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Studies on *M. lepraemurium* and *M. leprae* in Tissue Culture

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STUDIES ON *M. LEPRAEMURIUM*

Despite many attempts over the years, neither the rat leprosy bacillus (*M. lepraemurium*) nor the human leprosy bacillus (*M. leprae*) has been grown in ordinary bacteriologic media. *In vivo*, the organisms are obligate intracellular parasites; to thrive *in vitro*, like viruses, they may require an intracellular environment. Tissue culture, therefore, seemed an obvious approach to the problem. To begin our studies of *M. lepraemurium* in tissue culture we used monocytes, because, in the natural host, the bacilli have a predilection for cells of the reticulo-endothelial system. In 1956 and 1957 monocytes were kept in good condition for 80 days in a medium of embryo extract, and Balanced Salt Solution (BSS), or Parker's 199 in which albumin and globulin were substituted for the usual serum component. This medium required renewal only every 10 days. However, unlike Chang (1), we did not obtain multiplication of *M. lepraemurium* when using quantitative methods of assessment. Spleen explants from mice infected *in vivo* were also tried, and, although assessment of these cultures was difficult, doubling numbers were observed (9). Because the life of the explants was limited, and because explants, like monocytes, could not be transferred, neither appeared to be a suitable host cell for continuous multiplication of *M. lepraemurium in vitro*. We turned our attention therefore to some of the established cell lines.

The first cell line used was L strain, a mouse fibroblast which is phagocytic and ingests *M. lepraemurium* very well. In 17 experiments, however, results were negative. Wallace, Elek and Hanks (11) reported doubling numbers in L strain cultures with added hydrocortisone, but we found their concentration of hydrocortisone toxic and obtained no multiplication.

We next used 14pf cells—a rat fibroblast line—obtained from Dr. George O. Gey. This strain was established in 50 per cent human cord serum (CS_{50}). Because the latter is rather difficult to obtain, we attempted to adapt the cells to simpler media, but with no great success. Therefore, we began our experiments using 50 per cent human cord serum and 50 per cent Hanks' BSS ($CS_{50}H_{50}$). In eight of nine experiments an increase in the number of bacilli was obtained, and in three of these the bacterial populations increased 5.5, 4.4 and 3.1 fold (Table 1), representing significantly greater multiplication than had previously been obtained by Rees and Wong (4) in spleen cultures and by Wallace, Elek and Hanks (11) with the L strain. Hydrocortisone did not enhance the bacillary multiplication, and a combination of streptomycin and isoniazid was inhibitory (2).

These results were encouraging, but multiplication was limited to the initial 13 day period of incubation, or, where it was more continuous, the rate of multiplication diminished, probably because of deterioration in the quality of the cells, since the conditions used were adopted primarily for accurate enumeration of bacilli. In particular the infected cells were kept 2 to 6 weeks without transfer to avoid loss of bacilli.

A procedure for obtaining continuous multiplication of *M. lepraemurium* in tissue culture. Procedures were then modified to provide more favorable conditions for maintaining healthy cells. Before the results of these successful experiments are

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TABLE 1. *Multiplication of* M. lepraemurium *in nonsubcultured rat fibroblasts grown for 26 to 39 days.*

Experiment number	Group	Maximum increase
1	A	2.2
	B + 0.1 mgm. HC[a] per ml.	2.1
2	A	2.2
	B + 0.1 mgm. HC per ml.	1.4
3	A	1.2
	B + 0.05 mgm. HC per ml.	1.0
4	A	5.3
	B + 0.05 mgm. HC per ml.	5.5
5	A	2.0
	B + 100γ SM[b] + 0.1γ INH[c] per ml.	1.1
6	A	4.4
	B + 100γ SM + 0.1γ INH per ml.	1.6
7	A	2.0
	B + 100γ SM + 0.1γ INH per ml.	1.1
8	A	2.5
	B + 100γ SM + 0.1γ INH per ml.	1.5
9	A	3.1
	B + 100γ SM + 0.1γ INH per ml.	1.4

[a]hydrocortisone (free alcohol).
[b]streptomycin.
[c]isoniazid.

presented, the methods used will be outlined briefly.

Cultures of fibroblasts on glass were infected by overlaying them for 24 hours with a partially purified suspension of *M. lepraemurium* in low concentrations of albumin or serum in BSS. The bacilli were freshly obtained from homogenized livers of heavily infected mice; approximately 90 per cent of the fibroblasts ingested bacilli. The infected cells were washed, trypsinized and transferred to fresh bottles. A medium consisting of 50 per cent cord serum and 50 per cent Hanks' BSS, plus 25 units of penicillin per ml. ($CS_{50}H_{50}$+25 pen./ml.) was added. Cultures were incubated at 34°C, and the medium was changed approximately every 10 days. Cells were subcultured if signs of deterioration were seen —usually at 20 to 30 days. The bacillary population was followed by counting the total number of acid-fast organisms in samples of cells taken at the beginning and end of each subculture period and in the samples of medium removed at 10-day intervals from the cultures. To count the total number of bacilli accurately in each culture it was necessary first to release them completely from the cells and so obtain a well dispersed suspension of organisms. This was achieved by exposing the suspension of cells to ultrasonic vibration ([3]). A standard method described by Hart and Rees ([4]) was used for counting the numbers of acid-fast bacilli in smears prepared from the suspensions. When a high proportion of the cells, about 75 per cent, was transferred, we could initiate and maintain continuous multiplication of *M. lepraemurium* in 14pf, but if, to avoid overcrowding, only 25 per cent of the cells were transferred, soon there were too few bacilli for enumeration ([3]). This illustrates the problem of attempting to culture an intracellular parasite with a much longer division time than the host cell.

Figure 1 shows a cumulative bacterial increase of 2.7×10^{29} over a total period of 1,618 days, of which 1,525 (or 4.4 years) were counted. This represents 99.5 generations, giving an average generation time of 15.3 days, which closely approximates the generation time of *M. lepraemurium in vivo*, suggesting that the cell cultures are providing the necessary growth factors or enzyme systems as efficiently as the animal host cells.

Subcutaneous inoculation of mice showed that bacilli recovered from the tissue cultures retained their viability and their

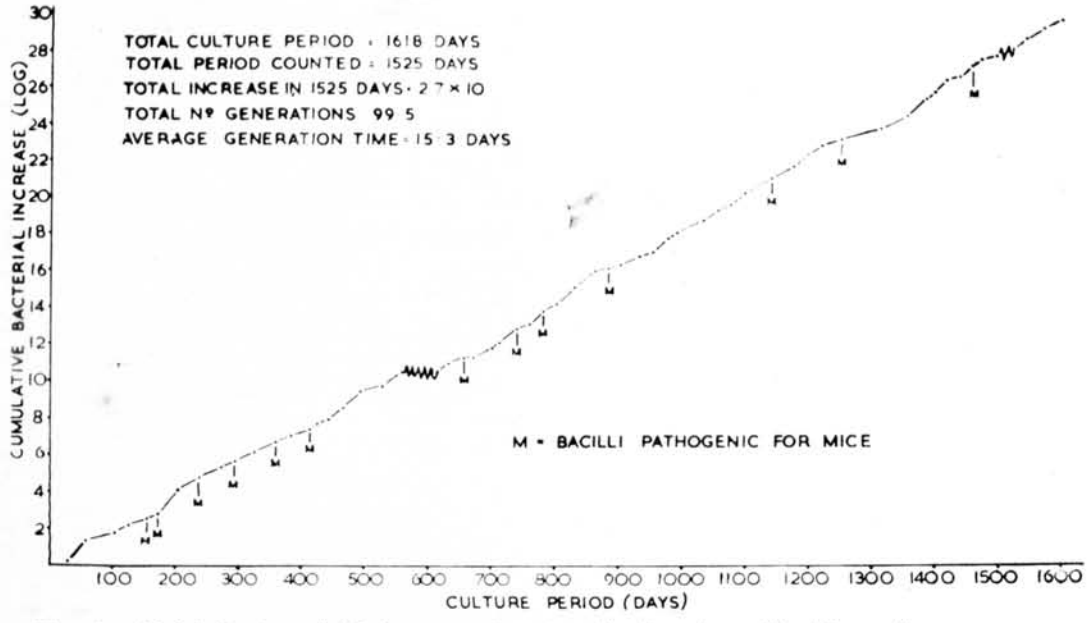

FIG. 1.—Multiplication of *M. lepraemurium* in subcultured rat fibroblast cells.

pathogenicity, for they produced typical leprosy nodules. At regular intervals we also inoculated the bacilli into bacteriologic media and, over a period of more than four years, never obtained growth of an acid-fast organism.

We have also shown that the bacilli grown in tissue culture can be used to infect new cells (Fig. 2). This can be done in two ways, either by releasing the bacteria from the cells by ultrasonic vibration and using them to infect fresh cells, or by mixing the heavily infected cells with uninfected cells. There is less danger of contamination when the latter method is used, and bacilli released from heavily infected cells are readily ingested by adjacent cells. Multiplication in these cultures seems to progress at the same rate as in the parent culture.

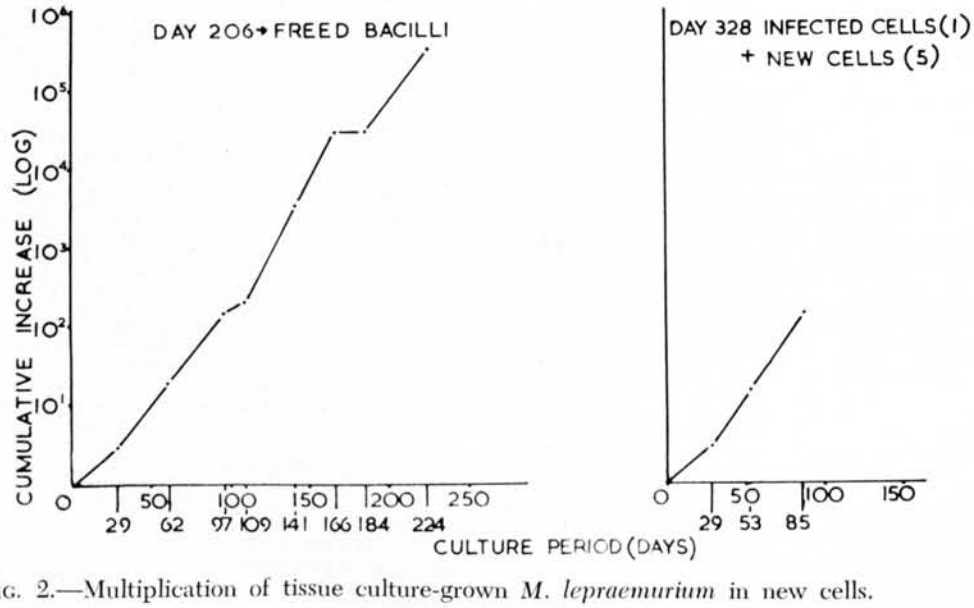

FIG. 2.—Multiplication of tissue culture-grown *M. lepraemurium* in new cells.

Concentrated filtrates of medium from heavily infected cultures were examined for the presence of soluble mycobacterial antigens, and small amounts have been detected in 14 of 24 filtrates screened ([7]).

Throughout the experiments the percentage of normal and degenerate bacilli has been followed, by use of electron microscopy or, latterly, by the method of Rees and Valentine ([8]), in which degenerate organisms can be identified by their irregular staining properties (Fig. 3).

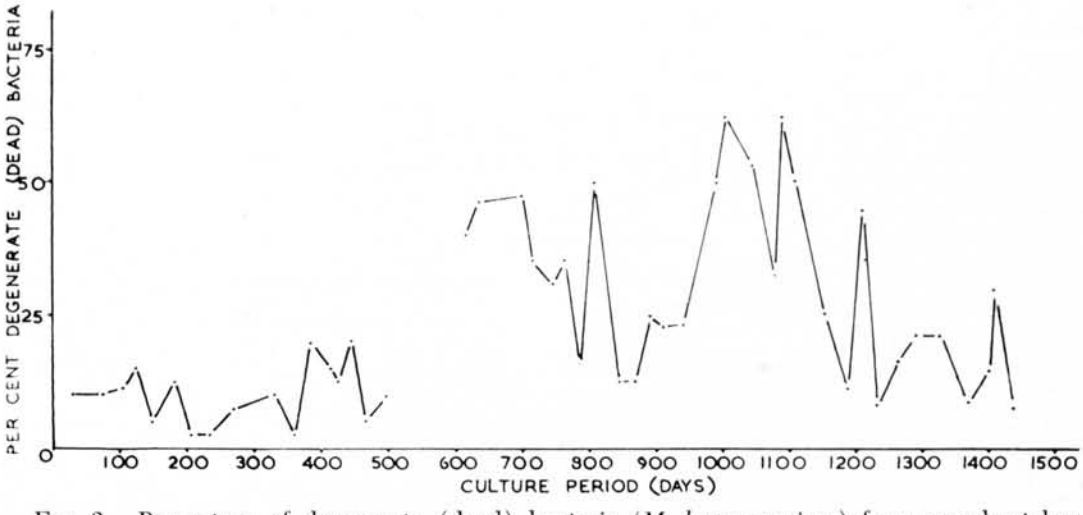

FIG. 3.—Percentage of degenerate (dead) bacteria (*M. lepraemurium*) from samples taken at the beginning of each subculture.

Analysis of some of the conditions required for continuous multiplication of *M. lepraemurium*. To obtain continuous multiplication of *M. lepraemurium* in 14pf we had deliberately slowed down growth of the cells in three ways: (1) by having dense cell populations, (2) by changing media infrequently, and (3) by incubating the cultures at 34° instead of 37°C. Reversal of these procedures produced faster growing cells, a fact that made the technic more laborious and unsuitable for continuous multiplication of *M. lepraemurium*. Nevertheless, the bacilli will multiply in these faster growing cells.

In the past two years we have adapted 14pf for growth without cord serum in Eagle's medium with added peptone plus 10 per cent newborn calf serum (CaS_{10} EP_{90}). In three experiments, however, using *M. lepraemurium* in these adapted cells, we observed not only that there was no multiplication of bacilli but that there was actual destruction and disappearance of organisms from cultures followed for 131, 139 and 173 days respectively. Thus it appears that *M. lepraemurium* is a particularly fastidious organism with subtle growth requirements dependent on both the host cell type and the tissue culture medium. Therefore we tested the rate of degeneration of *M. lepraemurium* in tissue

TABLE 2. *Survival of* M. lepraemurium *in cell-free media at 34°C.*

Culture period (days)	Percentage degeneration of *M. lepraemurium*		
	$CS_{50}H_{50}$	$CaS_{10}EP_{90}$	PO_4 Buffer
0	8/100 = 8	8	8
7	16/100 = 16	12/33 = 36	3/25 = 12
14	46/104 = 44	64/100 = 64	23/100 = 23
20	55/66 = 83	91/100 = 91	32/66 = 48
28	92/100 = 92	100/100 = 100	94/100 = 94

culture media at 34°C, comparing the rates in $CS_{50}H_{50}$, $CaS_{10}EP_{90}$ and PO_4 buffer over a period of 4 weeks. The results are shown in Table 2. From previous work ([6]) we know that organisms, even after regular and continuous growth within tissue culture cells, died when inoculated into $CS_{50}H_{50}$, i.e., the same medium used to maintain their host cells, but the death rate was not as rapid as that obtained with $CaS_{10}EP_{90}$.

STUDIES ON *M. LEPRAE*

Human embryo lung cells. Because *M. lepraemurium* had been cultivated successfully only in cells from susceptible hosts, it was felt that species specificity might be important for *M. leprae* as well. Therefore we chose a line of human diploid cells from embryo lung that grew well in $CaS_{10}EP_{90}$ at 34°C, requiring renewal of medium only every 10 days. The diploid cells were phagocytic for *M. leprae* but required a 72 hour exposure to the organisms for a satisfactory bacillary uptake.

The bacillary suspensions of *M. leprae* were prepared from leprosy nodules obtained at biopsy from untreated lepromatous patients. The nodules were minced with scissors and ground to a thin cream in 1 per cent albumin-saline in a glass tissue grinder. This was centrifuged at 1,500 rpm for 5 minutes. The supernatant was then removed and centrifuged at 4,500 rpm for 5 minutes. Its supernatant, in turn, was centrifuged at 4,500 rpm for 30 minutes. The two resulting deposits were then pooled in 1 to 2 ml. of 1 per cent albumin-saline and counted. All procedures were carried out at 4°C.

In experiments with *M. lepraemurium* the 14pf cells were overlaid with a suspension of 4×10^9 bacilli per ml. The counts of the *M. leprae* suspensions, however, were much lower, and in the three following experiments the diploid cells were overlaid in full culture medium with 2×10^8, 1.1×10^9 and 1.7×10^7 bacilli per ml. respectively. A 3 or 4 day infection period, instead of 24 hours, was adopted. The infected cells were washed and fresh medium was added. Because of their fragile appearance, due, perhaps to the toxic effect of tissue debris in the *M. leprae* suspension, the cells were not transferred immediately after infection. They were allowed a recuperation period of 4 to 14 days before being transferred and the day of transfer became the baseline of the experiment. The first experiment (B) was terminated after 196 days because of contamination. There were four transfers, the first being on day 17. The total calculated bacillary increase to day 141 was 148.6 fold, or 7.1 generations, giving a generation time of 17.4 days *in vitro*. On the third transfer from day 110 to day 141 there was an increase of 21 fold. Although the numbers counted throughout were small, it was felt that multiplication of bacilli was occurring; otherwise bacilli would have disappeared from the cultures before day 110. Furthermore, after the 21 fold increase there was a corresponding increase in the baseline count, and the percentage of degenerate bacilli had fallen from 61 per cent at day 0, and 84 per cent at day 17, to 45 per cent at day 141.

In the second experiment (A), by 160 days there was an increase of 45 fold, or 5.4 generations, giving a generation time of 27 days. Again, numbers were small, but all samples were collected and processed in new lusteroids, thus eliminating the possibility of carry-over of contaminating acid-fast bacilli from previous experiments.

The third experiment of this type (C) was maintained for 224 days, and there was a cumulative increase of 139 fold, or 7.08 generations, giving a generation time of 30.6 days. This was followed by a culture period of 98 days and one of 31 days, which have not been assessed as yet because the ultrasonic vibrator is out of order. Once again, whenever a culture period showed a substantial increase, there was a corresponding increase in the number of bacilli found in the baseline samples. Figure 4 summarizes these results, and, except for a variation in what might be considered the lag phase of growth, curves for the three experiments are almost identical. This so-called lag phase appears to be from 2 to 4 months *in vitro*.

14pf rat fibroblasts. In addition to the diploid cells, *M. leprae* was tried in 14pf cells, which had proved so successful for

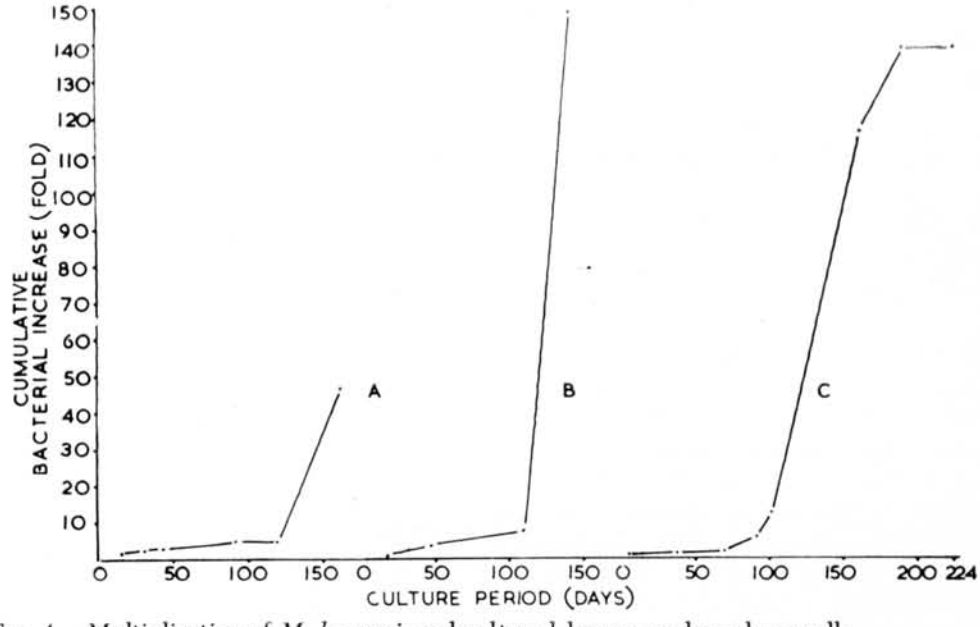

FIG. 4.—Multiplication of *M. leprae* in subcultured human embryo lung cells.

continuous multiplication of *M. lepraemurium*. New lusteroids were used throughout. A suspension of 7.3×10^8 bacilli per ml. was added for 24 hours. After 12 transfers over 473 days bacilli could still be counted in the cultures, and some of them were solid-staining, or nondegenerate, organisms. There was a cumulative increase of 1.62×10^6 fold, or 20.5 generations, over 452 days, giving a generation time of 22.0 days (Fig. 5). As in the case of the human embryo lung cells, there appeared to be a lag phase of approximately 3 months before *M. leprae* adapted to the new environment and began to multiply actively. Sometimes a period of little or no growth was followed by

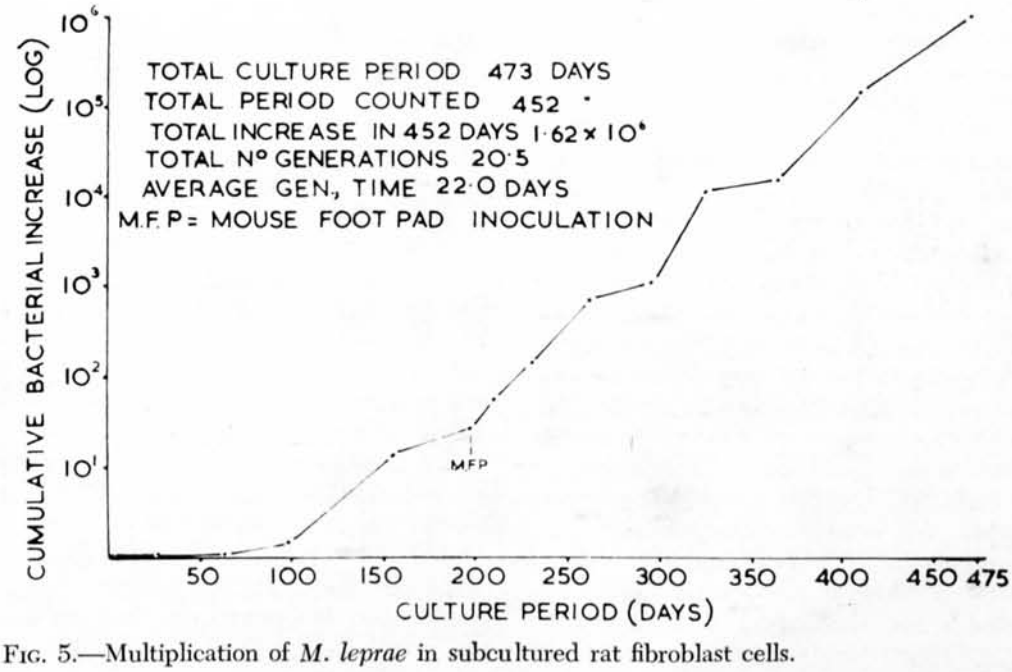

FIG. 5.—Multiplication of *M. leprae* in subcultured rat fibroblast cells.

one of marked increase in bacillary numbers, a phenomenon that had been observed also with *M. lepraemurium* in 14pf and for which as yet we have no explanation.

At day 196, following two periods of 7 fold and 3.9 fold increases respectively, bacilli released from the cells by ultrasonic vibration were injected into mouse foot pads. The suspension was counted before inoculation and 12 mice received approximately 1.6×10^4 bacilli in 0.03 ml. in the right foot pad. The morphology of the few bacilli seen was normal. The results of the foot pad infection are shown in Table 3.

TABLE 3. *Multiplication of tissue culture-grown* M. leprae *in the mouse foot pad (inoculum* 1.6×10^4 *bacilli/foot pad).*

Time (months)	Yield of bacilli	Bacterial increase (fold)
6.5	1.2×10^5	7.5
8.75	neg.	0
10	neg.	0
14	2.4×10^5	15.0
14	neg.	0
14.5	1.2×10^5	7.5
14.5	2.4×10^5	15.0
14.6	neg.	0
14.6	1.2×10^5	7.5
14.75	neg.	0
14.75	3.6×10^5	22.5

While the yields of bacilli from the foot pads are not as high as might have been expected if fresh or mouse-passaged *M. leprae* at a corresponding count had been inoculated ([5,10]), still 6 out of 11 mice showed increases of between 7.5 and 22.5 fold from bacilli that had been maintained within tissue culture cells for 196 days. Moreover, more than 50 per cent of the bacilli recovered from the foot pads were solid-staining, or viable organisms.

Human skin fibroblasts. Studies are also proceeding in our laboratories to attempt confirmation of Yoshie's work with *M. leprae* introduced in lines of cells derived from the skin of lepromatous and tuberculoid patients ([12]). Yoshie asserts that *M. leprae* is destroyed in the tuberculoid lines but not in the lepromatous lines. We are using several different media and the results are as yet inconclusive.

On four different occasions we discarded experiments because the Loewenstein-Jensen slope, blood agar, or broth inoculated with the *M. leprae* suspensions, grew short, plump acid-fast bacilli. Such organisms could not have been *M. leprae*, because for such changes or adaptations to have occurred we would have had to assume that the majority had arisen from mutations. It would seem very unlikely that, with such slowly dividing organisms, mutations could occur sufficiently often to result in large populations of culturable acid-fast bacilli. Thus it cannot be too highly stressed that bacteriologic cultures must be included in order to exclude these culturable mycobacteria, which, in error might be called *M. leprae*.

SUMMARY

Our studies show that continuous multiplication of *M. lepraemurium* can occur in rat fibroblasts that are subcultured regularly. There is also some evidence that *M. leprae* will grow in regularly subcultured cells, both human and rat, provided the cultures are kept a sufficiently long time to overcome an apparent bacillary lag phase of between 2 and 4 months.

The cell type used for growing rat leprosy bacilli may be important, because, in our laboratories, multiplication did not occur in mouse monocytes, mouse fibroblasts (L strain), HeLa cells, or monkey kidney cells. It may be significant that growth of *M. lepraemurium* has been achieved only in cells derived from animal species susceptible to the infection. The growth requirements do not seem to be limited to the cell alone; the medium also is of importance. This is illustrated by the destruction of bacilli in cultures of 14pf cells in cell serum and peptone ($CaS_{10}EP_{90}$) while bacilli in 14pf grown in cord serum and Hanks' solution ($CS_{50}H_{50}$) are capable of continuous multiplication.

With *M. leprae* some multiplication has been achieved in rat fibroblasts and human diploid cells, and tissue culture-grown bacilli from the fibroblasts have been pas-

saged successfully in mouse foot pads. The common denominator of these experiments has been a human element—either human cells grown in medium containing non-human serum, or rat cells grown in human cord serum. This raises the possibility that growth of *M. lepraemurium* might be obtained in cells other than those from rats or mice if rat or mouse serum were used. This has not been attempted, however.

Tissue-culture-grown *M. lepraemurium* has shown all the characteristics of its parent organism, and it is suggested that *M. leprae* grown in tissue culture systems, would be expected to do the same.

Acknowledgments. This work is part of a series of joint studies with my colleagues, Dr. R. J. W. Rees and Miss Celia Fildes, on attempts to apply cell cultures for the growth of *M. leprae*. I am particularly grateful to Miss Fildes for maintaining some of the longer term cultures during my absence.

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Dr. Hart. Thank you very much. A most encouraging paper. I shall now ask Dr. Chang to give his paper on "*Mycobacterium* and *Mycobacterium leprae* in cultures of mouse peritoneal macrophages."