Use of the Mouse Foot Pad in Studying Thermoresistance of *Mycobacterium leprae*

Stefaan R. Pattyn, M.D.[1]

With the aid of the mouse foot passage technic as a means of evaluation, we have made observations on the survival of *M. leprae* under different conditions of temperature and suspending media, which are the subject of this paper.

MATERIALS AND METHODS

The observations were made on strain 2401 of *M. leprae*, which was received from Dr. Shepard of the Communicable Disease Center in Atlanta, and strain 22491 obtained in our laboratory from a lepromatous nodule from Leopoldville, Republic of Congo. Inoculations, harvests and quantitative evaluations were made by the technic described by Shepard ([4,5]). As suspension and diluting fluids we used Hanks' balanced salt solution (BSS) containing 160 mgm. of penicillin and 25 units of Nystatin (Mycostatin, Squibb) per ml. The harvests from mouse foot pads were eventually diluted in other fluid media, as described in the text. The mice used were of the NMRI strain reared in our Institute.

RESULTS

Conservation of suspensions of *M. leprae* at —25°C. In the course of our isolation work on strains of *M. leprae* we made one unplanned observation on the conservation of *M. leprae* at a low temperature. One of the biopsy specimens had been kept for a period of 42 days at −13°C. We found

[1]Professor of Bacteriology, Laboratory of Bacteriology, Prince Leopold Institute for Tropical Medicine, 155 Rue Nationale, Antwerp, Belgium.

TABLE 1. *Multiplication of* M. leprae (*strain 22491*) *in mouse foot pads after freezing at* $-25°C$.

Months of incubation in mice	Period of conservation			
	6 weeks[a]	3 months[a]	6 months[a]	3 months[b]
7	0		0	+[c]
8				5.0×10^4
9	0			
10	1.2×10^5 (2)	3.7×10^5 (2)		
11		3.7×10^5 (2)		
12	3.0×10^5 (4)	0 3.7×10^5 5.0×10^5 (4)		

Number of mice—in parenthesis.
[a]Done in one experiment, inoculation 2×10^3.
[b]Inoculation 10^3.
[c]AFB present, less than 10^3.

that the leprosy bacilli it contained did give rise to multiplication in the mouse foot pads.

In the course of passage work with the above mentioned strains we made more observations on the conservation of *M. leprae* at low temperature. Mouse pad harvests were diluted in Hanks' BSS in order to contain 10^3 to 5×10^3 acid-fast bacilli (AFB) per 0.63 ml. as inoculum for the mouse foot pad. The suspensions were kept in screw-capped vials in our electric deep-freeze refrigerator at $-25°C$. After different periods of conservation they were inoculated into groups of mice, and multiplication of the bacilli was followed. Results on conservation periods ranging from 6 weeks to 6 months are presented in Tables 1 and 2. They show clearly that suspensions of *M. leprae* remain viable at $-25°C$ for these periods of time. The multiplication of bacilli in the mouse foot pads was not significantly different from that observed after passage of fresh harvests.

Inactivation at 45°C. In one experiment a suspension of *M. leprae* (strain 2401) containing 2.3×10^3 AFB per 0.03 ml. was immersed in a water bath at 45°C for one hour, before inoculation into mouse foot pads. We were unable to observe any multiplication during the 11-month observation period that followed.

Incubation at room temperature in Hanks' BSS. Several suspensions of *M. leprae* were incubated at room temperature in Hanks' BSS for periods from 1 to 4 weeks. The room temperature in our laboratory varies from 18° to 23°C. In the first experiment (Table 3) a suspension containing 4×10^3 AFB per 0.03 ml. showed a considerable loss of viable organisms between the third and fourth weeks; after 4 week's of incubation only 1 out of 6 animals showed any multiplication. In a second experiment, however, carried out with a different harvest, which contained 10^4 AFB per 0.03 ml., survival was better, for in this case 2 animals out of 2 available after 10 months showed a normal increase in the number of bacilli.

The results indicate that one month of conservation of *M. leprae* under these conditions seems to be a critical period for their survival, although this would necessarily be influenced by the percentage of solidly staining bacilli at the start of the incubation.

TABLE 2. *Multiplication of* M. leprae (*strain 2401*) *in mouse foot pads after 4 months at* $-25°C$.

Months of incubation in mice	Multiplication after 4 months at $-25°C$	
7	0	
8	+	
9	1.4×10^5 +, 2.0×10^5	2.4×10^5 2.0×10^5
10	4.2×10^5	

TABLE 3. *Multiplication of* M. leprae (*strain 2401*) *in mouse foot pads after incubation at room temperature in Hank's BSS.*

Months of incubation in mice	Controls	Time at room temperature				
		Experiment 1[a]				Experiment 2[b]
		1 week	2 weeks	3 weeks	4 weeks	4 weeks
5	+					
6	+					
7	+					
9	2.4×10^4					
10	2.4×10^5	4×10^4	5×10^4	2.6×10^4	0.0×10^4	8×10^5; 3.7×10^5
11	9.0×10^5			2.9×10^4		
12		2.2×10^6	2×10^6		0, 0, 0	

[a]Inoculation = 4×10^3.
[b]Inoculation = 10^4.

TABLE 4. *Multiplication of* M. leprae *in mouse foot pads after incubation at room temperature in different media.*

Months of incubation in mice	Atmospheric air				Air enriched with CO_2			
	Hanks BSS	Sula	Dubos	Hanks glycerol serum	Hanks BSS	Sula	Dubos	Hanks glycerol serum
8	10^5	5.6×10^4	0; 0	0	0; 0; +	+	0; +	10^5; 0; +
9		3.8×10^4 6.8×10^5 (2)	0; 0	0; 0	0; 0	1.4×10^5 0	+	2.6×10^6 10^5
10		4.2×10^5 (2)						10^5, 0, +

In parenthesis, the number of mice pooled if more than 1 animal examined.

Incubation of *M. leprae* suspensions at room temperature in different media. In this experiment a suspension of *M. leprae* (strain 2401) was diluted in a double series of four different media: Hanks' BSS, Hanks' BSS containing 5 per cent calf serum and 5 per cent glycerol; Dubos' liquid serum medium; and Sula medium containing 5 per cent calf serum. All tubes were stoppered with aluminum caps and incubated at room temperature. One set of media was left in a humidified jar in atmospheric air. The second series was set up in a jar in which a candle had been lighted before sealing. The dilutions were made so that 0.03 ml. of the medium contained 2.4×10^3 AFB. After one month these suspensions were inoculated into mouse foot pads, and the bacilli were harvested after 8-10 months of observation. Table 4 shows the results.

Unfortunately only one animal survived in the group inoculated with the suspension incubated in Hanks' BSS in atmospheric air. It showed, however, that at least some of the bacilli were still living when inoculated in mice. Sula medium gave quite good results with respect to survival. No multiplication was observed after incubation in Dubos' liquid medium and Hanks BSS containing both glycerol and serum. Atmosphere enriched with CO_2 might have been beneficial in connection with the Hanks-glycerol-serum medium. In fact, microscopic examination of the suspensions immediately before inoculation into mice had shown that in this condition more bacilli were solidly stained, although the ratio of solid to nonsolid organisms was not determined. The observed effect from CO_2 may also be explained through a pH effect in this bicarbonate-buffered solution.

Incubation at 30° and 33°C. Part of the suspension of *M. leprae* used in the experiment summarized in Table 3 was incubated also in Hanks' BSS at 33°C for one month. Inoculation of this suspension in mouse foot pads gave negative results in 5 mice examined 10 and 11 months after inoculation (Table 5).

Other observations at 30° and 33°C were made in connection with experiments on the behavior of *M. leprae* in tissue culture. In one experiment DE40 cells (of rat origin) were infected with *M. leprae* and incubated at 33°C. After 1 and 2 weeks respectively, the supernatant fluid was centrifuged, and the sediment added to the trypsinized cells. These were resuspended in Hanks' BSS and this suspension was inoculated in foot pads of mice. As Table 5 shows, some living bacilli were present after one week in this tissue culture system, but no multiplication of *M. leprae* was obtained in mouse foot pads with bacilli that had been in the suspension for 2 weeks. Other experiments, with suspensions of *M. leprae* that had been kept for one month at 30° and 33°C in tissue culture cells, or in the nutrient medium without cells, showed no evidence of multiplication in mouse foot pads.

DISCUSSION

Our results show that suspensions of *M. leprae* can remain viable at −25°C for at least 6 months. This is in accord with the facts for most of the mycobacteria as shown by Tarshis ([6]), and with experience gained

TABLE 5. *Survival of* M. leprae *(strains 2401 and 22491) at 33°C in Hanks' BSS and tissue culture.*

Months of incubation in mice	Strain 2401	Strain 22491	
	Hanks BSS at 33°C	Tissue culture DE 40 cells	
	1 month	1 week at 33°C	2 weeks at 33°C
8		10^4	
9		0	0
10	0 (2)	+	0 (2)
11	0 (3)	1.6×10^3 (3)	
12			0 (2)

in our own laboratory with our collection of mycobacteria. *M. ulcerans* can be kept in these conditions for a year at least, and our experience with a strain of *M. lepraemurium* covers a period of a year and a half.

A room temperature of 18-23°C permits survival of *M. leprae* in Hanks' BSS for a period of 3 weeks. An acid pH may be beneficial, as shown in Hanks' BSS incubated in a CO_2 enriched atmosphere. It may be worthwhile to note in this connection that increased CO_2 tension is beneficial for the growth of *M. tuberculosis*, as shown by Cohn and Middlebrook ([1]).

Loss of visibility is more rapid at 30° and 33°C than at room temperature. It may be that in our tissue culture experiments the disappearance of viable mycobacteria was due to some noxious effect from the cultured cells or the trypsinization procedure. However, in experiments where the cells were "versenized," instead of being treated with trypsin, the results were also negative.

Our results may at best be interpreted as made with resting cells, but the observation that they seem to be rapidly inactivated by a temperature of 45°C, and viable for a longer time at room temperature than at 30° or 33°C, can be considered as an indication of their sensitivity to temperature. This is in accord with their ability to grow in mouse foot pads, the temperatures of which are only a little higher than room temperature.

Count of the ratio of bacilli staining solidly in the acid-fast stain was shown by the work of Rees and associates ([3]) and that of Shepard and McRae ([5]) to offer a means for making immediate estimates of the viability of *M. leprae*. It might render obsolete such observations as those related in this paper. But Shepard and McRae calculated that half time required for dead bacilli to degenerate into nonsolid forms varies between 8 and 20 days; this is a rather long period between the death of the bacilli and their appearance as "nonsolids" and may justify our observations.

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DISCUSSION

Dr. Mason. Dr. Pattyn has brought a new note in this conference, survival time, which has implications for much of the work to be reported later. I know that Dr. Shepard has given much attention to this factor in the past. We have a short period for discussion.

Dr. Weiser. Has anyone tried to lyophilize this organism? If this could be accomplished the lyophilized material could be stored at low temperature and we should be able to keep the organism to eternity.

Dr. Middlebrook. Dr. Pattyn, did you shield your suspensions from ordinary visible daylight when you carried out the experiments at low temperature?

Dr. Pattyn. We did not.

Dr. Shepard. I would like to say that where we have used the same temperatures as Dr. Pattyn we have had just about the same results. We have examined our re-

sults with the mice in a slightly different way, but there seems to be very little discrepancy, if any at all. We did not do experiments at higher temperatures, and I am sure that this is useful information to have in the long run, certainly from the standpoint of epidemiologic experience. To view these results in the proper background we really need to go back a few years to the time in the work on *M. leprae* in tissue culture, mice, and so on, when we were all afraid that the viability of *M. leprae* might be gone in the first day or two. I used to work at Carville all day, then go back to Montgomery on the plane and work all night inoculating mice in an attempt to avoid deterioration.

A question was asked about the half-life we had calculated. These results need to be viewed in this background. The half-life that we estimated in intact mice was not measuring quite the same thing. We did not know exactly when the organisms in the mice died. The calculation was the rate at which the organisms lost their solid staining. This is not necessarily the rate at which they lost their viability in the mice.

Dr. Rees. The results of Dr. Pattyn and those reported by Dr. Shepard yesterday, have a most practical application for pharmaceutical firms. Hitherto one had to have available freshly isolated strains of *M. leprae;* now although nobody has yet lyophilized the bacilli, the experiments of Drs. Shepard and Pattyn show that *M. leprae* can be stored at minus 75° or 170° (in liquid nitrogen). This would obviously enable pharmaceutical firms to have readily available supplies of these bacilli for testing against drugs—something we all hope that the pharmaceutical firms will take up in the search for new antileprosy drugs. Dr. Pattyn has been using the morphologic *appearance* of *M. leprae* to judge their viability. Our own experience would suggest that this is unreliable when the bacilli are kept at lower temperatures. The degenerate changes result from loss of cytoplasmic content following death of the bacilli—a process that would not occur at lower temperatures. Therefore, for this type of work, I would think that the Morphologic Index would be less precise in determining viability and we should rely at this stage on the mouse foot pad test for determining viability (equated with infectivity).

Dr. D'Arcy Hart. I want to ask Dr. Pattyn a question. I cannot remember if you used Sula's medium at room temperature. Did you use it at 33°, and, if so, what were the results?

Dr. Pattyn. We did not use Sula's medium at another temperature than the room temperature except in that one experiment. I would not be sure if that was the end of the story. It seemed that Sula's was a good medium.

Dr. Mason. If there are no other questions we shall go on to the next speaker, Dr. J. P. Wiersema.