Maintenance of Infectivity of *M. lepraemurium in Vitro* for Sixteen Years by Means of Lyophilization

Determination by Bacteriologic and Morphologic Observation of The Infecting Bacilli[1]

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Since murine leprosy bacilli are obligate intracellular parasites, they should best be preserved *in vivo* by inoculating from animals to animals, rats or mice. In a previous paper ([2]), however, maintenance of infectious activity of *M. lepraemurium in vitro* for 10 years by means of lyophilization was reported.

The present paper describes additional data indicating that the infectious activity of the bacilli can be maintained *in vitro* for 16 years by lyophilization.

MATERIALS AND METHODS

M. lepraemurium. The Fukuoka strain (R-38) of *M. lepraemurium,* which was isolated by Urabe and Yoshimura ([6]) in Fukuoka, Japan, in 1938, was used as the lyophilized material.

Material tested for lyophilization. A leproma from a strain developed in the subcutaneous tissue of a rat experimentally inoculated was homogenized in a sterile mortar and suspended in sterile physiologic saline to make a 20 per cent suspension. This suspension was divided into four aliquots, and these were added respectively to equal volumes of physiologic saline, 10 per cent inactivated bovine serum-water, 4 per cent glycerine water, and Kirchner medium ([1]) containing 10 per cent bovine serum. Thus the concentrations of both bacillary suspension and media for dilution were diluted to twice the original volume.

Procedure for lyophilization. One ml. of each mixed material was poured into separate ampoules for lyophilization and immediately frozen in a dry ice-acetone mixture, which was then dried by a rotary pump for about two hours in the case of the saline and serum solutions, and about three hours in the case of glycerine-water and the Kirchner medium. After drying, these ampoules were sealed and stored in a refrigerator at 4°C.

Infectivity test. The ampoules that had been stored in a refrigerator for 16 years after lyophilization were opened and the dried material in each ampoule was suspended in 1 ml. of sterile distilled water. One-tenth ml. of each suspension was inoculated subcutaneously into normal mice (dd strain). These mice were killed at appropriate periods after inoculation, and the persisting infectious activity of the lyophilized bacilli was determined by bacteriologic observations on smears of subcutaneous tissues of mice.

RESULTS

Morphology of the bacilli. Lyophilization of *M. lepraemurium* was performed on 26 April 1951. The ampoules stored in a refrigerator were opened on 1 December 1967, and the dried bacilli were suspended in sterile distilled water. Morphologic characteristics in the bacilli were observed under a light microscope and an electron microscope. The results of preservation for 10 years were reported previously ([2]). In the present paper the results obtained from the material after 16 years of preservation are described. No significant morphologic differences were observed between the lyophilized bacilli and bacilli that were obtained from a fresh leproma. The morphologic appearances of the lyophilized bacilli as observed under an electron microscope are shown in Figures 1 and 2.

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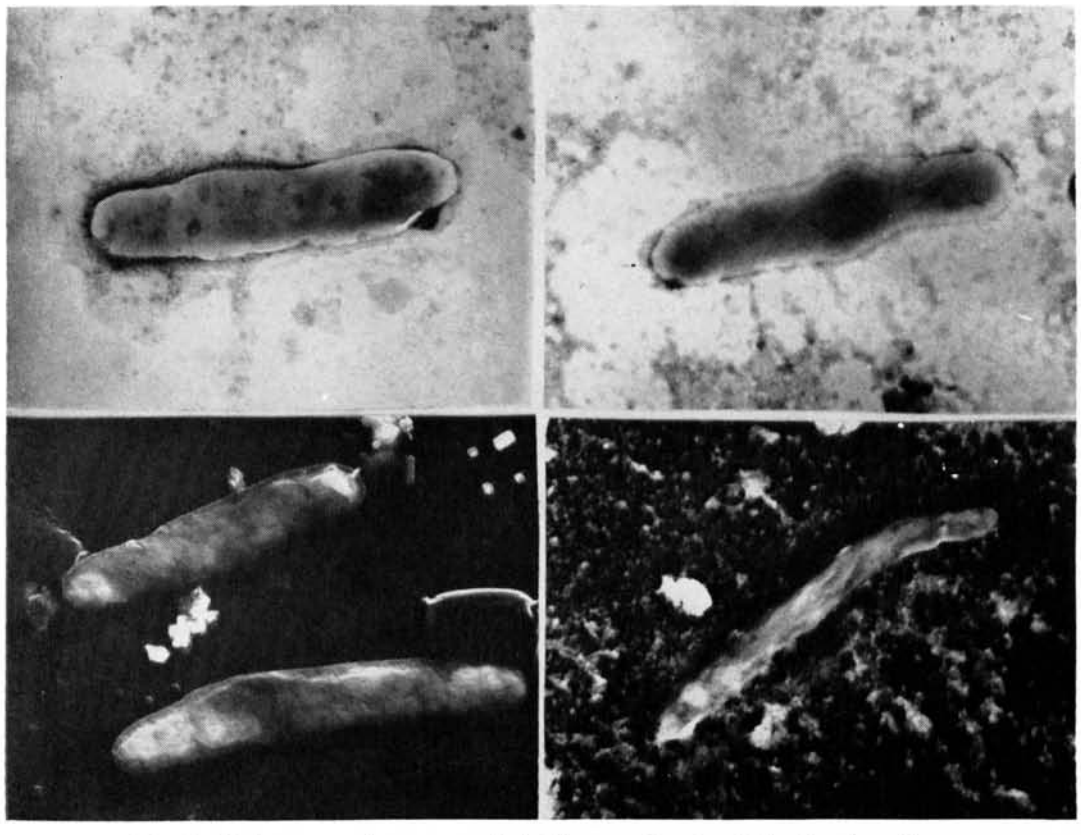

FIG. 1. *M. lepraemurium* suspended 16 years after lyophilization in saline.
FIG. 2. *M. lepraemurium* suspended 16 years after lyophilization in 10 per cent bovine serum-water.
Upper: negatively stained (PTA). *Lower*: Chrome shadowed. (Magnification X33,000)

Determination of persisting infectivity. Results obtained with the bacilli lyophilized for 10 years and for 16 years are summarized in Table 1. As already described in a previous paper ([2]), infectious activity of *M. lepraemurium* lyophilized in the media tested could be maintained *in vitro* for more than 10 years. Particularly in the case of physiologic saline and 10 per cent bovine serum-water, high potency infectivity of the bacilli was demonstrated. In the case of 16 years' preservation also, it was demonstrated that infectious activity, as measured by morphologic appearance, was maintained in the media tested, viz., physiologic saline and bovine serum-water. From the results obtained, however, it could be presumed that infectious potency of the bacilli was progressively decreasing with time during the last preservation periods.

DISCUSSION

Recently successful transmission of *M. leprae* to mice has been established by Shepard ([3]), and it is possible by this means to maintain infections by transmission of *M. leprae* from mouse to mouse. This significant experiment has contributed to a number of studies of human leprosy, including chemotherapy ([5]) and prophylaxis ([4]). However, it is not always easy to obtain fresh human leprosy bacilli when they are needed. It is necessary, therefore, to develop a mouse-adapted strain of *M. leprae*, and keep it in mice. On the other hand, it would be convenient for experiments if the bacilli could be obtained any time they were wanted, without waiting until they had multiplied enough for use. It would be helpful, for this purpose, if the bacilli could be kept *in vitro*. The results

TABLE 1. *Determination of infectious activity of lyophilized* M. lepraemurium *by animal experiments.*

Medium for lyophilization	Preservation periods of lyophilized ampules at 4°C (yrs.)	Animal experiments for determining infectivities			Decision of infectious activity
		No. of mice tested	Survival of mice after inoculation (days)	Bacteriologic results in smears of inoculated tissues	
Physiologic saline	10	1	133	++++[a]	+
		2	133	++++	+
		3	133	++	+
		4	133	++++	+
		5	133	++++	+
	16	1	200	−	+
		2	200	−	−
		3	200	++++	+
2% glycerine water	10	1	133	−	−
		2	133	−	−
		3	133	++	+
		4	133	−	−
		5	133	−	−
5% bovine serum-water	10	1	131	+++	+
		2	133	++++	+
		3	133	++++	+
		4	133	−	−
		5	133	++++	+
	16	1	158	−	−
		2	158	−	−
		3	158	++++	+
		4	158	++++	+
Kirchner medium (twice diluted)	10	1	133	+	+
		2	133	+	+
		3	133	−	−
		4	133	−	−

[a] The suspended bacilli in the lyophilized ampoules were inoculated subcutaneously into dd mice, and persisting infective activity of the bacilli was determined by autopsy on the number of days indicated after inoculation.

recorded in the present paper indicate that infectious activity of *M. lepraemurium* can be maintained *in vitro* for 16 years and that no morphologic changes in the bacilli are noted after that length of time.

It can be said, therefore, that application of lyophilization technics is to be recommended for the preservation of *M. lepraemurium*, as well as *M. leprae, in vitro.*

When the bacilli are lyophilized, one of the most important factors influencing the maintenance of their activity during storage seems to be the dryness of the solutions intended to suspend materials.

It was unfortunate that in the experiments here reported the infectivity of the bacilli lyophilized in 2 per cent glycerine-water and Kirchner medium could not be

tested. These ampoules were lost six years ago. It might be presumed from the results recorded in the previous paper ([2]) that their infectivity was completely lost during the additional six years, for their infectivity after 10 years' preservation was remarkably reduced.

SUMMARY

It has been confirmed that the infectious activity of *M. lepraemurium*, as indicated by morphologic appearance, can be maintained *in vitro* for 16 years when the bacilli are frozen, dried, and stored in a refrigerator. No morphologic change in bacilli thawed after this length of time was demonstrated, on comparison with fresh bacilli obtained from a recently developed murine leprosy leproma.

RESUMEN

Se ha confirmado que la actividad infecciosa del *M. lepraemurium*, como lo indica el aspecto morfológico, puede mantenerse *in vitro* por 16 años cuando el bacilo es congelado, desecado y almacenado en un refrigerador. Ningún cambio morfológico fué demostrado en el bacilo después de este prolongado período de tiempo, en comparación con el bacilo fresco obtenido de lepromas leprosos murinos recientemente desarrollados.

RÉSUMÉ

Il a été confirmé que l'activité infectieuse de *M. lepraemurium* telle qu'elle est révélée par son aspect morphologique, peut être maintenue *in vitro* pendant 16 ans lorsque les bacilles sont congelés, séchés, et entreposés dans un refrigérateur. Lorsque les bacilles étaint dégelés après cette période, aucune modification morphologique n'a pu être mise en évidence, quand on les comparait avec des bacilles frais obtenus à partir de lépromes récents de lépre murine.

REFERENCES

1. KIRCHNER, O. Die Leistungsfähigkeit der Tieferkultur des Tuberkelbazillus bei Verwendungs besonders geeigneter flüssiger Nahrboden. Zentr. f. Bakt. Parasitenk. **1** (1932) 403-412.
2. NAKAMURA, M. Maintenance of infectivity of *Mycobacterium lepraemurium* for ten years by lyophilization. Kurume Med. J. **11** (1964) 46-47.
3. SHEPARD, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. J. Exper. Med. **112** (1960) 445-454.
4. SHEPARD, C. C. Vaccination against human leprosy bacillus infections of mice: Protection by BCG given during the incubation period. J. Immunol. **96** (1965) 279-283.
5. SHEPARD, C. C. and CHANG, Y. T. Effect of several anti-leprosy drugs on multiplication of human leprosy bacilli in foot-pads of mice. Proc. Soc. Exper. Biol. & Med. **109** (1962) 636-638.
6. URABE, K. and YOSHIMURA, K. Relation between environment and duration of infectivity of *Myc. lepraemurium in vitro*. Tokyo Ijishinshi **3152** (1939) 2377-2380 (in Japanese).