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Use of Cycloheximide for Cultivating *Mycobacterium lepraemurium* in Cell Culture[1]

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One of the most important procedures for cultivating *Mycobacterium leprae* and *M. lepraemurium* in cell culture seems to be how to keep the infected cells in good condition as long as possible. Cycloheximide, an antibiotic, is known to specifically suppress protein biosynthesis by eukaryotic cells, and has currently been recommended for isolating *Chlamydiae* by cell culture from clinical specimens ([4]). In a preliminary experiment, the effect of cycloheximide on an established cell line, A31 cells, was investigated, and this antibiotic was found to delay the division of the cells at a low concentration without killing them. Cycloheximide treatment of cell cultures of *M. leprae* and *M. lepraemurium* therefore seemed worthwhile. The present report deals with the application of this technique to the cultivation of *M. lepraemurium* in cell cultures.

MATERIALS AND METHODS

Bacillary suspension. The bacillary-tissue suspension was prepared from an homogenate of a subcutaneous nodule of a mouse infected 15 weeks previously with the Hawaiian strain of *M. lepraemurium*. The suspension was centrifuged at 650 × *g* for 5 min. The supernatant fluid was decanted, mixed with 1 N NaOH, incubated in a water bath at 37°C for 20 min, and then neutralized with 1 N HCl. The mixture was centrifuged at 20,000 × *g* for 30 min at 0°–4°C. The pellet was washed once with distilled water and suspended in an appropriate volume of the culture medium.

Host cells. A31 cells were provided by Dr. Nomaguchi, Institute for Microbial Diseases, Osaka University, Osaka, Japan, and have been maintained at the author's laboratory in medium L15 (GIBCO Laboratories, Grand Island, New York, U.S.A.) containing 10% fetal calf serum (GIBCO Diagnostics, Madison, Wisconsin, U.S.A.).

Culture medium. The culture media used were R-L15FCS10 and R-L15FCS10-Cy. The medium R-L15FCS10 was medium L15 containing per ml 400 μg of reduced glutathione (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), 200 μg of DL-cysteine hydrochloride (Tokyo Kasei, Japan), 100 μg of dithiothreitol (Boelinger Mannheim, West Germany), and 0.1 ml of fetal calf serum. The medium supplemented with cycloheximide (Wako Chemical, Japan) at a concentration of 0.1 μg per ml was referred to as R-L15FCS10-Cy.

Infection and cultivation. A confluent monolayer of A31 cells grown in a 25 cm^2 culture flask was infected with 4 ml of the bacillary suspension and incubated at 30°C for three days to allow phagocytosis to occur. The infected cells were then trypsinized, washed once, and suspended in 8 ml of medium R-L15FCS10. A 4 ml portion of the cell suspension was poured into a new culture flask of the same size. A small portion of the remaining suspension was used for the counting of acid-fast bacilli, and the rest was placed onto several coverslips in Leighton tubes. All the cultures were maintained at 30°C throughout the experiment. As soon as the cells developed in an approximately 80% confluent monolayer, the medium was replaced with R-L15FCS10-Cy. They could then be held for ten days or more prior to changing the medium. At the time of subculture, the cells in the culture flask were trypsinized and suspended in a volume of R-L15FCS10 two times as large as that used for the preceding culture. The cell suspension was poured into a new culture flask and a few Leighton tubes in the same way as the primary culture.

Assessment of bacillary multiplication. The number of bacilli at the time of transfer was enumerated from counts made on a known proportion of the cell suspension by

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THE TABLE. *Cultivation of* Mycobacterium lepraemurium *with cycloheximide treatment.*

Culture	Cyclo-heximide (0.1 μg/ml)	Duration (days)	Number of AFB (× 10^6)				Fold increase	Generation time (days)
			0 time	Harvest				
				Medium[a]	Cell[b]	Total		
Primary	+	79	4.37	2.7	52.5	55.2	12.6	21.4
	−			2.9	12.8	15.7	3.6	43.9
Secondary	+	85	20.5	20.7	195.3	216.0	10.5	25.0
Tertiary	+	81	53.2	66.2	839.3	905.5	17.0	19.8

[a] The total number of acid-fast bacilli in the withdrawn medium during the cultivation.
[b] The number of acid-fast bacilli harvested from the cells at the subculture.

the method of Shepard and McRae (5), and was referred to as the number at 0 time of the culture. The number of the bacilli in the withdrawn medium was counted on each occasion that the medium was changed. A sum of the number of acid-fast bacilli in the withdrawn medium and that in the cells at the time of subculture represented the total number harvested, and was compared with the number at 0 time. Coverslips removed from the Leighton tubes were stained for acid-fast bacilli and viewed under a microscope to observe the intracellular appearance of the bacilli.

RESULTS

The bacterial increases in the primary, secondary, and tertiary cultures are tabulated in The Table. The primary culture was continued for 79 days with or without adding cycloheximide. The acid-fast count increased 12.6-fold in R-L15FCS10-Cy, representing a generation time of 21.4 days. In the secondary and tertiary cultures, the number of acid-fast bacilli further increased 10.5- and 17.0-fold with a generation time of 25.0 and 19.8 days, respectively. In the medium without cycloheximide the bacterial increase was only 3.6 fold in 79 days.

The intracellular appearance of *M. lepraemurium* at the secondary culture in R-L15FCS10-Cy is illustrated with a series of photomicrographs in Figure 1 and Figure 2. Figure 1a shows part of the four-week-old culture. Figure 1b exhibits an enlargement of the rectangular area of Figure 1a, and Figure 1c exhibits that of Figure 1b. The cells contain scattered bacilli and a few small clumps. The cells of the eight-week-old culture (Figs. 1d, 1e, 1f) are loaded with an increased number of acid-fast bacilli with relatively regular arrangements, which surround the nuclei of the cells. Figure 2 represents a low-to-high magnification of the cells in the 12-week-old culture. Most of the cells are filled with hundreds of bacilli in bundles arranged very close to each other, and many of them appear to have formed globi (Figs. 2c to 2f).

DISCUSSION

Previously the author (2) reported a serial increase in the number of *M. lepraemurium* with successful subcultures for a period of 1225 days in cultures of mouse foot pad (MFP) cells *in vitro*. Special attention had been given to keeping the infected cells for a longer period by lowering the incubation temperature as well as reducing the serum concentration in the culture medium. Nonetheless, it was difficult to determine the optimum time for subculture, since those cells which were heavily loaded with bacilli were apt to become damaged and often subcultures were made too late to recover them because they had floated off the substratum.

Recently, cycloheximide has conveniently been used at concentrations of 1 μg to 2 μg per ml in the culture medium for isolating *Chlamydiae* from clinical specimens in cell culture systems in place of iododeoxyuridine treatment of the cells (4). Cycloheximide is known to suppress protein biosynthesis in eukaryotic cells but not in prokaryotic cells. A preliminary experiment showed that cycloheximide at concentrations of 0.5 μg or more per ml completely suppressed the division of A31 cells. This was accompanied by gradual death of the

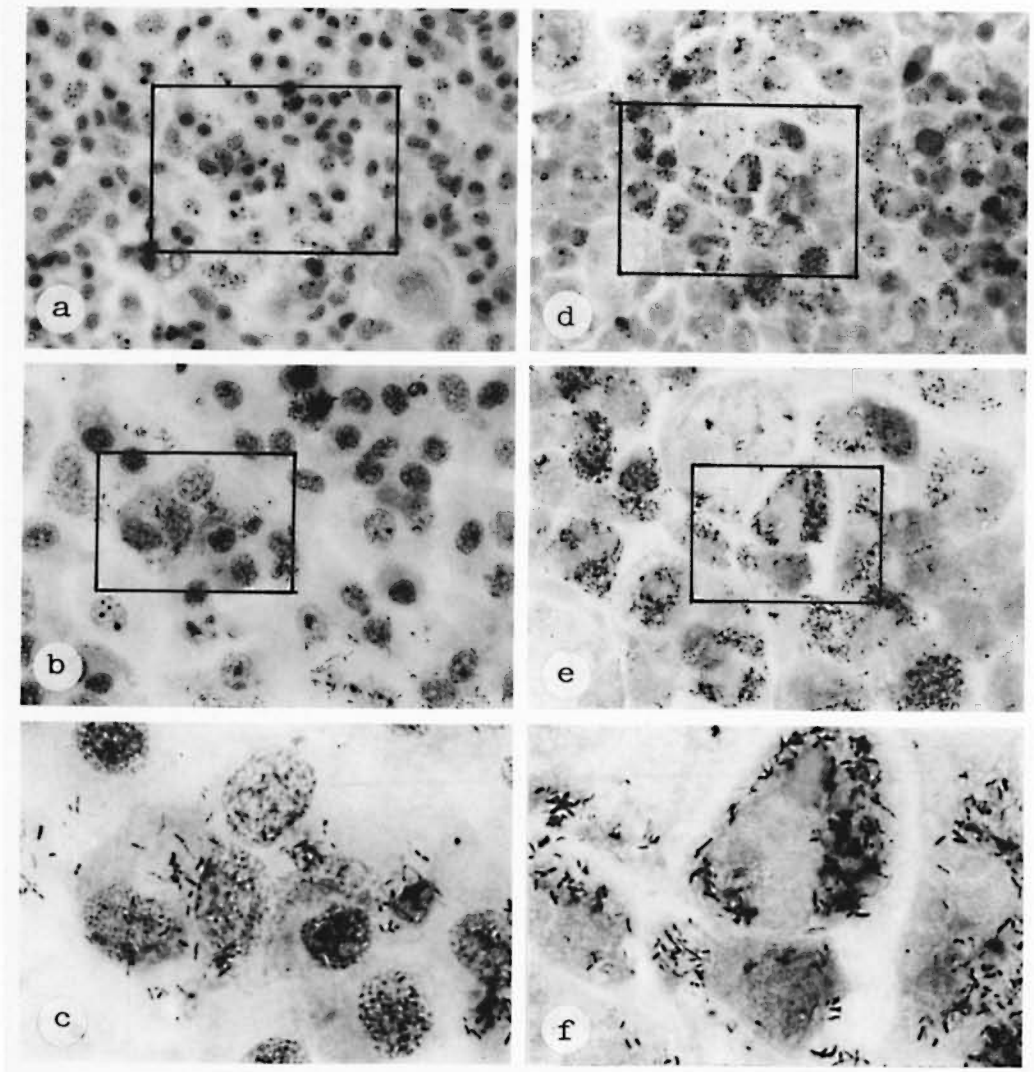

FIG. 1. A31 cells with *Mycobacterium lepraemurium* at the secondary culture maintained in medium R-L15FCS10-Cy.

a) A four-week-old culture (×200). Scattered bacilli and a few small clumps are seen within the cells.
b) Enlargement of the rectangular area of Fig. 1a (×400).
c) Enlargement of the rectangular area of Fig. 1b (×1000).
d) An eight-week-old culture (×200). The cells contain an increased number of bacilli.
e) Enlargement of the rectangular area of Fig. 1d (×400).
f) Enlargement of the rectangular area of Fig. 1e (×1000).

cells. A concentration of 0.3 μg per ml was also suppressive but sometimes killed the cells; while 0.1 μg per ml delayed cell division considerably but resulted in prolonged incubation of the cells in good condition without changing the medium. These findings encouraged the author to investigate the efficacy of this antibiotic for cultivating *M. lepraemurium* in infected cells. The results obtained are satisfactory for the present. The stained coverslips revealed far better intracellular multiplication of the bacilli than those observed in the MFP cell cultures previously reported [1, 2]. Successful subcultures have been continued up to the tertiary culture, and the fourth one is in progress. The generation time of the bacilli was 21.4 days in the primary, 25.0 days in the secondary, and 19.8 days in the tertiary culture, respectively. Overall the generation

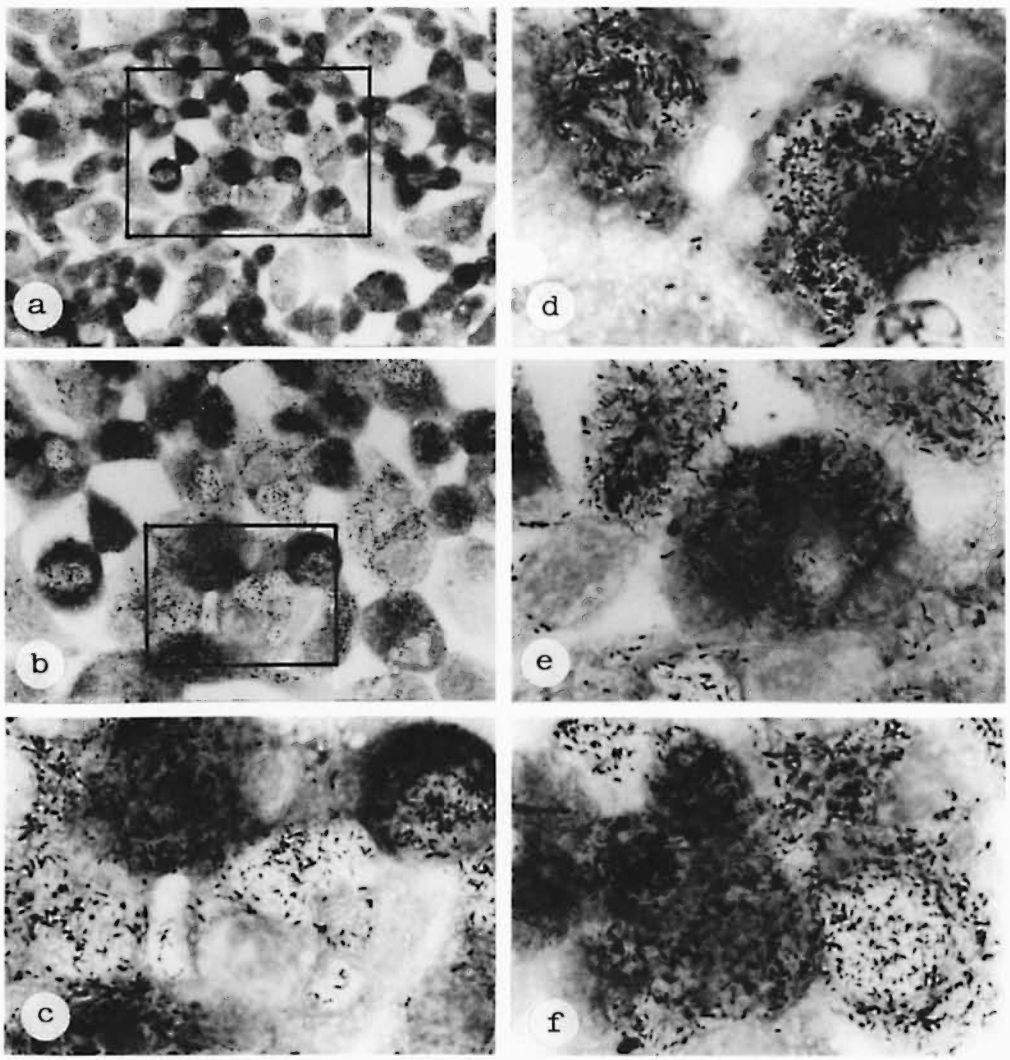

FIG. 2. A 12-week-old culture of A31 cells with *Mycobacterium lepraemurium* at the secondary culture maintained in the medium R-L15FCS10-Cy.

a) Low magnification (×200). Most of the cells are filled with hundreds of acid-fast bacilli in bundles.
b) Enlargement of the rectangular area of Fig. 2a (×400).
c) Enlargement of the rectangular area of Fig. 2b (×1000).
d–f) Cells with numerous bacilli, resembling the formation of globi (×1000).

time was 22.1 days over a period of 35 weeks. These values are very close to the results previously reported in which the generation times were 22.0 days to 24.5 days in the MFP cell cultures ([2]). This rate of multiplication is, however, not as rapid as that reported by Nomaguchi and her colleagues ([3]) who found generation times of 5.3 days to 9.4 days, using the same cell line maintained in a conditioned medium. Although the reason for this discrepancy remains unknown, it may be attributed in part to differences in the culture media and incubation temperatures.

The advantages of cyloheximide treatment are that the technique is very simple, infrequent changes of the medium are needed, and the results are highly reproducible. Attempts at cultivation of *M. leprae* in this cell culture system are under investigation.

SUMMARY

A serial increase in the number of *Mycobacterium lepraemurium* with successful

subcultures has been obtained in cell culture with cycloheximide treatment. The infected cells seldom floated off the culture vessel. They could be maintained and would support the bacillary multiplication in good condition for ten weeks or more without changing the medium frequently. An overall generation time of the intracellular bacilli up to the tertiary culture for the total period of 35 weeks was 22.1 days.

RESUMEN

Usando cultivos de células tratados con cicloheximida se logró un incremento progresivo en el número de *Mycobacterium lepraemurium* en los subcultivos que fueron exitosos. Las células infectadas raramente flotaron en los recipientes de cultivo. Las células pudieron mantenerse en buena condición y permitieron la multiplicación bacilar durante 10 semanas o más sin cambios frecuentes del medio. El tiempo promedio de generación de los bacilos intracelulares hasta el cultivo terciario durante un periodo total de 35 semanas fue de 22.1 días.

RÉSUMÉ

Une augmentation successive dans le nombre de *Mycobacterium lepraemurium*, lors de sous-cultures successives réussies, a été observée dans une culture cellulaire traitée au cycloheximide. Les cellules infectées flottaient rarement hors du tube de culture. Elles pouvaient être maintenues, et supportaient la multiplication bacillaire dans de bonnes conditions pendant dix semaines ou plus, sans qu'il soit nécessaire de changer le milieu de culture fréquemment. On a estimé que le temps de génération global des bacilles intracellulaires, jusqu'à la troisième culture, sur une période totale de 35 semaines, était de 22.1 jours.

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REFERENCES

1. MATSUO, Y. Multiplication of *Mycobacterium lepraemurium* in mouse foot-pad cell culture. Jpn. J. Microbiol. **14** (1970) 233–240.
2. MATSUO, Y. Studies of *Mycobacterium lepraemurium* in cell culture. I. Continuous multiplication in cultures of mouse foot pad cells. Jpn. J. Microbiol. **18** (1974) 307–312.
3. NOMAGUCHI, H., NYEIN, M. M., KOHSAKA, K., YONEDA, K. and MORI, T. The growth and drug sensitivity of *M. lepraemurium* by tissue culture applying monolayer and agar suspension technique. Int. J. Lepr. **48** (1980) 277–284.
4. SCHACHTER, J. *Chlamydiae.* In: *Manual of Clinical Microbiology.* 3rd ed. Lennette, E. H., *et al.*, eds. Washington, D.C.: American Society for Microbiology, 1980, pp. 357–364.
5. SHEPARD, C. C. and MCRAE, D. H. A method for counting acid-fast bacteria. Int. J. Lepr. **36** (1968) 78–82.