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ATTEMPTS AT CULTIVATION OF MYCOBACTERIUM LEPRAE IN CELL CULTURE

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In the last decade I have attempted to establish successive cultivation of *Mycobacterium lepraemurium* in cell cultures of various sources (1). As reported previously, the successful cultivation of this organism has been maintained in the MFP cell culture more than five years (2, 3). This encouraged me to investigate the further possibility of culturing *Mycobacterium leprae* in the same cell culture system.

PERCENT PHAGOCYTOSIS OF *M. LEPRAE* BY THE MFP CELLS

Five ml of bacillary suspension containing 10^8 cells of *M. leprae* were inoculated onto monolayer growth of the MFP cells in several 50-ml culture flasks and incubated at 25, 31, 33 and 37 C in duplicate for 96 hr. After the incubation period, the medium was withdrawn and the cell sheet was washed thoroughly with balanced salt solution to remove the unphagocytized bacteria. The cells were then trypsinized and suspended in 5 ml of the medium. Bacterial counts were made on a known portion of the cell suspension after a gentle ultrasonication.

Table 1 shows percent phagocytosis of *M. leprae* by the MFP cells at different temperatures. The phagocytosis was the best at 33 C, although it seemed somewhat inferior to those in tissue cultures of mouse origin reported by Dr. Fieldsteel in 1972 (4). From the results, incubation at 33 C was tentatively decided upon to be the choice condition for phagocytosis in my study.

Table 1. Phagocytosis of *M. leprae* by MFP cells at different incubation temperatures

Temperature	Percent recovery of *M. leprae*
37 C	31.2
33 C	38.4
31 C	22.8
25 C	15.4

96 hr for incubation time

METHODS FOR CELL CULTURE

The sources of M. leprae were the cutaneous nodules of patients with lepromatous leprosy and the footpads of CF#1 mice previously inoculated with M. leprae. The MFP cells were inoculated with 10^7 to 10^9 acid-fast bacilli and incubated at 33 C for phagocytosis. After phagocytosis for 72 hr, the cells were washed, trypsinized and suspended in an appropriate volume of the culture medium, 15 ml of which was transferred to a 250-ml culture flask and incubated at 30 C.

In the initial experiments, the culture medium employed was Eagle's minimum essential medium containing fetal calf serum without antibiotic. In later experiments, however, L-15 (Leibovitz) medium was added to the experimental series. In all the experiments, the concentration of serum in the culture medium was reduced from 10 to 2% following the establishment of a monolayer growth of the infected cells. The medium was changed about once a week.

Cultures were examined microscopically at least twice a week. When the cell sheet began to peel off and curl in the culture flask, the cells were trypsinized and transferred to a new culture flask of the same size. A portion of cell suspension was gently ultrasonicated and the bacterial counts were made on this suspension. This was also used for inoculation of mouse footpads.

RESULTS

As indicated in Table 2, cell culture experiments with M. leprae have been carried out without contamination 14 times thus far.

Table 2. M.leprae in the MFP cell culture

Source of bacilli	Duration (days)	Number of AFB		Bacterial increase
		Base-line	Harvest	
human	37	2.26×10^8	2.53×10^8	/
"	98	3.40×10^7	2.00×10^7	/
"	103	2.10×10^7	3.78×10^7	1.8-fold
mouse	128	1.17×10^7	5.10×10^7	4.4-fold
human	70	5.80×10^7	3.70×10^7	/
"	54	3.60×10^7	3.33×10^7	/
"	56	2.80×10^7	1.40×10^7	/
"	70	5.90×10^7	5.80×10^7	/ *
mouse	135	1.21×10^7	1.13×10^8	9.3-fold
"	54	1.28×10^7	3.60×10^7	2.8-fold *
"	100	7.00×10^6	5.92×10^6	/
human	62	8.21×10^7	6.95×10^7	/
mouse	60	2.46×10^7	4.40×10^7	1.8-fold
human	142	3.33×10^7	3.86×10^7	/

* Positive culture in the mouse footpads

Although increase in number of acid-fast bacilli seen at harvests of the primary culture was obtained in five of these experiments, no evidence of successive bacterial increase was observed in the subcultures. In two of 14 experiments, however, M. leprae was found to have survived in the MFP cell cultures at least for 54 and 70 days, respectively, by the mouse footpad technique.

The next several slides showed photomicrographs taken in one of the experiments. The intracellular patterns of the bacilli were quite similar to those of M. lepraemurium previously reported, but I am not sure that

M. leprae had really multiplied in the MFP cells for the following reasons.

1. The subcultures completely failed to support bacterial increase.

2. With a few exceptions, the acid-fast bacilli did not multiply in the mouse footpads even in the instances where there had been a significant bacterial increase.

3. Increase in bacilli in the primary culture might have resulted from release of single bacillus from bacterial clumps or fragmentation during the course of cell culture.

Although my effort to demonstrate multiplication of M. leprae in cell culture has not been rewarding, it is interesting to note that M. leprae survived in the cell cultures for several weeks in some instances.

In August, 1974, a piece of an armadillo leproma was supplied by Dr. Storrs through the National Institute for Leprosy Research, Tokyo. The acid-fast bacilli from this specimen were inoculated into the mouse footpads as well as onto the MFP cells.

Fig. 1 shows that the pattern of multiplication of the acid-fast bacilli was the same as that of M. leprae obtained from human leprosy or from mouse footpads. Furthermore, the bacilli recovered from the infected footpads failed to grow on bacteriologic culture media. Their acid-fastness was removed with pyridine extraction but under this condition they remained gram-positive. Thus, the nature of the bacilli from this armadillo leproma was identical with that of M. leprae.

In the course of cell culture using L-15 medium, however, the culture medium turned turbid and numerous acid-fast bacilli were demonstrated in the culture medium. A portion of the withdrawn liquid was transferred onto a medium for mycobacteria 60 days after cell culture. Following three weeks' incubation, a slow-growing, scotochromogenic mycobacterium was isolated.

This distinguishing characteristics of this organism are indicated in Table 3.

Table 3. Characteristics of the cell culture isolate

Cultures / Characters		Cell culture isolate	M.scrofulaceum ATCC 19981	M.gordonae ATCC 14470
Colonial morphology		S	S	S
Growth at 37 C		Slow	Slow	Slow
Pigment	in dark	Orange-yellow	Orange-yellow	Orange-yellow
	photo-active	+	+	++
Tween 80 hydrolysis		-	-	+
Tolerance to 5 mcg/ml ethambutol		+	+	-

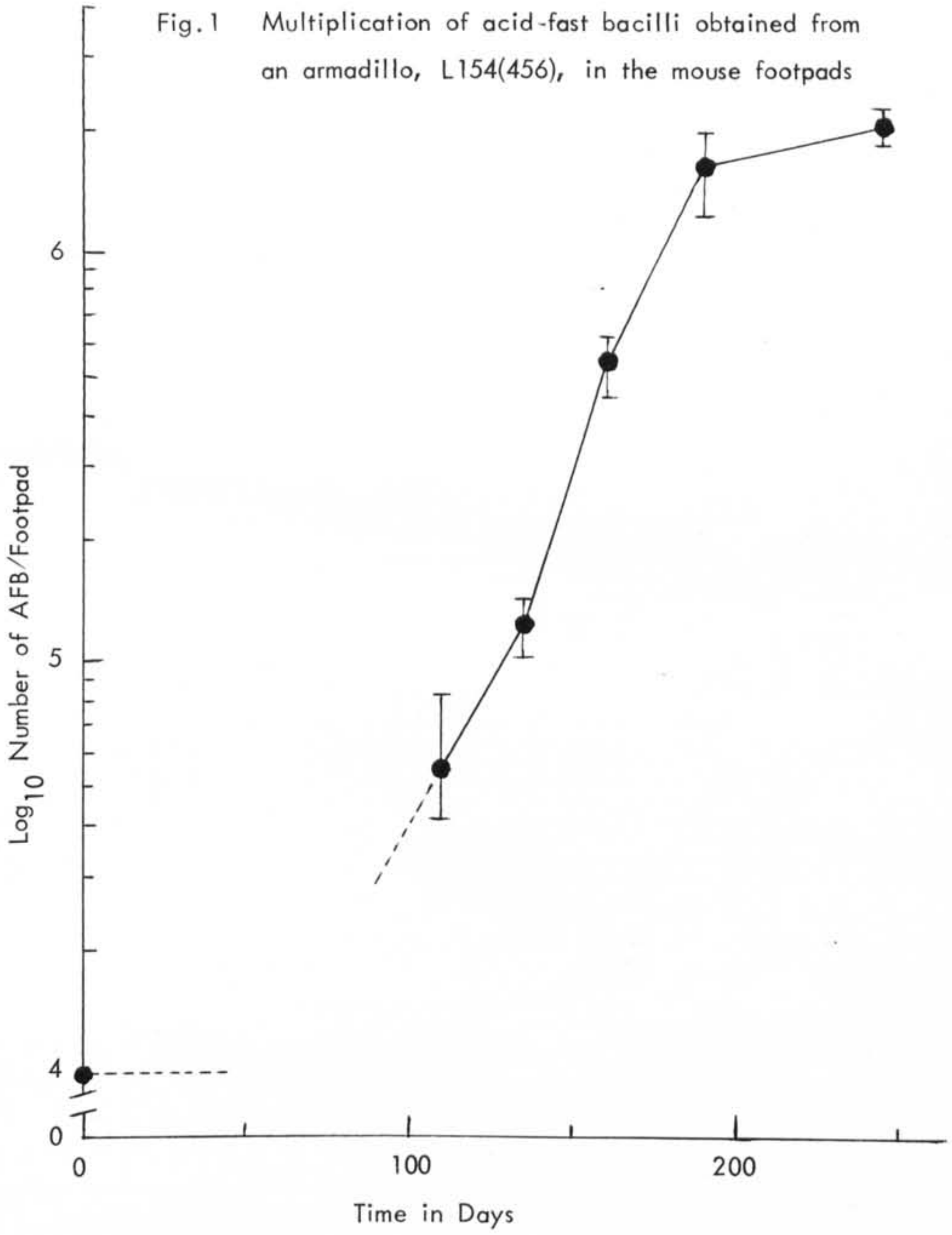
Fig. 1 Multiplication of acid-fast bacilli obtained from an armadillo, L154(456), in the mouse footpads
Log10 Number of AFB/Footpad
6
5
4
0
0
100
200
Time in Days

The tests of Tween 80 hydrolysis and tolerance to ethambutol are known to be the most reliable ones for differentiating M. scrofulaceum from M. gordonae, both included in Runyon's Group II mycobacteria. The cell culture isolate was negative for Tween 80 hydrolysis and tolerant of 5 mcg/ml of ethambutol. These characters were consistent with those of M. scrofulaceum. The bacilli inoculated into the mouse footpads disappeared without any grossly visible changes at the site of inoculation. The acid-fastness was not extracted by pyridine.

It is likely that the armadillo leproma had been contaminated with a small amount of M. scrofulaceum. The significance of this demonstration is that a new problem might be offered for cultivation attempts of M. leprae using an armadillo leproma.

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