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# THE GROWTH RATE OF TUBERCLE BACILLI FROM SOUTH INDIAN AND BRITISH PATIENTS*.

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## INTRODUCTION.

CULTURES of tubercle bacilli from Indian patients have been shown to be, on average, less virulent in the guinea-pig and to have a wider range of virulence than cultures obtained from British patients (Frimodt-Moller, Mathew and Barton, 1956 ; Mitchison *et al.,* 1960 ; Bhatia *et al.,* 1961). In the study of Bhatia *et al. (loc. cit.)* about one-third of the Indian cultures were as virulent as British cultures, the remainder being less virulent. In these studies the extent of disease in the organs of the guinea-pig was scored at intervals after the intramuscular injection of the organisms. In consequence, the measure of virulence was based upon the rate of development of the lesions and, by inference, the rate of multiplication of the bacilli in the organs. It was, therefore, considered of interest to compare the growth rates *in vitro* of Indian and British cultures of tubercle bacilli.

## MATERIALS AND METHODS.

*Cultures.*– A total of 34 cultures of tubercle bacilli from the same number of Indian patients selected at random from amongst those attending the Tuberculosis Chemotherapy Centre, and a total of 33 cultures from the same number of British patients attending a number of chest clinics in Britain were studied. All these patients had previously untreated pulmonary tuberculosis and were aged 12 years or more. All the cultures from the British patients were sensitive to isoniazid, streptomycin and PAS. The 29 Indian cultures which were tested for sensitivity to streptomycin and isoniazid, were sensitive to both these drugs ; sensitivity tests were not done on the remaining 5 Indian cultures.

*Control strain.*– The generation time of a culture of *Myco-tuberculosis,* strain H37Rv, which had recently been passaged through a guinea-pig was determined on one occasion. This culture was obtained from Dr. D.A. Mitchison of the Post-graduate Medical School of London.

*Media.*– The Lowenstein-Jensen medium referred to in this paper did not contain potato starch (Jensen, 1955). The 7H-10 Tween-albumin liquid medium was prepared as described by Cohn, Middlebrook and Russell (1959) but without glycerol. Double strength 7H-10 medium was used for viable counts as described by Subbaiah, Mitchison and Selkon (1960).

*Sputum culture and drug sensitivity tests.*– The sputum specimens from Indian patients were cultured at the Tuberculosis Chemotherapy Centre, Madras, and

* From the Tuberculosis Chemotherapy Centre. Madras, India. The **Centre is under the joint auspices** of the Indian Council of Medical Research, the Madras **State Government, the World** Health Organisation, and the Medical Research Council of Great Britain.

those from the British patients at the Post-graduate Medical School, London. In both laboratories, the sputum specimens were homogenised and decontaminated (Tuberculosis Chemotherapy Centre, 1969) by treatment with 4 per cent NaOH and then, after washing with distilled water, cultured on Lowenstein-Jensen medium. The sensitivity tests were carried out as described elsewhere (Tuberculosis Chemotherapy Centre, *loc. cit.).*

*Determination of the rate of growth.*– A representative sample of the growth on the primary diagnostic culture on Lowenstein-Jensen medium was inoculated into 6 c.c. of 7H-10 Tween-albumin liquid medium. After 8 days incubation at 37°C, 2 drops of the resulting growth was inoculated into a 5 c.c volume of 7H-10 Tween-albumin medium. After 7 days incubation at 37°C., the turbidity of the subculture was measured using an 'EEL' nephelometer. The number of viable units was estimated from a previously prepared standard curve and the suspension diluted to contain $5 \times 10^6$ viable units/c.c. One c.c of this suspension was inoculated into 09 c.c. of 7H-10 Tween-albumin medium in a 250 c.c. Erlenmeyer flask, which had a side tube for nephelometry, to give a final concentration of $5 \times 10^4$ viable units/c.c.

*Viable counts.*– Viable counts were set up by adding 0.2 c.c. of the appropriate dilutions of the suspension adjusted to contain $5 \times 10^6$ viable units/c.c. at the start of the experiment and from Erlenmeyer flasks after 4, 6 and 8 or 9 days incubation at 37°C., to 3.1 c.c. of double-strength 7H-10 liquid medium which was then solidified with 3.3 c.c. of silica sol (Selkon and Mitchison, 1957). After 4 weeks incubation at 37°C. the number of colonies was counted. Since there was occasional contamination of one of the triplicate counts set up, duplicate counts chosen at random were used in all analyses.

*Nephelometric measurements of turbidity.*– The turbidity of 8 of the Indian and 8 of the British cultures was measured daily for 8 days using an 'EEL' nephelometer.

*Generation time.*– The generation time was estimated from the viable counts and turbidimetric measurements as follows : A straight line with the equation $y=a+b\ x$ (y is the log viable count or turbidimetric measurment and x is the day on which the viable count or turbidimetric measurement was done) was fitted by the method of least squares. The slope of the straight line, represented by b in the equation, estimated the rate of growth. The generation time (G) in hours was then calculated by using the formula :

$$G = \frac{\log_{10} 2 \times 24}{b}$$

*Statistical note.*– The viable counts at 4, 6 and 8 or 9 days were examined by analysis of variance after transformation to logarithmic units. The transformation was necessary since the untransformed viable counts did not satisfy the assumption of homogeneity of variance. The logarithmic transformation, on examination, was found to produce as good an approximation towards homogeneity of variance as the square-root transformation and, in addition, had the advantage of simplifying the

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regression analysis since there is genenerally a linear relation between log counts and time.

**RESULTS.**

*Viable counts on Indian and British cultures.*– Of the 34 Indian and 33 British cultures selected for this experiment 4 Indian and 1 British cultures failed to grow when inoculated into 7H-10 Tween-albumin liquid medium for the first time and 3 Indian and 1 British cultures failed to grow in subsequent subcultures in this medium. This suggests that the 7H-10 medium is possibly unsatisfactory for some cultures, particularly some Indian cultures. Viable counts were set up on 23 Indian and on 27 British cultures after 4, 6 and 8 days incubation at 37°C., and on the remaining 4 Indian and 4 British cultures after 4, 6 and 9 days incubation.

The mean log viable counts at 4, 6 and 8 days for the 23 Indian and 27 British cultures are presented in Table I, along with their estimated generation times. Of

TABLE I.

*Mean log viable units c.c. after 4, 6 and 8 days incubation at 37°C. and the generation time of Indian and British cultures of tubercle bacilli.*

| INDIAN CULTURES : | | | | | BRITISH CULTURES: | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Culture number. | Mean log viable units/cc.* | | | Generation time, hours. | Culture number. | Mean log viable units/cc.* | | | Generation time, hours. |
| | 4 days. | 6 days. | 8 days. | | | 4 days. | 6 days. | 8 days. | |
| 45,490 | 5.20 | 5.98 | 6.97 | 16.37 | I 1,205 | 5.06 | 6.22 | 6.82 | 16.42 |
| 45,413 | 5.52 | 6.30 | 7.09 | 18.47 | I 971 | 5.46 | 6.32 | 7.15 | 17.05 |
| 51,916 | 5.44 | 6.34 | 6.96 | 19.01 | I 1,079 | 5.65 | 6.54 | 7.26 | 17.95 |
| 39,253 | 5.94 | 6.61 | 7.42 | 19.59 | I 977 | 5.89 | 6.54 | 7.48 | 18.18 |
| 39,294 | 5.79 | 6.58 | 7.26 | 19.73 | I 1,152 | 5.61 | 6.44 | 7.16 | 18.70 |
| 43,226 | 6.05 | 6.85 | 7.50 | 20.00 | I 1,439 | 4.98 | 5.48 | 6.50 | 18.95 |
| 49,996 | 5.01 | 5.60 | 6.40 | 20.79 | I 1,196 | 4.55 | 4.70 | 6.05 | 19.27 |
| 39,312 | 5.58 | 6.06 | 6.96 | 20.87 | I 1,210 | 5.29 | 6.12 | 6.75 | 19.79 |
| 39,069 | 5.26 | 5.70 | 6.54 | 22.58 | I 1,513 | 5.57 | 6.28 | 6.98 | 20.42 |
| 52,610 | 5.52 | 6.72 | 6.79 | 22.76 | I 1,215 | 5.58 | 6.32 | 6.98 | 20.57 |
| 49,498 | 5.82 | 6.32 | 7.09 | 22.84 | I 1,418 | 4.86 | 5.30 | 6.24 | 20.87 |
| 52,108 | 4.90 | 5.24 | 6.08 | 24.49 | I 1,419 | 5.20 | 5.52 | 6.55 | 21.49 |
| 49,317 | 4.80 | 5.35 | 5.94 | 25.24 | I 1,500 | 5.70 | 6.38 | 7.03 | 21.65 |
| 49,552 | 5.70 | 6.10 | 6.78 | 26.88 | I 1,497 | 5.64 | 6.32 | 6.96 | 21.98 |
| 43,262 | 5.30 | 5.69 | 6.35 | 27.52 | I 1,326 | 5.76 | 6.30 | 7.10 | 22.06 |
| 51,077 | 5.06 | 5.58 | 6.09 | 28.06 | I 1,448 | 5.02 | 5.45 | 6.30 | 22.58 |
| 53,079 | 5.56 | 6.03 | 6.56 | 28.76 | I 1,329 | 5.30 | 5.94 | 6.56 | 22.84 |
| 52,662 | 5.30 | 5.90 | 6.29 | 29.19 | I 1,142 | 5.44 | 6.05 | 6.67 | 23.50 |
| 42,980 | 5.98 | 6.38 | 6.96 | 29.49 | I 1,131 | 5.34 | 5.99 | 6.55 | 23.88 |
| 52,505 | 5.02 | 5.65 | 6.00 | 29.64 | I 1,072 | 5.63 | 5.90 | 6.83 | 24.08 |
| 51,377 | 4.98 | 5.20 | 5.94 | 30.10 | I 1,446 | 5.12 | 5.72 | 6.30 | 24.49 |
| 43,509 | 5.98 | 6.94 | 6.93 | 30.26 | I 1,437 | 5.24 | 5.45 | 6.42 | 24.70 |
| 52,326 | 5.32 | 5.54 | 6.22 | 31.76 | I 1,107 | 5.64 | 5.90 | 6.80 | 24.81 |
| | | | | | I 1,304 | 5.43 | 6.04 | 6.52 | 26.39 |
| | | | | | I 1,330 | 5.40 | 6.14 | 6.45 | 27.39 |
| | | | | | I 1,478 | 6.00 | 6.58 | 7.00 | 28.90 |
| | | | | | I 1,332 | 5.44 | 5.92 | 6.36 | 31.24 |
| All cultures | 5.44 | 6.03 | 6.66 | 23.70† | All cultures | 5.40 | 5.99 | 6.73 | 21.70† |

*Arithmetic mean of the logarithms of 2 replicate counts.

†Estimated by fitting a straight line to mean log viable units/c.c. of all cultures at 4, 6 and 8 days.

the 23 Indian cultures 8 (35 per cent) had a generation time of 16 to 21. hours, 7 (30 per cent) 22 to 27 hours and 8 (35 per cent) 28 to 33 hours. The numbers of British cultures with corresponding generation times were 12 (44 per cent), 13 (48 per cent) and 2 (7 per cent), respectively. There was, thus, a suggestion that a larger proportion of British cultures had shorter generation times than Indian cultures.

The results were further examined by the method of analysis of variance (Table II). A straight line was a good fit for the mean log viable counts at 4, 6 and 8 days for both the Indian and British cultures (Table II, terms e and f) indicating that the Indian and British tubercle bacilli were multiplying logarithmically between 4 and 8 days. The mean generation time, estimated from the slopes of these lines was 23.7 hours for the Indian cultures and 21.7 hours for the British cultures. This small difference between the mean generation times of Indian and British cultures was found not to attain statistical significance (Table II, term c, P=0.1). The mean generation time estimated from the slope of the joint regression line, (term b) was 22.6 hours, the 96 per cent confidence interval being 20.7 hours to 24.9 hours.

TABLE II.

Viable counts on Indian and British cultures of tubercle bacilli.

**Analysis of variance**

| Term. | Source. | Sum of squares. | D.F. | Mean square. | Term tested against. | F | P |
|---|---|---|---|---|---|---|---|
| a | Cultures | 45·5191 | 49 | 0·9290 | | | |
| b | Joint regression | 82·0480 | 1 | 82·0480 | d+g | 1508·24 | 0·001 |
| c | Difference between regressions of Indian and British cultures | 0·1540 | 1 | 0·1540 | d+g | 2·82 | 0·10 |
| d | Deviations from joint regression line | 0·1956 | 2 | 0·0978 | g | 1·83 | 0·18 |
| e | Deviations from regression of Indian cultures | 0·0107 | 1 | 0·0107 | g | ... | N.S.* |
| f | Deviations from regression of British cultures | 0·1849 | 1 | 0·1849 | g | 3·46 | 0·07 |
| g | Residual | 5·1344 | 96 | 0·0535 | | | |
| | Total | 133·0511 | 149 | | | | |

*N.S. indicates that the variance ratio is less than 1.

Similar analyses (not tabulated here) were done on the viable counts obtained with the 4 Indian and 4 British cultures after incubation at 37°C. for 4, 6 and 9 days. The growth of both the Indian and British cultures was exponential during this period and their mean generation times again did not differ significantly.

*Generation time estimated from nephelometric measurements of turbidity.*—On 8 of the 27 Indian cultures and 8 of the 31 British cultures, nephelometric measurements were also made daily for 8 days. Since the nephelometer was unable to detect the presence of turbidity at 4 days in all the cultures, the generation times were estimated by fitting straight lines to the nephelometric measurements at 5, 6, 7 and 8 days (Table III). The mean generation time was 25·5 hours for the Indian

**TABLE III.**

*Comparison of the generation times estimated from viable counts and turbidity measurements of Indian and British cultures of tubercle bacilli.*

| INDIAN CULTURES: | | | BRITISH CULTURES : | | |
|---|---|---|---|---|---|
| Culture number. | Generation time (hours). | | Culture number. | Generation time (hours). | |
| | Estimated from turbidity at 5, 6, 7 and 8 days. | Estimated from viable counts at 4, 6 and 8 days. | | Estimated from turbidity at 5, 6, 7 and 8 days. | Estimated from viable counts at 4, 6 and 8 days. |
| 43,226 | 19•79 | 20•00 | I 1,152 | 16.65 | 18.70 |
| 42,980 | 20•82 | 29•49 | I 1,142 | 20.52 | 23.50 |
| 39,069 | 23•00 | 22•58 | I 1,131 | 22.16 | 23.88 |
| 43,262 | 23•00 | 27•52 | I 1,072 | 22.42 | 24.08 |
| 39,253 | 24•00 | 19•59 | I 1,107 | 22.86 | 24.81 |
| 43,509 | 33•76 | 30•26 | I 1,079 | 29.25 | 17.95 |
| 39,294 | 34•08 | 19•73 | I 971 | 33.14 | 17.05 |
| 39,312 | 35•94 | 20•87 | I 977 | 39.92 | 18.18 |
| All cultures | 25•50* | 23•00† | All cultures | 24.10* | 20.40† |

*Estimated by fitting a straight line to the mean log nephelometrlc units of all cultures at 5, 6, 7 and 8 days.

† Estimated by fitting a straight line to the mean log viable units/c.c. of all cultures at 4, 6 and 8 days.

cultures and 24.1 hours for the British cultures, a difference that does not attain statistical significance. The mean generation times for these cultures, estimated from the viable counts at 4, 6 and 8 days, were 23.0 hours for the Indian and 20.4 hours for the British cultures. The difference between the mean generation times determined by the two methods does not attain statistical significance.

*Generation time of strain H37Rv*.– The rate of growth of strain H37Rv was determined on one occasion. The log viable counts after 4, 6 and 8 days incubation at 37°C. were 5.90, 6.56 and 7.40, respectively. These counts were found to fit a straight line and the generation time was estimated at 19.3 hours.

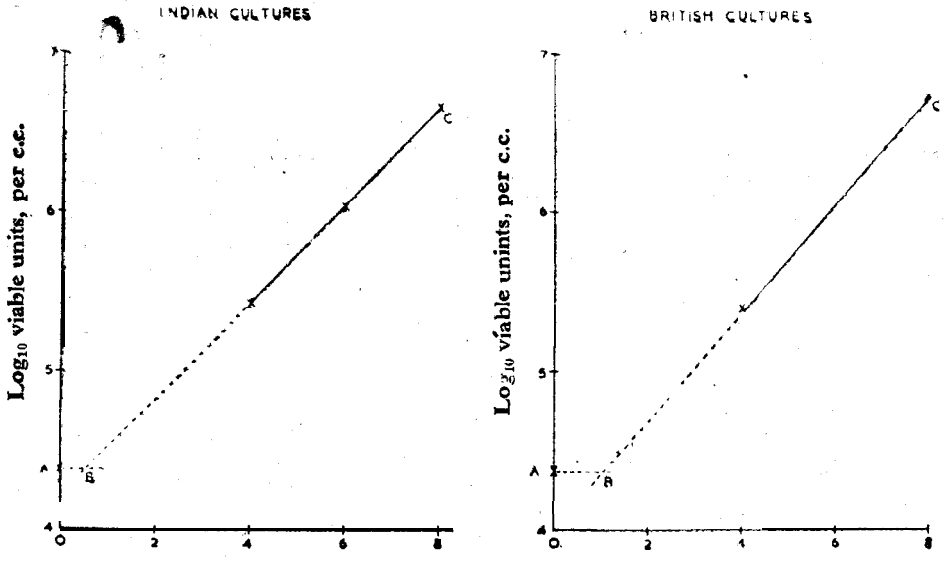
*Lag phase*– The mean viable counts of the Indian and British cultures at the start of the experiment were 4.38 and 4.37 log viable units, respectively. The duration of the lag phase for the Indian cultures as a whole and for the British cultures as a whole were estimated as follows : The straight line fitted to the mean viable counts at 4, 6 and 8 days for the culture of each race was extrapolated (Graph BC) to intersect with the horizontal line drawn through the initial mean viable count. The time between the initial viable count and the point of intersection (Graph AB) was taken as the lag phase (Squires and Hartsell, 1955). For the Indian cultures the mean lag phase was 13.4 hours and for the British cultures 23.8 hours.

## DISCUSSION.

In the present investigation, the rate of growth of Indian and British cultures of tubercle bacilli has been studied by viable counts and nephelometric estimations of the growth of cultures in 7H-10 Tween-albumin liquid medium. The medium

**GRAPH.**

Lag phase of Indian and **British** cultures of tubercle bacilli.




A=Initial viable count. AB=Lag phase. BC=Extrapolated regression line.

was incubated in shallow layers, about 2 cm. deep, in Erlenmeyer flasks plugged with cotton wool, and was shaken gently once daily. Aeration by continuous mechanical agitation or bubbling sterile air through the culture was not used because of conflicting reports of its value in enhancing the rate of growth of tubercle bacilli and because of its tendency to produce granular growth (Sachaefer, Marshak and Burkhart, 1949 ; Kull and Grimm, 1952 ; Volk and Myrvik, 1953 ; Halpern and Kirchheimer, 1964; Miller and Roessler, 1956). Logarithmic growth of the cultures was nevertheless obtained in the present experiments as shown by the close fit to a straight line of the log viable counts at 4, 6 and 8 or 9 days and the log nephelometric units at 5, 6, 7 and 8 days. The 7H-10 medium was considered suitable for the comparison of Indian and British cultures in view of the report by Holmgren and Youman (1952) that the addition of albumin fraction V to Proskauer and Beck medium stimulated the growth of virulent H37Rv more markedly than the attenuated strain H37Ra, resulting in a significant, difference in their generation times.

The estimates of the generation time of strain H37Rv, obtained in this study (19.3 hours) was similar to the estimates of 18 hours (range 18 to 23 hours) obtained by Fenner and Leach (1953) in Dubos Tween-albumin medium, of 17.3 hours obtained by Youmans & Youmans (1949) in Proskauer and Beck medium containing albumin fraction V and of 20.5 hours by Miller and Roessler *(loc. cit.)* for a stationary culture in Dubos Tween-albumin medium. Thus, the method used for determining

the generation-time of tubercle bacilli in the present investigation is comparable to that used by other authors.

The virulence in the guinea-pig of the Indian and British cultures studied in the present investigation could not be determined owing to the shortage of guinea-pigs. Bhatia *et al. (loc. cit.)* found that approximately two-thirds of Indian cultures were less virulent than the British cultures. Since the 27 Indian cultures studied in this investigation were obtained from 27 patients chosen at random from amongst those attending this Centre, it is reasonable to assume that they are representative of Indian cultures in respect of their virulence in the guinea.pig. It can, therefore, be inferred that the difference between the virulence of Indian and British cultures is unlikely to be due to a difference in their *in vitro* growth rates. However, there remains the possibility that the medium and the conditions of aeration used in this investigation did not simulate the complex biochemical conditions present *in vivo* sufficiently closely to demonstrate a difference in the generation times.

SUMMARY.

1. The rate of growth *in vitro* of cultures of tubercle bacilli obtained from 27 Indian and 31 British patients was determined from the viable counts of the growth after 4, 6 and 8 or 9 days incubation at 37%.

2. The rate of growth of both the Indian and British cultures was exponential between 4 and 8 or 9 days.

3. The mean generation times for the 23 Indian cultures and 27 British cultures which had viable counts set up at 4, 6 and 8 days, were 23.7 and 21.7 hours, respectively. The difference between the generation times does not attain statistical significance.

4. The generation times of 8 of the 27 Indian and 8 of the 31 British cultures were in addition estimated from nephelometrid measurements of the turbidity of cultures. The generation times estimated by nephelometry were slightly longer than those estimated from the viable counts, but again no significant difference was found between the generation times of Indian and British Cultures.

5. The lag phase for the Indian cultures was 13.4 hours compared with 23.8 hours for the British cultures.

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