

A Comparison of the Virulence in the Guinea-Pig of Tubercle Bacilli from Thai and British Patients*

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Recent work carried out at the Tuberculosis Chemotherapy Centre, Madras, and at the Postgraduate Medical School of London has shown that isoniazid-sensitive cultures of tubercle bacilli obtained before the start of chemotherapy from South Indian patients with pulmonary tuberculosis are of lower average virulence than corresponding cultures from British patients. The present paper from the Centre extends these studies to areas outside India by comparing isoniazid-sensitive cultures from 81 Thai patients and 67 British patients for their guinea-pig virulence. As assessed by the amount of disease visible at post-mortem examination six weeks after intramuscular injection of the cultures, by the mortality of the guinea-pigs and by the results of spleen culture, the Thai cultures were less virulent than the British cultures. However, a retrospective comparison with the earlier studies suggests that the Thai cultures were intermediate in their virulence between British and Indian cultures. These findings contribute to the over-all study of the geographical distribution of attenuated tubercle bacilli.

INTRODUCTION

Frimodt-Møller (1957), Mitchison et al. (1960) and Bhatia et al. (1961) have shown that isoniazid-sensitive cultures of tubercle bacilli obtained before the start of treatment from South Indian patients with pulmonary tuberculosis were, on the average, of lower virulence in the guinea-pig and had a wider range of virulence than corresponding cultures obtained from British patients. Singh (1957) has also reported that cultures of tubercle bacilli obtained from patients in North India were less virulent than cultures obtained from France and from the USA. It was therefore considered of interest to extend this study of the virulence in the guinea-pig of tubercle bacilli to cultures obtained from patients resident in other areas. In 1960, an opportunity arose to study cultures of tubercle bacilli obtained from patients in Bangkok, Thailand. This paper presents the results of a comparison of the virulence in the guinea-

pig of cultures of isoniazid-sensitive tubercle bacilli obtained before the start of treatment from Thai patients and of corresponding cultures obtained from British patients.

METHODS

Cultures from Thai patients

A single culture of tubercle bacilli was obtained from each of 109 Thai patients selected at random from those who had been detected in a sample survey of the prevalence of pulmonary tuberculosis in Bangkok, Thailand, or who had attended the Central Clinic, Bangkok, because of their symptoms. These patients were aged 12 years or more, showed radiographic evidence of pulmonary tuberculosis, had yielded cultures of tubercle bacilli and had denied having previously received any antituberculosis chemotherapy. After excluding 28 (26%) of the 109 cultures because they were resistant to isoniazid (for definition, see page 484), there remained 81 isoniazid-sensitive cultures in the study.

Cultures from British patients

A single culture was obtained from each of 68 British patients, aged 12 years or more with newly diagnosed and previously untreated pulmonary tuberculosis, attending a number of chest clinics or hospitals in Great Britain (see "Acknowledge-

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ments", page 489). After the exclusion of one isoniazid-resistant culture there remained 67 cultures in the study.

Preparation of cultures

The cultures of tubercle bacilli for the Thai patients were obtained at the laboratory of the Central Chest Clinic, Bangkok, from sputum specimens, laryngeal swabs or, in the case of one patient, from a pleural fluid. Some of the sputum specimens and the pleural fluid were treated with 4% sodium hydroxide, centrifuged and the deposit neutralized. The remaining sputum specimens and the laryngeal swabs were treated by the swab method of Nassau (1958). The specimens of sputum obtained from the British patients were cultured at the Unit for Research on Drug Sensitivity in Tuberculosis (Medical Research Council of Great Britain), Postgraduate Medical School of London; they were treated with 4% sodium hydroxide and the deposits were washed with water (Tuberculosis Chemotherapy Centre, 1959). All specimens were cultured on slopes of Löwenstein-Jensen medium which were incubated at 37°C and examined weekly for eight weeks for the presence of growth. Positive cultures were sent by air to Madras, where they were subcultured on to slopes of Löwenstein-Jensen medium for the isoniazid sensitivity tests, the identification tests and the virulence tests described below. The average period between the cultures first becoming positive and their subculture in Madras was four weeks (range, two to 26 weeks) for the 81 Thai cultures and five weeks (range, one to 12 weeks) for the 67 British cultures.

Isoniazid sensitivity tests

The method for testing the sensitivity of cultures to isoniazid has been described in detail elsewhere (Tuberculosis Chemotherapy Centre, 1959). In brief, a suspension containing 4 mg (moist weight) per ml of a representative sample of the growth on Löwenstein-Jensen medium was prepared in distilled water and a 3-mm loopful of this suspension was inoculated on to a slope of Löwenstein-Jensen medium without isoniazid and on to a series of slopes containing 0.2, 1, 5 and 50 µg/ml of isoniazid. Cultures were regarded as resistant to isoniazid if, after 4 weeks' incubation at 37°C, they yielded growth of 20 or more colonies on 1 µg/ml isoniazid or more, or if they yielded 20 or more colonies on 0.2 µg/ml isoniazid and did so again in a repeat test.

Identification tests

The following identification tests were employed to determine whether the cultures were of tubercle bacilli: (a) bacterial morphology in a smear from the growth on Löwenstein-Jensen medium stained by the Ziehl-Neelsen method; (b) colonial morphology on Löwenstein-Jensen medium slopes and 7H-10 oleic-acid-albumin agar plates; (c) pigmentation of the growth on Löwenstein-Jensen medium in the dark and after exposure to daylight; (d) a qualitative catalase test; and (e) the niacin test. The methods employed and the interpretation of these tests have been described elsewhere (Thomas et al., 1961).

Virulence tests

Guinea-pigs. The origin and diet of the M-breed mixed-colour guinea-pigs used in this study has been described in detail by Mitchison et al. (1960). A total of 162 guinea-pigs (52% males) of average weight 493 g (range, 280-735 g) were infected with the 81 Thai cultures and 134 guinea-pigs (48% males) of average weight 482 g (range, 280-711 g) with the 67 British cultures.

Infection of the guinea-pigs. An aqueous suspension of the growth containing 2 mg (moist weight) bacilli per ml was prepared from each subculture after incubation at 37°C for three weeks. Two guinea-pigs were each injected intramuscularly in the right thigh with 0.5 ml (1 mg (moist weight) bacilli) of this suspension.

Measure of virulence. Six weeks after infection the surviving guinea-pigs were killed and the amount of visible disease at post-mortem examination was assessed as a score ranging from 0 to 100. The maximum score for the spleen was 40, for the liver 30, for the lungs 20, and for the site of inoculation, and its draining lymph-nodes, 10. Animals that died before six weeks were autopsied and scored in the same way. The total score for each animal (whether dead from tuberculosis or killed) was divided by its survival time in days to give an index. This index is a measure of the rate at which lesions developed in the organs and, by inference, it also measures the approximate rate at which tubercle bacilli multiply in the animal body. Further, it combines the results of the score and the mortality. For reasons given by Mitchison et al. (1961), the mean of the square-roots of the indices for the two guinea-pigs infected with each culture, termed the "root-index of virulence", has been employed as the measure of virulence of the culture.

Spleen cultures. At autopsy, approximately a quarter of the spleen was homogenized in about 1 ml of water and a loopful of the suspension was inoculated on to two slopes of Löwenstein-Jensen medium. The slopes were incubated at 37°C for eight to nine weeks and examined weekly for the presence of growth. The number of colonies was recorded.

Mantoux tests. Four weeks after infection, 0.1 ml of 1 : 100 Old Tuberculin was injected intracutaneously into the abdominal wall. The reactions were read after 48 hours and the results were expressed as the mean of the two diameters of the area of erythema taken at right angles to each other.

Arrangement of experiments

The virulence in the guinea-pig of the 81 Thai and 67 British cultures of tubercle bacilli that were sensitive to isoniazid was estimated in eight experiments. These eight experiments were set up successively at approximately 6-week intervals, starting in July 1960. The number of Thai and British cultures tested in the different experiments is set out in Table 1.

TABLE 1
NUMBERS OF CULTURES IN EACH VIRULENCE EXPERIMENT

Experiment No.	Number of Thai cultures	Number of British cultures	Total
1	14	4	18
2	10	6	16
3	12	6	18
4	7	7	14
5	9	15	24
6	10	12	22
7	11	11	22
8	8	6	14
Total . . .	81	67	148

RESULTS

Identification tests

Seventy-nine of the 81 isoniazid-sensitive Thai cultures were examined by all the identification tests, one by all the tests except pigmentation after expo-

sure to light and one only by colonial morphology on 7H-10 oleic-acid-albumin agar plates. Of the 67 isoniazid-sensitive British cultures, 57 were examined by all the identification tests, two by all the tests except that for colonial morphology on 7H-10 oleic-acid-albumin agar plates, one by all the tests except that for catalase activity and one only by the niacin test. The six British cultures that were tested in the second of the eight virulence experiments were, by mistake, not examined by any of the tests. All of the cultures gave results that were typical of *Mycobacterium tuberculosis* and all that were tested for niacin production were niacin-positive and were, therefore, presumably human strains.

Mortality of guinea-pigs

The frequencies with which Thai and British cultures caused death of the guinea-pigs from tuberculosis within six weeks of infection are compared in Table 2. A result in which neither of the two guinea-pigs infected with each culture died was obtained with 77 (95%) of the 81 Thai cultures and with 58 (87%) of the 67 British cultures. This difference does not attain statistical significance.

TABLE 2
ABILITY OF THAI AND BRITISH CULTURES OF TUBERCLE BACILLI TO CAUSE DEATH FROM TUBERCULOSIS IN GUINEA-PIGS

Number of guinea-pigs per culture which died from tuberculosis	Thai cultures		British cultures	
	No.	%	No.	%
0	77	95	58	87
1	3	4	8	12
2	1	1	1	1
Total . . .	81	100	67	100

Root-indices of virulence

The distributions of the root-indices of virulence of the 81 Thai and 67 British cultures are set out in Table 3. Root-indices of virulence of 0.8 or more, indicating extensive disease in the visceral organs, were obtained with 61 (75%) of the 81 Thai cultures and with 63 (94%) of the 67 British cultures. Ten (12%) of the Thai and one (1%) of the British cultures were of moderate virulence, with root-indices of virulence of 0.60-0.79, and 10 (12%) and

TABLE 3
VIRULENCE IN THE GUINEA-PIG OF THAI AND
BRITISH CULTURES OF TUBERCLE BACILLI

Root-index of virulence	Thai cultures		British cultures	
	No.	%	No.	%
0.0-	0	0	0	0
0.2-	2	2	0	0
0.4-	8	10	3	4
0.6-	10	12	1	1
0.8-	13	16	4	6
1.0-	30	37	14	21
1.2-	16	20	38	57
1.4-	2	2	6	9
1.6 or above	0	0	1	1
Total . . .	81	99	67	99
Weighted mean ^a	1.00		1.20	
Standard deviation ^b	0.22		0.15	

^a Weighted to allow for the disproportion in the numbers of Thai and British cultures in the different experiments.

^b Estimated after allowing for the differences between experiments.

three (4%), respectively, had values of less than 0.60, indicating a low degree of virulence with disease mainly confined to caseation at the site of inoculation and its draining lymph-nodes.

The proportion of Thai and British cultures tested in the eight experiments differed and so the data were examined by analysis of variance appropriate for disproportionate sub-class numbers (Snedecor, 1957).

The results of the analysis are presented in Table 4. It can be seen that there was a significant variation in the mean virulence of the Thai and British cultures from experiment to experiment ($P < 0.001$). If the

TABLE 4
ROOT-INDICES OF VIRULENCE OF THAI AND BRITISH
CULTURES: ANALYSIS OF VARIANCE

Source of variation	DF	Mean square	F	P
Races	1	2.9072	26.87	<0.001
Experiments	7	0.4961	4.59	<0.001
Residual	139	0.1082		

mean root-indices of virulence for Thai and British cultures had been compared directly, bias could have been introduced by experiments where high root-indices were found, if they contained particularly large proportions of cultures from one of the races. The analysis for disproportionate sub-class numbers used here makes it possible to avoid the bias by suitably weighting the mean virulences of the two races.

The weighted means of the root-indices were 1.00 for the 81 Thai cultures and 1.20 for the 67 British cultures. The respective standard deviations after allowing for inter-experimental variations were 0.22 and 0.15, and this difference does not attain statistical significance. The difference in the weighted means of the Thai and British cultures attains statistical significance ($P < 0.001$) thus indicating that the Thai cultures were, on the average, less virulent than the British cultures.

Spleen cultures

The results of the cultures from the spleens of guinea-pigs infected with Thai and British cultures are set out in Table 5. Heavily positive (3-plus or 2-plus) spleen cultures were obtained from 115 (72%) of the 160 guinea-pigs infected with Thai cultures and from 110 (84%) of the 131 guinea-pigs infected with British cultures. Thus, the results of spleen cultures again suggested that the British

TABLE 5
RESULTS OF SPLEEN CULTURES FROM GUINEA-PIGS
INFECTED WITH THAI OR BRITISH CULTURES OF
TUBERCLE BACILLI

Result of spleen culture ^a	Thai cultures		British cultures	
	No.	%	No.	%
3-plus	7	4	8	6
2-plus	108	58	102	78
1-plus	31	19	12	9
Negative	14	9	9	7
Total . . .	160 ^b	100	131 ^c	100

^a 3-plus: innumerable discrete colonies; 2-plus: 20-100 colonies; 1-plus: 1-19 colonies.

^b Excluding one guinea-pig which yielded a contaminated result on spleen culture, and another which died of a non-tuberculous condition.

^c Excluding two guinea-pigs for which the result on spleen culture was not available and one which yielded a contaminated result on spleen culture.

cultures were more virulent than the Thai cultures, but the differences between the two distributions do not attain statistical significance.

Mantoux reactions

The distribution of the mean diameters of the Mantoux reactions are set out in Table 6 for the 81 Thai and for 66 of the 67 British cultures. The result for one of the British cultures was not available because the two guinea-pigs infected with this strain died from tuberculosis before the fourth week. The mean diameters of the Mantoux reactions were closely similar—namely, 16.3 mm for the Thai cultures and 15.4 mm for the British cultures. When the variation in reaction size from culture to culture in the same experiment was compared with the variation between duplicate guinea-pigs by analysis of variance, the results with both the Thai and the British cultures appeared homogeneous.

TABLE 6
RESULTS OF MANTOUX REACTIONS IN GUINEA-PIGS
INFECTED WITH THAI OR BRITISH CULTURES
OF TUBERCLE BACILLI

Mean diameter of reaction (mm)	Thai cultures		British cultures	
	No.	%	No.	%
11-	0	0	3 ^a	5
13-	19 ^a	23	20 ^a	30
15-	33 ^a	41	32 ^a	48
17-	26 ^a	32	11 ^b	17
19-20	3	4	0	0
Total . . .	81	100	66 ^c	100
Weighted mean ^d	16.3		15.4	

^a Including one culture for which the result was based on one guinea-pig only, the other having died before 30 days.

^b Including two cultures, for each of which the result was based on one guinea-pig, the other having died before 30 days.

^c Excluding one culture for which both guinea-pigs died before 30 days.

^d Weighted to allow for the disproportion in the numbers of Thai and British cultures in the different experiments.

Retrospective comparison with Indian cultures

Since Indian cultures of tubercle bacilli were not tested in the present series of experiments, the Thai cultures could only be compared retrospectively with Indian cultures. An earlier report from this centre (Bhatia et al., 1961) compared 55 Indian and 28

British cultures for their virulence in the guinea-pig, using the same breed of guinea-pigs and the same method of measuring virulence except that one of the two guinea-pigs infected with a culture was killed at six weeks and the other at 12 weeks. In consequence, for the retrospective comparison, one of each of the two guinea-pigs, infected with the 81 Thai and 67 British cultures and killed at six weeks in the present experiment, was chosen at random for comparison with the 6-week guinea-pig in the earlier study. The distributions of the root-indices of virulence obtained in these two studies are set out in Table 7.

The mean root-indices of virulence were 1.01 for the 81 Thai cultures and 0.92 for the 55 Indian cultures, a difference which suggests that the Thai cultures were, on the average, of slightly higher virulence. However, the mean root-index of virulence was 1.21 for the 67 British cultures tested in the present study and 1.34 for the 28 British cultures tested previously—a significant difference ($P=0.03$). This difference suggests that the scoring of the disease in the present study was lower than that in the previous study and that, therefore, the difference between the mean virulence of Thai and Indian cultures (0.09) is underestimated. It can be concluded that the mean virulence of Thai cultures lies intermediate between the means for the Indian and the British cultures.

DISCUSSION

The present study has compared the virulence in the guinea-pig of isoniazid-sensitive cultures isolated, before the start of treatment, from 81 Thai and 67 British patients. Three assessments of virulence were used: (1) the extent of the disease produced in the guinea-pig as assessed by a scoring system which took into account the period of survival of the animals; (2) the proportion of cultures that caused death from tuberculosis within six weeks after infection; (3) the degree of positivity of cultures made from the spleens of the guinea-pigs that were killed at six weeks. There was a statistically significant difference between the Thai and British cultures in respect of the extent of the disease produced in the guinea-pigs, the Thai cultures having been, on the average, less virulent than the British cultures. There was, however, only a suggestion that the Thai cultures less frequently caused the death of guinea-pigs within six weeks and that guinea-pigs infected with Thai cultures had smaller numbers of

TABLE 7
 VIRULENCE IN THE GUINEA-PIG OF THAI AND BRITISH CULTURES OF TUBERCLE BACILLI
 IN THE PRESENT SERIES COMPARED WITH THOSE OF BRITISH AND INDIAN CULTURES
 IN A PREVIOUS SERIES

Root-index of virulence	Present series ^a				Previous series ^b (Bhatia et al., 1961)			
	Thai cultures		British cultures		British cultures		Indian cultures	
	No.	%	No.	%	No.	%	No.	%
0.0-	0	0	0	0	0	0	0	0
0.2-	2	2	0	0	0	0	1	2
0.4-	9	11	2	3	0	0	5	9
0.6-	8	10	3	4	1	4	19	35
0.8-	11	14	5	7	1	4	8	15
1.0-	29	36	14	21	7	25	10	18
1.2-	14	17	28	42	5	18	7	13
1.4-	8	10	12	18	9	32	5	9
1.6 or above	0	0	3	4	5	18	0	0
Total . . .	81	100	67	99	28	101	55	101
Mean ^c . . .	1.01		1.21		1.34		0.92	

^a One guinea-pig selected at random.

^b Root-index on 6-week guinea-pig.

^c Unweighted mean.

tubercle bacilli in their spleens than those infected with British cultures.

Since Indian cultures were not tested in the present study, the Thai cultures could only be compared retrospectively with Indian cultures that had previously been tested by Bhatia et al. (1961); a link between the two studies was provided by tests on samples of British cultures which were included in each study. This comparison showed that the Thai cultures were, on the average, probably more virulent than the Indian cultures. This difference between the virulence of Thai and Indian cultures is interesting in view of the close geographical proximity of Thailand to India. Since Thai cultures have an intermediate degree of virulence between Indian and British tubercle bacilli, it suggests that the eastern geographical limit of the distribution of Indian-type tubercle bacilli may be somewhere in this region. It would therefore be of interest to extend this type of investigation to a study of the virulence of cultures of tubercle bacilli from other countries surrounding India, in order to delineate

the geographical distribution of pulmonary tuberculosis due to attenuated tubercle bacilli.

The possibility that some of the Thai patients may have received previous chemotherapy does not invalidate the present virulence comparison. All the 81 Thai and 67 British cultures used for this comparison were isoniazid-sensitive and it has been shown by Subbaiah et al. (1961) that, in the absence of the development of isoniazid-resistance, the degree of virulence is not affected by three months of treatment with isoniazid plus PAS or with isoniazid alone. The Thai cultures were obtained, in part, from patients detected as having tuberculosis in a sample survey, whereas the British and Indian cultures were obtained from patients attending chest clinics because of their symptoms. Any consequent differences in the severity of disease in the patients of the three races is unlikely to have affected the comparisons of virulence, since Ramakrishnan et al. (1961) have shown that the virulence of isoniazid-sensitive pretreatment cultures from Indian patients is not related to the severity of their disease.

SUMMARY

1. Isoniazid-sensitive cultures of tubercle bacilli obtained from 81 Thai and 67 British patients with pulmonary tuberculosis have been compared in respect of their virulence in the guinea-pig.

2. The Thai cultures produced, on the average, less extensive disease in the guinea-pig than the British cultures. There was also a suggestion that the Thai cultures less frequently caused the death of guinea-pigs within six weeks and that guinea-pigs

infected with Thai cultures had smaller numbers of tubercle bacilli in their spleens than those infected with British cultures.

3. A retrospective comparison with Indian cultures suggested that the virulence in the guinea-pig of Thai cultures was intermediate between that of Indian and that of British cultures.

4. The mean diameters of the Mantoux reactions in the guinea-pigs infected with the Thai and British cultures were closely similar.

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RÉSUMÉ

Des travaux récents effectués au Centre de Chimiothérapie de la Tuberculose de Madras et à la Postgraduate Medical School de Londres ont montré que les cultures de bacilles tuberculeux sensibles à l'isoniazide prélevés, avant la mise en œuvre de la chimiothérapie, chez des tuberculeux originaires du Sud de l'Inde ont une virulence moindre que celles de bacilles homologues provenant de malades résidant en Angleterre.

Le présent travail a pour but d'étendre ces études à des régions situées en dehors de l'Inde. C'est ainsi que des cultures de bacilles tuberculeux sensibles à l'isoniazide provenant de 81 malades Thaïs et de 67 Anglais atteints de tuberculose pulmonaire ont été comparés selon leur virulence pour le cobaye.

Les cultures thaïes ont produit chez le cobaye une maladie moins envahissante que les cultures anglaises. L'on a eu aussi l'impression que les cultures thaïes entraînaient moins souvent la mort en l'espace de 6 semaines et que les rates de cobayes infectés par des cultures thaïes contenaient moins de bacilles tuberculeux que celles infectées par des cultures anglaises.

Une comparaison rétrospective avec les cultures indiennes incite à penser que la virulence des cultures thaïes pour le cobaye se situe entre celle des cultures indiennes et celle des cultures anglaises.

Le diamètre moyen des réactions de Mantoux est sensiblement le même chez les cobayes infectés par des cultures thaïes et chez ceux infectés par les cultures anglaises.

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