, although the numbers of animals were too small to allow a firm conclusion. It has also been reported in mice in three out of four experiments (Barnett *et al.*, 1953b).

Strains growing on Löwenstein slopes containing 1 μg. of isoniazid per ml. did not respond to 4 mg. per kg. per day. The serum isoniazid concentrations of guinea-pigs receiving this dose were similar to those of patients on the same dosage, used in the Medical Research Council (1952) trials. Thus it is reasonable to assume that such strains would also not respond to treatment with isoniazid when present in human lesions. Unfortunately some of the strains growing on 1 μg. per ml. but not on 5 μg. per ml. were still of fairly high virulence (Table IV). An increase in the dosage of isoniazid would be unlikely to inhibit these strains in patients, since they did not respond to a treatment level of 20 mg. per kg. per day in guinea-pigs (Barnett *et al.*, 1953b).

Strains growing on 0.2 μg. per ml. but not on 1 μg. per ml. have been classified as of doubtful resistance in the Medical Research Council (1953) trials. There is justification for this classification, since on 4 mg. of isoniazid per kg. per day such a strain partially responded to treatment, and on 20 mg. per kg. per day there was almost complete response (Barnett *et al.*, 1953a).

Summary

In a study of eight pairs of strains of tubercle bacilli sensitive and resistant to isoniazid it was found that the higher the degree of resistance the lower was the virulence in guinea-pigs. Treatment with isoniazid in a dose comparable to that used in man protected animals infected with sensitive strains, and partially protected animals infected with a resistant strain growing on 0.2 μg. per ml. but not on 1 μg. per ml. It did not protect animals infected with resistant strains growing on 1 μg. per ml., and may, indeed, have increased the virulence of some of these strains.

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BACTERICIDAL ACTIVITY OF STREPTOMYCIN AND ISONIAZID AGAINST TUBERCLE BACILLI

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Garrod (1950) concluded that streptomycin, in concentrations attainable therapeutically in the body, was rapidly bactericidal to cultures of *Mycobacterium tuberculosis* growing in Tween–albumin liquid medium. The speed of killings became more rapid with increasing concentrations of streptomycin. More recently, Mackaness and Smith (1953) have described the bactericidal action of streptomycin, isoniazid, and a combination of these two drugs on similar cultures.

The purpose of the present investigation was to investigate the action of streptomycin and isoniazid, both alone and in combination, on Tween–albumin cultures of tubercle bacilli. An attempt was made to use concentrations of these drugs comparable to those found in treated patients.

Methods

Screw-capped bottles containing 20 ml. of the H37Rv strain of *Myco. tuberculosis* grown for nine days in modified Dubos and Davis Tween–albumin medium (Medical Research Council, 1948) were centrifuged for 10 minutes at 1,000 r.p.m. to remove the larger aggregates of bacilli. The supernatant fluid was removed, and stained films from it showed that 80% of the organisms were single or in pairs.

A volume of 0.5 ml. of this supernatant was added to 4.5 ml. of Tween–albumin medium containing various concentrations of streptomycin, isoniazid, or a combination of both drugs in screw-capped bottles. A control bottle containing no drug was included in each experiment. The bottles were incubated at 37° C. in a hot-air room. At intervals, 0.5-ml. samples were removed from these bottles for viable counts on oleic acid–albumin–agar plates, using a calibrated dropping pipette and loop as described by Mitchison (1953). The diluent employed was Tween–albumin medium. The plates were sealed with wax-coated "cellophane" tape and were incubated for four weeks at 37° C. The numbers of colonies were then counted with a plate microscope.

Where the presence of resistant organisms was suspected sensitivity tests to streptomycin and isoniazid were carried out by the Medical Research Council (1953b) methods. In one experiment the cultures which appeared to contain dead organisms were injected into guinea-pigs. These were killed after 10 weeks and examined for macroscopic evidence of tuberculosis.

Results

Streptomycin.—The bactericidal action of streptomycin alone in concentrations of 2, 20, and 200 units per ml. is shown in Fig. 1. The number of viable units—that is, discrete organisms or clumps developing into single colonies—in the cultures was diminished by a thousandfold after contact with two units per ml. for seven days, 20 units for three days, and 200 units for one day. Thus the higher the concentration of streptomycin the more rapid was the

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