

The Virulence in the Guinea-pig of Isoniazid-Sensitive Tubercle Bacilli Isolated from South Indian Patients before Treatment and after Three Months of Chemotherapy

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In order to find out whether chemotherapy with isoniazid affects the virulence in the guinea-pig of tubercle bacilli that do not develop resistance to the drug, virulence tests were carried out on isoniazid-sensitive cultures obtained from 20 South Indian tuberculous patients before treatment and after three months of chemotherapy with isoniazid. No significant difference in virulence was observed between the cultures obtained on admission to treatment and those obtained after three months of chemotherapy. This is a finding with important implications for large-scale surveys of the distribution of attenuated strains of tubercle bacilli from untreated patients in India and other countries. Detailed and repeated inquiries as to previous chemotherapy are not important in such surveys, provided that sensitivity tests are done on all the cultures.

INTRODUCTION

In confirmation of the findings of Frimodt-Møller, Mathew & Barton (1956) and Frimodt-Møller (1957), Mitchison et al. (1960) and Bhatia et al. (1961a) have shown that cultures of tubercle bacilli isolated from South Indian patients, resident in Madras City, were, on the average, less virulent in the guinea-pig and had a wider range of virulence than cultures from British patients. All of the Indian cultures studied were fully sensitive to isoniazid and almost all were obtained from patients before the start of antituberculosis chemotherapy. However, a small proportion of them were from patients who had received short periods of treatment, probably with isoniazid. It is well established that the emergence of isoniazid-resistance is associated with loss of virulence in the guinea-pig (Middlebrook, 1957),

but there appears to be no evidence as to whether chemotherapy with isoniazid affects virulence in the absence of the emergence of resistance. In consequence, it was thought desirable to compare the virulence of *isoniazid-sensitive* cultures obtained from patients before treatment and after three months of chemotherapy with isoniazid.

An opportunity arose to make the comparison during the course of a concurrent study of four chemotherapeutic regimens in the treatment of pulmonary tuberculosis in South Indian patients (Tuberculosis Chemotherapy Centre, 1960). In brief, the regimens employed were:

PH. Isoniazid 3.9-5.5 mg/kg body-weight plus p-aminosalicylic acid (sodium salt) 0.2-0.3 g/kg daily, divided into two doses, by mouth.

HI-1. Isoniazid alone, 7.8-9.6 mg/kg daily, in one dose by mouth.

HI-2. Isoniazid alone, 7.8-9.6 mg/kg daily, divided into two doses, by mouth.

H. Isoniazid alone, 3.9-5.5 mg/kg daily, divided into two doses, by mouth.

Sensitive cultures were obtained from 20 patients participating in the study, both before treatment and

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TABLE 1
ROOT-INDICES OF ISONIAZID-SENSITIVE CULTURES OBTAINED ON ADMISSION TO TREATMENT
AND AFTER THREE MONTHS OF CHEMOTHERAPY

Patient number	0-month culture (on admission to treatment)				3-month culture *			Mean root-index of virulence
	Experiment number	6-week root-index	12-week root-index	Root-index of virulence	6-week root-index	12-week root-index	Root-index of virulence	
1	Por. 1 ^b	0.89	0.61	0.75	0.81	1.00	0.90	0.83
2	" 2 ^b	1.09	0.87	0.98	0.99	1.02	1.00	0.99
3	" 3 ^b	0.77	0.32	0.54	0.45	0.56	0.50	0.52
4	" 3 ^b	0.96	0.84	0.90	1.00	1.16	1.08	0.99
5	" 4 ^b	0.81	0.47	0.64	0.71	0.32	0.52	0.58
6	" 4	0.88	0.32	0.60	0.66	0.32	0.49	0.54
7	" 5 ^b	0.44	0.90	0.67	0.32	0.45	0.38	0.53
8	" 5	1.08 ^c	1.00	1.04	0.65	0.35	0.50	0.77
9	" 5	1.14	0.96	1.05	1.38	0.93	1.16	1.10
10	" 5	0.37	0.32	0.34	0.66	0.46	0.56	0.45
11	" 6	1.01	0.56	0.78	0.89	0.81	0.85	0.82
12	" 6	0.66	0.88	0.77	0.75	0.26	0.50	0.64
13	" 7	0.79	0.82	0.80	0.66	0.46	0.56	0.68
14	" 8	0.93	0.86	0.90	0.96	0.49	0.72	0.81
15	" 8	1.12	1.37	1.24	0.82	0.44	0.63	0.94
16	" 10	1.10	0.87	0.98	0.71	0.94	0.82	0.90
17	" 10	0.42	0.30	0.36	0.45	0.35	0.40	0.38
18	" 12	1.12	1.10	1.11	1.10	0.96	1.03	1.07
19	" 12	0.95	1.05	1.00	1.06	0.84	0.95	0.98
20	" 12	1.01	0.62	0.82	0.81	0.89	0.85	0.83
Mean		0.88	0.75	0.81	0.79	0.65	0.72	0.77

*All the 3-month cultures were stored in the deep-freeze and all were tested in Por. 13.

^bDeep-freeze stored culture.

^cNon-tuberculous death; missing observation estimated by statistical techniques (see Mitchison et al., 1961).

after three months of the allocated regimen. Twelve of the patients received the PH regimen, three the HI-1, two the HI-2 and three the H. We report here on the virulence of these cultures in the guinea-pig.

METHODS

Cultures

Sputum specimens were cultured on Löwenstein-Jensen medium and sensitivity tests were done on the cultures by the methods described previously (Tuberculosis Chemotherapy Centre, 1959). The

identification test procedures, which included the niacin test, were those of Thomas et al. (1961).¹ All except one of the cultures, which became contaminated, were identified as *Mycobacterium tuberculosis* var. *hominis*, and all were sensitive to isoniazid and streptomycin. Cultures were flown to England and were either tested for their virulence within eight weeks of having become positive or stored at -20°C for periods of up to 60 weeks until required.

¹ See article on page 747.

Storage at -20°C has been shown not to affect virulence (Mitchison et al., 1961).

Virulence tests

The measure of virulence was based on the rate of progression of the disease in the guinea-pig, and has been described in detail by Mitchison et al. (1960, 1961). In brief, 1 mg (moist weight) of a 3-week-old culture was injected into each of two guinea-pigs of the Duncan Hartley breed (mean weight, 435 g; range, 300-520 g) at the Microbiological Research Establishment, Porton, Wiltshire, England. One of these animals was killed at 6 weeks and the other at 12 weeks. At post-mortem examination, the total extent of tuberculous disease in the spleen, liver, lungs and local glands was assessed as a score ranging from 0 to 100. For reasons given elsewhere (Mitchison et al., 1961), the square root of the ratio of the score to the survival time in days (whether the guinea-pig died or was sacrificed) was determined for each animal, and was called the 6-week root-index or the 12-week root-index. The mean of the 6-week and 12-week root-indices of the two guinea-pigs was employed as the measure of virulence, and was termed the "root-index of virulence".

Some of the cultures were tested for virulence in four guinea-pigs (two killed at 6 weeks and two at 12 weeks). On grounds of simplicity, the analysis is based on the results on only two of these animals (one killed at 6 weeks and one at 12 weeks), selected at random.

Arrangement of experiments

The virulence tests were done in a series of experiments (which included tests on other cultures) at Porton and, in conformity with the nomenclature adopted by Mitchison et al. (1961), they are referred to as Por. 1, 2 The virulence of strain H37Rv and of five recently isolated British cultures was tested in each of these experiments, principally to detect inter-experimental variation (for a fuller account, see Mitchison et al., 1961). The cultures obtained from the patients before the start of the prescribed regimen were tested either fresh or after storage at -20°C in 10 of these experiments (Table 1), but those obtained after three months' treatment were all stored at -20°C and were tested together in experiment Por. 13. Of the 80 guinea-pigs used, one died from a non-tuberculous cause in experiment Por. 8, and the value of its root-index was estimated from the results on the remaining animals in the experiment, as described by Mitchison et al. (1961).

RESULTS

The root-indices obtained in the virulence tests on the 40 cultures from 20 patients are set out in Table 1. Inter-experimental variation in the series of experiments Por. 1 to Por. 13 was found to be very small (Mitchison et al., 1961), so that the results obtained in the different experiments have been considered as a unit and examined by analysis of variance (Table 2).

Variation between patients

The means of the root-indices of virulence for the two cultures from each patient are shown in the last column of Table 1. The variation between these means from patient to patient was significantly greater than the estimate of variation between the root-indices of virulence of duplicate cultures from the same patient (Table 2, terms a and c, $P < 0.005$). Thus, there were consistent differences between patients in the virulence of their cultures.

Difference between 0-month and 3-month cultures

The means of the root-indices of virulence on the 0-month and 3-month cultures from the 20 patients were 0.81 and 0.72 respectively (Table 1). The difference does not attain statistical significance (Table 2, term b, $P = 0.07$). Furthermore, the mean of the root-indices of virulence obtained with strain H37Rv in experiments Por. 4-12 was 0.85 (strain H37Rv was not set up in Por. 1-3), and in Por. 13 it was 0.82. The corresponding means with the British cultures were 1.05 in Por. 1-12 and 1.03 in Por. 13. These results suggest that the root-indices obtained in Por. 13, which included all the tests on 3-month cultures, were, on the average, very slightly lower than in the earlier experiments, among which the 0-month cultures were distributed. Thus, the difference between the means of the root-indices on 0-month and 3-month cultures, as well as being small and not statistically significant, may also have resulted in part from slight systematic underscoring in experiment Por. 13.

Variation between cultures from the same patient

An estimate of variation between duplicate cultures from the same patient was provided by the interaction of the difference between months and the differences between patients. From this estimate, the variation in virulence between pairs of cultures from the same patient did not appear to be greater than the residual variation in response of the guinea-pigs (Table 2, terms c and h, $P = 0.1$).

TABLE 2
ROOT-INDICES OF ISONIAZID-SENSITIVE CULTURES OBTAINED ON ADMISSION TO TREATMENT AND
AFTER THREE MONTHS OF CHEMOTHERAPY
ANALYSIS OF VARIANCE

Design of the investigation { 20 Patients; two cultures from each patient: each culture inoculated into two guinea-pigs; total of 80 guinea-pigs.

Term	Source of variation	Sum of squares	D F	Mean square	Term tested against	F	P
a	Patients (P)	3.5295	19	0.1858	c	3.83	10.005
b	0 and 3 months (M)	0.1739	1	0.1739	c	3.59	0.07
c	Interaction M x P	0.9214	19	0.0485	h	1.61	0.1
d	6 and 12 weeks(W)	0.3551	1	0.3551	h	11.80	<0.005
e	Interaction W x P	0.4778	19	0.0251	g	—	N S*
f	Interaction W x M	0.0013	1	0.0013	g	—	NS
g	Interaction W x P x M	0.6937	19	0.0365			
h	Residual (e+f+g)	1.1728	39	0.0301			
	Total	6.1527	79	0.0779			

*NS indicates that the variance ratio is less than 1.0.

Remaining terms in the analysis of variance

The difference between the means of the 6-week and 12-week root-indices is statistically significant (Table 2, term d, $P < 0.005$). This finding has been a consistent feature of all analyses of virulence tests done by the method reported here. It arises from a non-linear relationship between the post-mortem score and the period of survival of the guinea-pigs. As both the first-order interactions with this difference between the 6-week and 12-week results were small and did not attain statistical significance, they were pooled with the second-order interaction. The resultant residual mean square (Table 2, term h, 0.0301) was similar to the corresponding mean square (0.0264) in a larger sample of virulence tests on Indian cultures done by the same method (see Table 6 in Mitchison et al., 1961).

DISCUSSION

The main findings of the present study of *isoniazid-sensitive* cultures of tubercle bacilli are as follows: (a) there was considerable variation between patients in the virulence of their cultures in the guinea-pig; (b) sensitive cultures obtained from the patients before treatment and after three months of chemotherapy, including isoniazid, did not appear to differ

in their virulence. However, the probability that a difference existed was close to the 5% level; (c) the variation between duplicate cultures from the same patient was no greater than the natural variation in response of the guinea-pigs. Thus, in the present study, cultures from a given Indian patient had a characteristic and consistent degree of virulence, which was unaffected by a 3-month period of chemotherapy, so long as the cultures remained sensitive to isoniazid. These findings confirm and extend the observations of Bhatia et al. (1961b), who tested the virulence of three cultures obtained over a 6-week period before the start of chemotherapy from each of 12 patients, and who also found that the differences in virulence between patients were consistent.

In two earlier papers the virulence in the guinea-pig of 281 cultures obtained before the start of chemotherapy from the same number of South Indian patients in Madras is related to the extent and type of their disease before treatment (Ramakrishnan et al., 1961), and is compared with the virulence of cultures from British patients (Bhatia et al., 1961a). These cultures were all sensitive to isoniazid and were all regarded as being pretreatment cultures, although, at the time they were obtained, antituberculosis chemotherapy had probably been given to 11 of the 281 patients for up to two

weeks and certainly to one patient for three months; the remaining 269 patients had almost certainly had no previous chemotherapy (Tuberculosis Chemotherapy Centre, 1960). Our finding that three months of chemotherapy did not appear to influence the virulence of isoniazid-sensitive cultures provides good justification for considering all the 281 cultures as either genuinely pretreatment or having the same virulence as pretreatment cultures.

This finding is likely to be of general importance for epidemiological investigations in the future. Studies of the virulence of cultures from patients in other parts of India and in other countries would be of value in determining the geographical area in which attenuated cultures exist. Such studies might provide information on the spread of tubercle bacilli within India and from one country to another. In these investigations it will be a great advantage to be able to assume, from the evidence reported here, that the virulence of any isoniazid-sensitive culture is the same, or nearly the same, as that of a pretreatment (isoniazid-sensitive) culture from the same patient and that, even if the patient has had some chemotherapy, this has not altered the virulence of his strain. It is easy to do sensitivity tests, but difficult to get reliable histories of chemotherapy, since these

require detailed and repeated inquiries over a period of many months, during which increasing confidence can be established with the patient and his family (Tuberculosis Chemotherapy Centre, 1959, 1960). It is most unlikely that circumstances which would allow of such exhaustive inquiries would be available in widespread epidemiological surveys.

SUMMARY

1. Cultures of tubercle bacilli sensitive to *isoniazid* and streptomycin were obtained from 20 South Indian patients before and at three months after the start of chemotherapy with isoniazid plus PAS or with isoniazid alone. These cultures were tested for their virulence in the guinea-pig.
2. Statistically significant variation was found between patients in the mean virulence of their cultures.
3. No significant difference in virulence was found between the pretreatment cultures and the cultures obtained at the end of three months' chemotherapy.
4. The variation in virulence between duplicate cultures from the same patient was no greater than the natural variation in response of the guinea-pigs.

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RÉSUMÉ

Il a été démontré au Centre de Madras pour l'étude de la chimiothérapie de la tuberculose que les souches de bacilles tuberculeux isolés de malades en Inde méridionale étaient, en moyenne, moins virulentes pour le cobaye que les souches britanniques auxquelles on les a comparées, et qu'elles avaient une gamme de virulence plus étendue.

Les auteurs ont poursuivi ces études et ont comparé la virulence de souches de bacilles tuberculeux avant et après trois mois de traitement à l'isoniazide et à l'isonia-

zide + PAS, ces souches étant, au départ comme après trois mois de traitement, sensibles à l'isoniazide.

Les résultats ont montré qu'il y avait, d'un patient à l'autre, des variations de virulence pour le cobaye statistiquement significatives. En revanche, on n'observait pas de différences significatives de la virulence moyenne entre les souches du même malade, avant et après trois mois de traitement. La variation n'était pas plus importante que la variation naturelle du cobaye manifestée par sa réponse à l'infection.

REFERENCES

- Bhatia, A. L., Csillag, A., Mitchison, D. A., Selkon, J. B., Somasundaram, P. R. & Subbaiah, T. V. (1961a) *Bull. Wld Hlth Org.*, **25**, 313
- Bhatia, A. L., Devadatta, S., Mitchison, D. A., Radhakrishna, S., Selkon, J. B. & Subbaiah, T. V. (1961b) *Tubercle (Lond.)*, **42**, 317
- Frimodt-Møller, J. (1957) In: Indian Council of Medical Research, *Technical Report of the Scientific Advisory Board*, New Delhi, p. 153
- Frimodt-Møller, J., Mathew, K. T. & Barton, R. M. (1956) In: Tuberculosis Association of India, *Proceedings of the Thirteenth Tuberculosis Workers' Conference, held in Trivandrum . . .*, New Delhi, p. 151
- Middlebrook, G. (1957) In: *Proceedings of the Fifteenth Tuberculosis Conference held under the auspices of the International Union Against Tuberculosis and the Tuberculosis Association of India*, New Delhi, p. 71
- Mitchison, D. A., Bhatia, A. L., Radhakrishna, S., Selkon, J. B., Subbaiah, T. V. & Wallace, J. G. (1961) *Bull. Wld Hlth Org.*, **25**, 285
- Mitchison, D. A., Wallace, J. G., Bhatia, A. L., Selkon, J. B., Subbaiah, T. V. & Lancaster, M. C. (1960) *Tubercle (Lond.)*, **41**, 1
- Ramakrishnan, C. V., Bhatia, A. L., Fox, W., Mitchison, D. A., Radhakrishna, S., Selkon, J. B., Subbaiah, T. V., Velu, S. & Wallace, J. G. (1961) *Bull. Wld Hlth Org.*, **25**, 323
- Thomas, K. L., Joseph, S., Subbaiah, T. V. & Selkon, J. B. (1961) *Bull. Wld Hlth Org.*, **25**, 147
- Tuberculosis Chemotherapy Centre (1959) *Bull. Wld Hlth Org.*, **21**, 51
- Tuberculosis Chemotherapy Centre (1960) *Bull. Wld Hlth Org.*, **23**, 535