Studies of Mycobacterium lepraemurium in Cell Culture

I. Continuous Multiplication in Cultures of Mouse Foot Pad Cells

Yoshiyasu Matsuo

Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima

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ABSTRACT

A serial increase in the number of Mycobacterium lepraemurium with successful subcultures has been obtained in the mouse foot pad (MFP) cell culture. Special attention has been given to maintaining the infected cells for longer periods; 1) the infected cells were incubated at 30 °C rather than at 37 °C, and 2) the concentration of serum in the culture medium was reduced from 10 to 2%, as soon as a monolayer growth of the transferred cells was obtained. There have been cumulative bacterial increases of $1.47 \times 10^{17}$ and $1.84 \times 10^{15}$ fold for the Kurume-42 strain during a period of 1255 days, and $2.23 \times 10^{9}$ and $3.89 \times 10^{5}$ fold for the Hawaiian strain during periods of 831 and 572 days. The overall generation times were estimated at 22.0, 24.8, 26.8, and 30.8 days, respectively. All attempts to grow the acid-fast bacilli obtained in cell cultures on artificial culture media have failed. The ability of the organisms to produce typical lesions in mice has been well preserved.

Previously the author [5] reported that a successful multiplication of Mycobacterium lepraemurium had been obtained in the mouse foot pad (MFP) cell culture. The subcultures were, however, terminated at the tertiary culture. The major disadvantages experienced were cell deterioration at an early stage of the infection and faster growth of the cells than the bacteria. One of the most important subjects in cell culture of such bacteria as Mycobacterium leprae or M. lepraemurium with extremely long generation times would be how to adjust the relationship between the rate of multiplication of the organisms and that of the host cell. The cells usually multiply faster than the bacteria, therefore, prior to sufficient multiplication of the bacteria the cells develop into a monolayer growth and superannuate. This leads to a decrease of the number of bacteria per cell on every occasion of cell division. Contact inhibition of cells also occurs. Cells, especially those heavily infected with bacteria rapidly break down and the bacteria dilute out from the culture. Special attention should be given to maintaining the infected cells for periods long enough for them to permit continuous cycles of cellular infection. Slowing down of the metabolic activity of the cells may meet this purpose. With this in mind, the infected cells have been serially subcultured, and recent studies show that it has been possible to maintain more or less a continuous multiplication of the intracellular bacteria for more than 1000 days.

MATERIALS AND METHODS

Mouse foot pad (MFP) cell culture. Cells were isolated from the foot pad tissues of young C3H mice in 1967 [5] and have been maintained as a monolayer growth in tissue culture bottle over 80 subcultures thus far.
The medium used was Eagle's minimum essential medium (MEM) containing 0.292 g of glutamine and 1.2 g of sodium bicarbonate per liter and 2 or 10% fetal calf serum. Penicillin G (100 u/ml) was added.

Establishment and maintenance of cell culture infected with M. lepraemurium. A monolayer culture of the MFP cells was grown in a 250-ml culture bottle at 30 C for 10 days. Before infecting the cells the medium was removed and the cells washed with balanced salt solution (BSS). A fresh bacillary suspension was prepared, by a method already described [5], from skin nodules of CF #1 mice 4 months previously infected with the Kurume-42 or the Hawaiian strain of M. lepraemurium. Fifteen milliliters of the suspension containing about 10^9 acid-fast bacilli were added to the washed cell sheet which in turn was incubated at 30 C for 24, 48, 72, or 96 hr to allow phagocytosis to occur. At the end of the phagocytosis period, the medium was withdrawn and the cell layer washed thoroughly with warmed BSS to remove the extracellular organisms. The cells were then trypsinized and suspended in an adequate volume of fresh culture medium containing 10% serum. A 15-ml portion of the infected cell suspension was transferred to a new 250-ml culture bottle and 1 ml to each of several Leighton tubes, each containing a coverslip. These culture vessels were incubated at 30 C. At an appropriate interval of incubation, the cells were trypsinized and suspended in the culture medium (10% serum) two times as large as the preceding culture. The suspension was transferred to a new culture bottle and a few Leighton tubes in the same fashion as the primary culture. In primary as well as every subculture, as soon as a monolayer growth of the cells was obtained, concentration of the serum in the medium was reduced from 10 to 2% so as to suppress the metabolic activity of the cells. The medium was changed about once a week.

Assessment of bacterial multiplication. The number of bacteria transferred was enumerated from counts made on a known proportion of cell suspension by the microspot method, and was referred to as the number at 0-time of the experiment. The number of bacteria in the withdrawn liquid was counted on each occasion of changing the medium. A sum of the number of acid-fast bacilli in the withdrawn liquid and that in the cells at the time of subculture represented the total number harvested, and was compared with the number at 0-time. Portions of the cell suspension were inoculated onto Ogawa egg and into Dubos liquid media at harvesting. Similar portions were injected into the subcutaneous tissues of CF #1 mice and the sites inspected monthly for the presence of leprosy nodules. Coverslips removed from Leighton tubes were acid-fast stained and viewed under a microscope to observe the intracellular behavior of the bacteria.

RESULTS

Per cent phagocytosis of M. lepraemurium by the MFP cells at 30 C for different incubation periods are shown in Table 1.

The percentages in the column headed 'Medium' are referred to as those of bacteria recovered in the culture medium and washing liquid, and the ones in the column headed 'Cell' are those of bacteria liberated from the cells. Total recovery of bacteria ranged from 83.8 to 85.0%. Phagocytosis increased with a lapse of the incubation time, showing 30.0% for 72 hr. From the results, 72-hr incubation at 30 C was tentatively decided

<table>
<thead>
<tr>
<th>Incubation (hr)</th>
<th>Per cent recovery of AFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium a</td>
<td>Cell b</td>
</tr>
<tr>
<td>24</td>
<td>77.2</td>
</tr>
<tr>
<td>48</td>
<td>63.5</td>
</tr>
<tr>
<td>72</td>
<td>55.0</td>
</tr>
<tr>
<td>96</td>
<td>51.1</td>
</tr>
</tbody>
</table>

Fifteen milliliters of bacillary suspension containing 10^9 cells of M. lepraemurium (strain Kurume-42) were inoculated onto a monolayer growth of the MFP cells in a 250-ml culture bottle and incubated at 30 C. After the respective incubation periods, the medium was withdrawn and the cell sheet washed thoroughly with BSS. The cells were trypsinized and suspended in an appropriate volume of the medium. Bacterial counts were made on a known proportion of the withdrawn liquid as well as the cell suspension.

a) Percentage of acid-fast bacilli (AFB) recovered from the medium and washing liquid,

b) those liberated from the cells.
Fig. 1. Multiplication of *M. lepraemurium* (strain Kurume-42) in subcultured mouse foot pad (MFP) cells. Increases in numbers of acid-fast bacilli in the cultures were determined from counts on known aliquots of samples taken at the beginning and end of each period and also of samples of medium removed. a, Main culture. b, Duplicated culture from day 520 onwards.

Cumulative bacterial increases, including the duplicate culture from day 520 onwards with the Kurume-42 strain, are plotted in Fig. 1. There have been total increases in the number of bacteria of $1.43 \times 10^{17}$ and $1.84 \times 10^{15}$ fold for the Kurume-42 strain multiplying in the MFP cells during a period of 1255 days, which represent overall generation times of 22.0 and 24.8 days, respectively. As for the Hawaiian strain, as can be seen in Fig. 2, bacterial increases have been $2.23 \times 10^{9}$ and $3.89 \times 10^{5}$ fold during periods of 831 and 572 days, which represent overall generation times of 26.8 and 30.8 days, respectively. Table 2 summarizes average generation times of five successive subcultures of each experiment.

The intracellular appearance of *M. lepraemurium* is illustrated with a series of photomicrographs in Fig. 3. Figure 3a exhibits part of the 506th day culture. The cells are loaded with a large number of acid-fast bacilli, showing regular arrangements, which surround the nuclei of the cells. At the periphery of the bacterial mass, the bacilli appear to grow freely outward. The 610th day culture (Fig. 3b) gives the intracellular bacteria in bundles arranged very close to each other, resembling the globi-formation. The same pattern is observed in a photo of the 1240th day culture (Fig. 3c). Photo d

### Table 2. Average generation times of *M. lepraemurium* multiplying in subcultured MFP cells

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Kurume-42</th>
<th>Hawaiian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main</td>
<td>Duplicated</td>
<td>No. 1</td>
</tr>
<tr>
<td>1 to 5</td>
<td>32.1</td>
<td>35.9</td>
</tr>
<tr>
<td>6 to 10</td>
<td>21.2</td>
<td>35.5</td>
</tr>
<tr>
<td>11 to 15</td>
<td>28.8</td>
<td>33.4</td>
</tr>
<tr>
<td>16 to 20</td>
<td>25.3</td>
<td>27.9</td>
</tr>
<tr>
<td>21 to 25</td>
<td>16.7</td>
<td>22.8</td>
</tr>
<tr>
<td>26 to 30</td>
<td>27.0</td>
<td>18.0</td>
</tr>
<tr>
<td>31 to 35</td>
<td>13.2</td>
<td>20.9</td>
</tr>
<tr>
<td>36 to 40</td>
<td>16.8</td>
<td></td>
</tr>
</tbody>
</table>

Overall generation time (days) 22.0 24.8 26.8 30.8

Days of culture 1255 1255 831 572

Generation times were calculated based upon the cumulative bacterial increases in 5 successive cultures.

*a* Duplicated cultures from the 13th subculture (day 520) onwards.
Fig. 3. The figures are photomicrographs of growth of *M. lepraemurium* within the MFP cells. 

**a**, The 506th day culture. The cells are heavily infected with acid-fast bacilli which appear to grow freely outward.

**b**, The 610th day culture, resembling globi-formation.

**c**, The 1240th day culture. The same appearance as the photo **b**.

**d**, Low magnification of the 1240th day culture. Most of the cells contain a few or numerous acid-fast bacilli.

**e** and **f**, Enlargement of rectangular areas 1 and 2 of the photo **d**. The rectangular area 2 reveals mitosis of a cell accompanied with bacterial multiplication.

The bar represents 10 μm.
Table 3. Multiplication of M. lepraemurium in MFP cell cultures at 37 C

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Generation of subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8 (32) 1.3 (33) 0.6 (31)</td>
</tr>
<tr>
<td>2</td>
<td>1.7 (23) 1.4 (23) 1.6 (26) 1.3 (27) 0.9 (25)</td>
</tr>
</tbody>
</table>

a) Fold in bacterial increase. The number in parenthesis represents days of incubation.

is a low magnification of the 1240th day culture. Most of the cells have ingested few to numerous acid-fast bacilli. Photos e and f are enlargements of the rectangular areas 1 and 2. Rectangular area 2 reveals mitosis of a cell accompanied with bacterial multiplication.

All attempts to grow the acid-fast bacilli obtained from the cells on each occasion of subcultures on artificial culture media have failed. In contrast, the bacilli multiplied in the subcutaneous tissues of mice and produced typical lesions of rat leprosy even after 1000 days of cultivation.

Cell cultures maintained at 37 C are recorded in Table 3. The number in parenthesis represents days of incubation. As is clear in the table, the degree of bacterial increase was not appreciable, especially after the secondary culture, and the cultures had to be terminated at the fifth subculture at the latest.

In order to promote the rate of multiplication of M. lepraemurium, decanoic acid, a-ketoglutaric acid and/or cytochrome c were added to the medium. No evidence was found that these substances provided better support for the bacteria.

DISCUSSION

The MFP cells infected with M. lepraemurium have been serially cultured in a medium with a reduced serum concentration at a relatively lower temperature, and the behavior of the bacteria inside the cells has been observed. Following evidences indicate that continuous multiplication of M. lepraemurium has taken place. (i) Serial increase in the number of acid-fast bacilli has been maintained in successive subcultures. (ii) All attempts to grow the acid-fast bacilli obtained from these cultures on artificial culture media have failed. (iii) Bacilli from the culture produced typical lesions of rat leprosy in mice. (iv) The growth pattern of the intracellular bacteria is similar to that observed in the subcutaneous tissues of mice infected with M. lepraemurium. (v) More or less acid-fast bacilli are found in various mitotic phases of the cells.

The temperature optimum for growth of the M. lepraemurium in vitro has not been well documented. Wallace et al [11] and Rees and Garbutt [9] observed multiplication of M. lepraemurium in tissue cultures at 34 C. Wiygul and Rightsel [12] reported that M. lepraemurium grew in cell-impermeable diffusion chambers placed within petri dish monolayer cultures of human embryonic skin cells at 32 C. Nakamura [7] observed a better elongation phenomenon of M. lepraemurium at 30 C than at 37 C in his cell-free culture medium enriched with a-ketoglutaric acid (EK medium) which supported growth of the bacteria slightly. Ogawa [8] declared that two strains of M. lepraemurium isolated on his egg-yolk medium grew at temperatures ranging from 30 to 37 C, and that optimal growth was obtained at 37 C. Chang et al [1] successfully cultured the organisms in mouse peritoneal macrophages at 37 C. As for M. leprae, Samuel and his co-workers [10] reported that its multiplication took place both at 33 and 37 C and suggested that M. leprae did not have a narrow range of temperature for optimal growth. The multiplication of M. lepraemurium in the MFP cells at 37 C was only slight and from the secondary culture onwards bacillary counts were beginning to decline. In contrast, continuous multiplication of the bacteria in the cells at 30 C was appreciable, possibly because of a longer survival of the infected cell populations than at 37 C.

According to Hanks, both the infectiousness [2] and hydrogen transfer capacity [3] of M. lepraemurium were sharply inhibited by serum from all animals tested, including that from the natural host. And Hanks and Gray [4] assumed that the susceptibility to serum inhibition accounted for the failure to multiply in cell cultures. There is no convincing evidence regarding the influence of the metabolic activity of the cells upon the ability of multiplication of M. lepraemurium,
except for the work of Wallace et al [11] who demonstrated that the incorporation of hydrocortisone in the culture medium resulted in a significant increase of the bacteria. A modification of the cell metabolism by this drug has been described earlier. In a preliminary experiment, the author observed that the MFP cells could be maintained for at least a few months by reducing the concentration of the serum in the culture medium. As far as the present study is concerned, it is likely that slowing down of the metabolic activity of the cells by means of incubation at a lower temperature as well as the lower concentration of serum in the medium provides favorable conditions for continuous cycles of cellular infection.

Kusaka (personal communication) observed that *M. lepraemurium* retained the valuable activity of forming long carbon-chain fatty acids from decanoic acid, and suggested that decanoic acid was one of the nutrients required for the cultivation of this organism *in vitro*. Nakamura [6] presented data in which growth of *M. lepraemurium* was promoted by the addition of α-ketoglutaric acid and cytochrome c to the NK-medium. In this communication, however, no appreciable effect of any of these substances has been proven.

Rees and Garbutt [9] maintained cultures of rat fibroblasts (strain 14pf) infected with *M. lepraemurium* for 425 and 477 days, giving mean dividing times of 14.2 and 15.3 days, respectively. Chang et al [1] reported more rapid and uniform growth of the organisms in cultures of mouse peritoneal macrophages with an average generation time of 7 days in serial transfers. The results at hand show much longer generation times. The reason for these discrepancies in the growth rate of *M. lepraemurium* is not clear. The addition of substances which would initiate and stimulate the multiplication of *M. lepraemurium in vitro* will be investigated further.

**ACKNOWLEDGEMENT**

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**REFERENCES**