Hiroshima Journal of Medical Sciences
Vol. 31, No. 2, June, 1982
HIJM 31-21

Attempts at Cultivation of *Mycobacterium leprae* in Cell Culture under Regulation of Redox Potential at Environment*)

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(Received February 25, 1982)

Key words: *Mycobacterium leprae, Cell culture, Redox potential*

ABSTRACT

Cultivation of *Mycobacterium leprae* in cell culture was attempted under lower redox potential by adding such reducing agents as reduced glutathione, DL-cysteine hydrochloride, and dithiothreitol to culture medium, and by reduced oxygen tension varying the depth of medium or applying agar suspension technique. During a five years' period since 1976, a slight increase in the number of acid-fast bacilli at harvests was occasionally observed. However, the viability of bacilli recovered was unable to be proven by the mouse foot pad method. It is uncertain whether lower redox potential at environment is strictly requested for cultivation of *M. leprae*.

Obtaining isolation and pure culture of *Mycobacterium leprae* has been one of the major concerns among leprologists, and a number of investigators have reported the cultivation of this fastidious microbe *in vitro*. Unfortunately, no convincing relationship of the organism thus obtained to the causative agent of the disease leprosy has been established so far. A cell culture system for *Mycobacterium lepramurium* where Matsuo[4] established a continuous multiplication of this organism in subpassages has been tested for cell culture of *M. leprae* in our laboratory since 1966. According to our results obtained over ten years, it appeared there had been no notable multiplication of *M. leprae* in this cell culture system[5].

The growth pattern of *M. lepramurium* in Nakamura system clearly points out that oxygen tension should be restricted to a certain extent in order to obtain a favorable multiplication of this mycobacterium in NC-5 medium[6]. Fieldsteel, Becker and Stout[2] demonstrated a prolonged survival of virulent *Treponema pallidum* in cell-free and tissue culture systems by reducing redox potential in media. Nomaguchi and her colleagues[7] recommended the agar suspension method for cell culture of *M. lepramurium*, and have employed this technique for cultivation of *M. leprae*. The application of lower redox potential in cell culture system for *M. leprae* seems to be a subject worth investigating. The present paper summarizes the results of our experiments carried out along this line from 1976 to 1980.

Nodules from untreated patients with lepromatous leprosy were provided by Dr. D. I. Kim, Korean Leprosy Institute, Republic of Korea, and the lepromatoid foot pad tissues of nude mice previously infected with *M. leprae* were provided by Dr. K. Kohsaka, Osaka University, Japan. Bacterial suspensions were prepared by a modification of the method described elsewhere[3]. Established mouse foot pad (MFP) cells[3] have been maintained in our laboratory in medium L15 (GIBCO, USA) containing 1% fetal calf serum (GIBCO, USA)-L15FCS1. The cells were infected with 10^7 to 10^8 organisms of *M. leprae* and incubated at 37°C for 48 to 72 hr for phagocytosis to occur. The cells were then washed, trypsinized and suspended in an appropriate volume of medium R-L15FCS10 which contained reduced glutathione (Sigma, USA), DL-cysteine hydrochloride (Tokyo-Kasei, Japan), and dithiothreitol (Boelinger

*) 松尾吉恭，辰川　博：酸化還元電位を調整した環境におけるらい菌の細胞培養のこころみ

Mannheim, W-Germany) at the concentration of 400, 200, and 100 μg per ml, respectively, in medium L15FCS10. A 5-ml portion of the infected cell suspension was transferred into a 25-cm^2 plastic flask and incubated at 30°C. The number of acid-fast becilli in the transferred cells was counted and referred to as the number in inoculum. As soon as a monolayer growth of the cells was obtained, the medium was replaced with R-L15FCS1. The medium was renewed approximately every 10 days. The total number of bacilli in the withdrawn medium and in the cells at the time of subpassage represented the total number harvested. Although a slight increase in the number of bacilli was noted in the primary and secondary culture occasionally (Table 1), it would be unreasonable to assume that *M. leprae* did multiply in this cell culture system, since the bacilli recovered were unable to grow in the mouse foot pad. The efficacy of reducing agents on the growth of *M. leprae* remains obscure.

Table 1. Effect of reducing agents on the growth of *M. leprae* in MFP cell culture

Culture	Days of cultivation	Number of AFB recovered		Fold increase
		Inoculum	Harvest	
Primary	67	1.12×10^7	2.06×10^7	1.84
Secondary	89	3.64×10^6	5.03×10^6	1.38
Tertiary	40	6.30×10^5	5.71×10^5	0.91

M. leprae-infected MFP cells were suspended in medium R-L15FCS10 and tranrferred to a new culture flask. After growing as a monolayer, the cells were maintained in medium R-L15FCS1. The data shows one of five experimental results.

A 0.1-ml of the infected MFP cell suspension was spread over a 15-mm round coverslip in a tissue culture dish and incubated overnight with 5%CO_2 in air. After washing, the coverslip was placed in the flat bottom of a shell vial. The depth of medium R-L15FCS1 in shell vials varied from 5 to 25 mm. The vials were sealed with silicone rubber stoppers and incubated at 30°C. The count of bacilli was made across the equator of the coverslip in fields selected every 0.5 mm with the scale on the mechanical stage which gave 30 fields per coverslip. The magnification factor (MF) was determined as 6.94×10^3 by dividing the coverslip area by the field area. The total number of bacilli on coverslip was calculated as follows: MF × (number of acid-fast bacilli detected/number of fields observed). The cells were kept well adhered on coverslips throughout the experiments in which they were immersed in the medium of 10 or 15 mm deep (Table 2). Neither increase nor decrease in the number of bacilli was observed. The depth of medium over 20 mm and at 5 mm caused an earlier deterioration of the cells, resulting in a significant reduction in the number of bacilli recovered. Thus, relatively lower oxygen tension failed to support the growth of *M. leprae*.

Table 2. Effect of depth of medium on the growth of *M. leprae* in MFP cell culture

Days of cultivation	Number of AFB ($\times10^5$)				
	5[a]	10	15	20	25
18	2.49	2.94	3.47	2.62	1.18
30	1.50	3.27	3.14	1.11	0.72

M. leprae-infected MFP cells were spread over a 15-mm round coverslip and immersed in varying depth of medium R-L15FCS1. The number of bacilli at O-time was 2.98×10^5. The deta represent averages of 4 coverslips in each case. a) Depth of medium (mm).

M. leprae-infected MFP cell suspension in R-L15FCS20 was mixed with an aliquot of 0.2, 0.4, or 0.6% agar in distilled water. Immediately after mixing, 1 ml of each agar-cell suspension was laid over 0.5% agar sheet in a Leighton tube. At the indicated time of incubation at 30°C, the count of bacilli was made and compared with that of O-time control. As shown in Table 3, the agar suspension method did not

Table 3. Recovery of *M. leprae* from agar suspension culture

Conc. of agar (%)	% recovery of AFB at indicated time		
	Day 15	Day 30	Day 45
0.1	67.4	65.0	41.5
0.2	75.9	62.7	77.7
0.3	70.0	64.7	71.5

M. leprae-infected cell suspension was mixed with an aliquot of 0.2, 0.4, or 0.6% agar in distilled water and laid over 0.5% agar sheet in Leighton tube. The data represent averages of 4 experiments in which the number of acid-fast bacilli at O-time ranged from 9.61×10^5 to 2.24×10^6.

help the bacillary multiplication, and the acid-fast bacilli recovered was extremely poor in every instance.

Peritoneal resident macrophages from female CF♯1 mice were adhered on coverslips in Leighton tubes according to the method by Chang[1], and infected with *M. leprae*. Four days after incubation for phagocytosis, the cells were washed and cultured at 30°C in medium L15FCS20, R-L15FCS10, or overlaid with 0.2% agar in L15FCS10. Coverslips stained at this time were referred to as O-time controls. Medium was changed at intervals of approximately 10 days except for the agar overlay culture. On the fortieth day of incubation, phagocytosis rate reached 100%. Most of the cells were proven viable by the dye exclusion test, but no significant increase in the number of acid-fast bacilli per cell was observed (Table 4).

Table 4. Growth of *M. leprae* in mouse peritoneal macrophages

Days of cultivation	Day 0	Day 20	Day 40
Phagocytosis rate of cells (%)	96.0	95.3	100.0
Average number of AFB/cell	12.2	10.3	14.1

Peritonal resident macrophages were adhered onto coverslips in Leighton tubes, and infected with *M. leprae*. Phagocitosis of the cells was allowed for 4 days. The data represent averages of the results carried out in triplicate.

ACKNOWLEDGEMENTS

This work was supported in part by the Japan-US Cooperative Medical Science Program and the Sasakawa Memorial Health Foundation. We gratefully acknowledge Drs. D. I. Kim and K. Kohsaka for providing us with *M. leprae*.

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