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CONSERVATION OF MYCOBACTERIUM LEPRAE IN LIQUID NITROGEN

by

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Summary — Suspensions of *M. leprae* can be successfully preserved if they are slowly frozen in buffered dimethylsulfoxide 7.5 per cent final concentration, and maintained in liquid nitrogen.

KEYWORDS : Mycobacterium leprae, Bacteriological technics; Freezing; Nitrogen; Dimethyl sulfoxide.

Introduction

The conservation of *Mycobacterium leprae* strains constitutes still a problem : lack of suitable method to maintain the organisms viable *in vitro* requires continuous animal passage, with many disadvantages : high cost in personel and experimental animals and their maintenance, possible loss of strains due to intercurrent disease in mice, mixture and substitution of strains, variability in lag phase and incubation time in inoculated animals.

M. leprae can be kept viable for 2-3 weeks at 4 °C and for about 3 months at − 25 °C (Pattyn 1965, 1968, Shepard and McRae 1965). Long term maintenance at low temperature has not been satisfactorly resolved, although most eukaryotic cells and many human and animal parasites can be readily maintained in liquid nitrogen, either after controlled cooling or after direct immersion, and in the presence of glycerol or dimethylsulfoxide.

For conservation in liquid nitrogen, tissue cultured cells are routinely mixed with dimethylsulfoxide (DMSO) at 10 per cent to attain a final concentration of 5 per cent.

When we learned that some protozoa (R. A. Neal, *cited in* Jadin *et al.* 1971) require a slightly higher concentration of dimethylsulfoxide we decided to try this technique with *M. leprae.*

This paper presents the results of this work.

Materials and Methods

Mouse passaged strains of *M. leprae* previously described (Pattyn *et al.*, 1972) and one suspension from a human biopsy specimen were used.

M. leprae was harvested in Hanks balanced salt solution (Hanks BSS). After counting (Shepard, 1960) and just prior to freezing, the suspension was mixed in equal parts (if necessary after dilution with Hanks BSS) with a solution of dimethylsulfoxide in sterile distilled water, or in buffered gelatin tris (BGT) (Mandel, 1958), so as to contain 5 to 7.10^3 acid fast bacilli per 0.03 ml. Control mice were inoculated immediately and the rest of the suspension was introduced into borosilicate ampules in 0.50 ml amounts sealed and slowly frozen. This was done with a Biological Freezer BF 4 (of Union Carbide) for 1 °C per minute to − 70°C, and for 5 °C per minute between − 70 °C and − 100 °C when the ampules were immersed in liquid nitrogen.

In a first series of experiments DMSO was used in a final concentration of 5 per cent, later in a final concentration of 7.5 per cent. Thawing was done by immersing the ampules in a water bath at 37 °C. Groups of 10 or 12 mice were inoculated immediately in the left hind foot pad.

Taking into account the incubation time in the control animals, one mouse was examined monthly either by counting (Shepard, 1960) or histologically. When a mouse was found positive, remaining animals were examined, if the monthly examination remained negative all surviving mice were examined after 11-12 months.

Evaluation of results is done by comparison of the incubation time between control animals and those inoculated with frozen samples and/or the regularity with which the inoculations with frozen bacilli gave positive results.

Results

A. *Freezing of* M. leprae *suspensions in DMSO at 5 per cent*

As can be seen in table 1, in this series many mice died from intercurrent disease, but results of inoculations of *M. leprae* suspensions kept in liquid nitrogen were very irregular and unpredictable.

The incubation times are not indicated in the table but these were always 2-3 months longer for the frozen suspensions as compared with the fresh ones.

Although the results are irregular it seemed that killing of the bacilli occurred during the cooling process, since negative results did not increase as conservation periods at low temperature increased. Therefore a second series of experiments were made.

B. *Influence of different circumstances during cooling on the survival of* M. leprae

Harvests were made in Hanks BSS, and part of it diluted so as to contain a suitable number of acid fast bacilli (AFB). An aliquot of this suspension was inoculated as a control and an aliquot directly immersed in liquid nitrogen (table 2).

TABLE 1

Results of freezing *M. leprae* suspensions in 5 per cent DMSO

	Strain N°				
	22491	2401	2401	144/62	6382
Control mice	9/9 (**)	6/6	9/9	—	6/6
3 weeks (*)	—	—	—	0/7	—
6 weeks	9/12	0/4	0/8	—	0/5
12 weeks	1/8	2/5	0/4	1/7	0/4
18 weeks	3/4	0/4	0/3	5/11	0/4
24 weeks	3/8	2/5	—	—	0/2
30 weeks	1/3	5/7	—	—	0/7
36 weeks	—	1/1	—	—	0/3
42 weeks	2/3	2/4	—	—	0/2
48 weeks	2/5	—	—	—	0/4

(*) Time of conservation in liquid nitrogen.
(**) Number of mice positive for *M. leprae* / Number of mice examined after appropriate incubation time (see Material and Methods).

TABLE 2

Influence of different circumstances of cooling on the survival of *M. leprae*

	Strain n°		
Conditions of cooling	22491	12445	17547
Controls	9/9 (*)	6/6	6/6
Susp. Hanks BSS, immersion	—	4/9	2/6
Buffered DMSO 5 %, immersion	—	0/7	1/7
Buffered DMSO 5 %, s.c.	0/6	1/5	3/8
Buffered DMSO 5 %, 4 hrs 4°C-1°/min. s.c.	—	5/8	5/9
Buffered DMSO 5 %, 4 hrs RT-1°/min. s.c.	—	0/7	5/8

(*) Number of mice positive for *M. leprae* / number of mice examined after suitable incubation time.
s.c. : slow cooling.

A second part of the original suspension was mixed with DMSO 10 per cent in BGT (calculated to contain the same number of AFB) and cooled in 4 different ways :

1) direct immersion in liquid nitrogen;
2) slow cooling;
3) the same procedure after keeping the suspension at 4 °C for 4 hours;
4) the same procedure after keeping the suspensions for 4 hours at room temperature (22 °C).

Six weeks later the ampules were thawed and inoculated into mice.

As can be seen from table 2 none of the different procedures gave better results. Particularly the use of buffered DMSO did not offer any advantage.

C. *Buffered DMSO 7.5 per cent*

In the meantime we had heard about the use of DMSO at 7.5 per cent for protozoa (Neal *cited in* Jadin *et al.*, 1971) and used this also in BGT.

It can be seen in table 3 that results were now very satisfactory. This was not only the case for the regularity with which the mice become positive but also in the incubation time : this did not differ significantly between the controls and the mice inoculated after the different conservation periods.

TABLE 3

Results of inoculation of *M. leprae* suspensions frozen in buffered DMSO at 7.5 per cent

	3/63	12445
Controls	7/7 (**)	5/5
3 weeks (*)	6/6	5/5
6 weeks	4/4	4/4
10 weeks	6/6	7/7
12 weeks	7/7	6/6
15 weeks	9/9	6/7

(*) Period of conservation in liquid nitrogen.
(**) Number of mice showing multiplication of *M. leprae* / number of mice examined.

Results on more strains and a suspension from a human biopsy (L 19) are shown in table 4. For some of these strains conservation in liquid nitrogen was near to 200 days. One strain (not included in the table) was a failure : none of the thawed ampules gave positive results. (Unfortunately no mice has been inoculated with the fresh suspension, so that suitable controls in fact were not available).

Discussion

Whereas lyophilisation is an excellent method to maintain bacteria viable (Seastone, 1971) including *M. lepraemurium* (Nakamura, 1969) this procedure is not satisfactory for *M. leprae* (Hilson, Shepard, personal communication).

Freezing at − 15 to − 25 °C in different media is a commonly applied method to maintain collections of mycobacteria, Kim and Kubica (1972) recently presented the methods applied at the Trudeau Institute. Since years mycobacteria in our laboratory are stored at − 25 °C in TB broth (Difco) with satisfactory results although detailed studies on survival rates were not undertaken.

As mentioned before (Pattyn, 1965, 1968) and from numerous unpublished results, storage of *M. leprae* at this same temperature be it in Hanks BSS, or BGT, or TB broth or fat containing medium (Pattyn, 1971) maintains this species viable for only about 3 months.

TABLE 4

Results of freezing *M. leprae* suspensions in 7.5 per cent DMSO in BGT

Strain 144/62		Strain 6382		Strain 12445		Strain 17847	
1 d (*)	8/8 (**)						
10 d	5/5	10 d	5/5	13 d	7/7	13 d	7/8
106 d	5/6	72 d	2/6	92 d	7/7	92 d	7/7
201 d	9/9	90 d	4/6	181 d	10/10	181 d	9/9
		197 d	6/6				
Strain 2401		**Strain 6382**		**Strain 3/63**		**L 19**	
10 d	5/5	8 d	5/5			0 d	6/6
100 d	3/3	30 d	7/7	51 d	7/7	58 d	8/10
195 d	9/9			92 d	8/8	210 d	8/8

(*) Conservation time in liquid nitrogen in days.
(**) Number of mice showing multiplication of *M. leprae* / number of mice examined after suitable incubation.

From his studies Levy (1969, 1971) concluded that at lower temperature (− 60 °C) prolonged storage did not decrease the viability of *M. leprae*, nor the rate of thawing, but that loss of viability resulted from freezing. This was also evident from our observations.

We agree with L. Levy (1971) that the suspending medium is the most important factor in the conservation of viability of *M. leprae.* Although no detailed adequate comparisons were made it is thought that the most important factor for the successfull conservation of *M. leprae* is the slightly increased concentration of DMSO and not the use of DMSO in buffer, since the latter did not improve the results with the lower concentration of

DMSO (see series B). It is very likely that DMSO in distilled water could be used as well.

The fragility of *M. leprae* to freezing distinguishes this organism considerably from other mycobacteria.

Slow freezing of suspensions of a strain of *M. ranae* (serotype fortuitum) in Hanks BSS with or without 5 per cent DMSO did not kill these organisms considerably.

The satisfactory solution of conservation of strains of *M. leprae* can lower considerably the work load in the experimental leprosy laboratory, allowing available man hours to be applied to other problem solving work.

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Samenvatting — **Bewaring van** *Mycobacterium leprae* **in vloeibare stikstof.**
Suspensies van *M. leprae* kunnen worden bewaard indien ze traag worden ingevroren in gebufferde dimethylsulfoxide aan 7,5 ten honderd en verder bewaard in vloeibare stikstof.

Résumé — **Conservation de** *Mycobacterium leprae* **en azote liquide.**
Des suspensions de *M. leprae* peuvent être conservées vivantes si elles sont refroidies lentement en présence de diméthylsulfoxide tamponnée et à la concentration finale de 7,5 p. cent., ensuite plongées dans l'azote liquide.

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