Observations on *in Vitro* Survival of *Mycobacterium leprae*

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At present two technics are available to determine the viability of *Mycobacterium leprae*. These are: (1) aspect of the bacilli under the electron microscope or after Ziehl-Neelsen staining, [Rees *et al* ([9, 10])] and (2) multiplication of *M. leprae* in the mouse footpad [Shepard ([13]) Rees ([8]), Pattyn *et al.* ([7])]. While the first technic gives results in a shorter time, it relies, as does all morphology, on a good deal of subjectivity; the second technic, on the other hand, takes much more time, but is completely reliable (as far as no evident mistakes are made). Both methods were used in our laboratory with the hope of obtaining some information in which direction culture media for *M. leprae* should be developed. In all instances bacilli harvested from mouse foot passages were used and all necessary controls, such as inoculation on Loewenstein medium, and eventually intravenous inoculation of mice (to exclude *M. lepraemurium*), were made.

Our first observations [Pattyn ([5])] showed that *M. leprae* suspended in Hanks balanced salt solution (BSS) at room temperature slowly die within three weeks, since the incubation time for positive takes in the subinoculated mice becomes progressively longer. After four weeks only occasional bacilli survive; only some of the inoculated mice become positive later on. When incubated at 33°C in Hanks BSS the bacilli also die within one month.

Some observations were made with Šula's medium. This medium differs primarily from other mycobacterial media in that it contains alanine instead of asparagine as a nitrogen source. In one case mice were inoculated with material that had remained four months in this medium at 33°C. Two out of four mice examined after nine months or longer showed a take. However none of the mice inoculated with bacilli that had been kept for five months in Šula's medium became positive.

As a result of the observations of Rees' group in London [Palmer *et al.* ([4]), Rees *et al.*([11])] and some of our own results [Pattyn([6])], showing that the muscle cell is a predilective if not exclusive site for the multiplication of *M. leprae* in the mouse, it was decided to add muscle fat to the media.

Lipids together with tweens are currently added to culture media for mycobacteria since the original observations made by Dubos. ([2]).

Rat skeletal muscle was extracted with methanol-chloroform as described by Folch *et al.* ([3]) and suspended with the aid of tween 80 in "diploid cell tissue culture medium" (GBL) containing 1 per cent sodium bicarbonate and bovine albumin fraction V. The following concentrations were used in all combinations.

Rat muscle fat 0.05, 0.1, 0.2, 1 mgm./-ml.
Tween 80 0.0025, 0.005, 0.01 per cent
Bovine albumin V 0.1 and 0.5 per cent

All incubations were at 33°C.

In 3 out of 5 experiments an increase in acid-fast elements was noted. This was the case in media containing the following additives:

A. B.A. 0.5 per cent, fat 0.2 mgm./ml.; tween 0.005 per cent
B. B.A. 0.5 per cent, fat 0.05 mgm./ml.; tween 0.01 per cent
C. B.A. 0.5 per cent, fat 0.05 mgm./ml.; tween 0.0025 per cent

Calculation of the respective generation times gave the following results:

Experiment	*Incubation Time*	*Generation Time*
A	122 days	19 days
B	119 days	18 days
C	60 days	12 days

At first sight these generation times are acceptable for *M. leprae*. However, there

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are at least four reasons why the observed phenomenon was not multiplication:

1. These generation times were calculated without taking into consideration an eventual lag phase. The existence of any such lag phase would greatly shorten these generation times, which would then become highly suspect.
2. The aspect of the acid-fast elements observed was also peculiar: viz., they were extremely small: 1-3 mu.
3. There was no further increase in numbers when the observation was pursued.
4. In only 3 out of 5 experiments were these results observed.

In order to obtain a definite answer these materials were inoculated into mouse footpads, with entirely negative results, as far as the counts were concerned (only 2-5 AFB could sometimes be found after long searching in a thick preparation (Table 1).

TABLE 1. *Incubation time and bacterial counts in mice.*

Exper.	Incubation time *in vitro*	Results in inoculation in mice	
		Histology	Counts
A	122 d.	0/6	0/4
	365 d.	0/6	0/3
B	173 d.	1?/6	0/3
C	1?5 d.	0/5	0/2

In some histologic preparations a few acid-fast bacilli were found. These were, however, certainly nonsolid forms or very ill looking elements. On the other hand in some instances the bacteria were located in a small group of densely packed histiocytes or pseudoepithelioid cells with rather voluminous nuclei, evidently the beginning of a granulomatous reaction. In one instance a granuloma without AFB was found. This picture is entirely different from the one seen after inoculation of living *M. leprae*, where a granulomatous reaction appears only after a greatly prolonged observation time, when the plateau level of the bacterial multiplication has long since passed, and a considerable proportion of the *M. leprae* present are dead. The explanation proposed for the phenomenon observed *in vitro* is that the apparent increase of acid-fast elements was the result of a fragmentation of the *M. leprae* originally inoculated in the media.

The conclusions to be drawn from these observations are:

1. When *M. leprae* are incubated in Šula's medium some bacilli can survive for periods up to four months. These are probably some bacilli from an otherwise asymptotically dying population.
2. The very short acid-fast bacilli appearing sometimes in greater numbers in some media after two to four months of incubation, must be considered as degenerate forms in accordance with the findings of Rees and Valentine ([10]), although this interpretation has recently been criticized by Chang and Andersen ([1]) on the basis of observations on *M. lepraemurium* grown in mouse macrophages; they compared such short forms with the "club forms" described by the Ridleys ([12]) in relapsing lepromatous disease. This may represent another difference between *M. leprae* and *M. lepraemurium*.
3. Inoculation of these degenerate bacilli in the mouse footpad may give rise to small granulomata. Since living *M. leprae* inoculated in suitable numbers by the same route, do not produce early granulomata, this may be a supplementary way to determine the nature of bacilli observed in some media.
4. In efforts to obtain multiplication of *M. leprae in vitro* the possibility that the bacteria may fragment must be kept in mind.

SUMMARY

Since 1966 the survival of *M. leprae* in different media and under different circumstances was followed. Two criteria were employed: the morphologic appearance of the bacilli and the result of inoculation of such suspensions into footpads of

mice to control the results of morphological observations.

Diploid cell medium GBL containing 1 per cent sodium bicarbonate was used as the basal medium to which were added rat muscular fat, tween 80, and bovine albumin in different concentrations. In some instances acid-fast staining cocco-bacillary forms appeared in these media. It could not be shown that these were viable *M. leprae*. The phenomenon was interpreted as the result of fragmentation of the mycobacteria originally inoculated.

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