INTERNATIONAL JOURNAL OF LEPROSY

Volume 61, Number 3
Printed in the U.S.A.

Preservation of *Mycobacterium leprae in Vitro* for Four Years by Lyophilization[1]

Kenji Kohsaka, Masanori Matsuoka,
Tsunehiko Hirata, and Masahiro Nakamura[2]

In 1985, one of us ([8]) reported that the infectivity of *Mycobacterium lepraemurium* could be completely maintained *in vitro* for as long as 30 years by means of lyophilization. Although cryopreservation techniques in liquid nitrogen for a wide range of animal cells and microorganisms have been developed and applied to *M. leprae* ([1, 13]), *M. leprae* still have been transmitted and maintained usually from mouse to mouse or from armadillo to armadillo in some institutes because of the absence of successful *in vitro* cultivation. Accordingly, the possibility of phenotypic changes in *M. leprae* during these animal passages might be considerable. If lyophilization is applicable to *M. leprae* as it is to *M. lepraemurium* and if lyophilization is as effective as cryopreservation in maintaining viability, then the lyophilization procedure would be preferable as a means of eliminating possible phenotypic changes in *M. leprae.* In 1989, we presented ([9]) the results on the survival of *M. leprae* under the process of the lyophilization procedure measured quantitatively by animal experiments.

The present paper is to describe the viabilities of *M. leprae* which were suspended in water with serum or with skim milk, lyophilized, and maintained *in vitro* for at least 4 years, as measured quantitatively by the nude mouse foot pad inoculation method.

[1] Received for publication on 27 July 1992; accepted for publication in revised form on 15 March 1993.

[2] K. Kohsaka, Ph.D.; M. Matsuoka, Ph.D.; T. Hirata, Ph.D., National Institute for Leprosy Research, 4-2-1 Aoba-cho, Higashi-Murayama, Tokyo 189, Japan. M. Nakamura, M.D., Ph.D., Professor Emeritus, Kurume University, Koga Hospital Medical Research Institute, 120 Tenjin-cho, Kurume 830, Japan.

Reprint requests to Dr. K. Kosaka, Sasakawa Research Building, Raj-Pracha-Samasai Institute, Leprosy Division, Soi Bamrasnaradoon Hospital, Tiwanond Road, Nonthaburi 11000, Thailand.

MATERIALS AND METHODS

Nude mice. Six-week-old, BALB/c, AJcl-nu female mice were obtained from CLEA, Japan, Inc. During the experiment, the mice were maintained in plastic isolators.

M. leprae. The *M. leprae* Thai-53 strain ([6]) was originally derived from an untreated lepromatous leprosy patient in Thailand and subsequently passaged four and six times in nude mouse foot pads. The fifth and seventh passages were used as the material for the experiments on the effects of serum and of skim milk, respectively, on the viability of *M. leprae* during lyophilization and storage.

***M. leprae* suspension.** Infected mouse foot pads 1 year after inoculation were minced with scissors, ground in a mortar, and then suspended in sterile distilled water. After centrifugation at 160 × *g* × 5 min, the supernatant was treated with 1% NaOH at the final concentration for 5 min at room temperature, and then centrifuged at 1500 × *g* × 20 min. The pellet was resuspended in phosphate buffered saline (PBS), pH 6.8, and then centrifuged at 1500 × *g* × 20 min. Thereafter, the pellet was washed twice with sterile distilled water by repeated centrifugation at 1500 × *g* × 20 min. According to the experimental design, the final bacillary pellet thus obtained was resuspended in sterile distilled water or in water containing 10% fetal calf serum (FCS) at the final concentration or in 10% skim milk-water. The dried skim milk (Bact® skim milk dehydrate; Difco, Detroit, Michigan, U.S.A.) was dissolved in distilled water 10% (w/v), and sterilized by autoclaving for 15 min at 121°C. The number of acid-fast bacilli (AFB) in the suspensions was counted just before inoculation by the method of Shepard and McRae ([15]).

Lyophilization procedure. Ampules containing 0.2 ml of bacillary suspension with 1.8×10^8 AFB/ml in water and serum-wa-

ter and with 9.0×10^8 AFB/ml in skim milk-water, respectively, were frozen at −60°C with dry ice-alcohol and then lyophilized for 4 to 16 hr until completely dry in a Freezvac-4c (Tohzai-tsusho Co., Japan) lyophilizer.

Maintenance of lyophilized ampules. After lyophilization, all ampules were stored in a refrigerator at 4°C until used. The experiment on the effect of water and serum-water was carried out separately at a time different from that of the skim milk-water experiment.

Estimation of viability of lyophilized *M. leprae*. An inoculum of the samples from nude mouse foot pads was used for estimating the number of *M. leprae* viable cells. The dried bacillary suspension in a lyophilized ampule was dissolved in 1 ml of distilled water (the number of bacilli was diluted five times and is referred to as 10^0 in the tables), and then diluted tenfold with PBS. An inoculum of 0.05 ml of each dilution was injected into the nude mouse foot pads. One year after inoculation, the number of AFB in the nude mouse foot pads was counted ([15]). As a control, the bacillary suspension before lyophilization was used for bacillary counting and for estimating viability. The size of the foot pad swelling at harvest time was not measured, and the morphological index (MI) of the bacilli also was not examined.

RESULTS

Lyophilization of *M. leprae* suspended in water with and without serum. The viable cells of *M. leprae* suspended in water with and without 10% FCS just after lyophilization and 4 years after preservation at 4°C are summarized in Table 1. It is clear that the number of viable *M. leprae* was reduced to approximately 10^{-2} to 10^{-3} of the starting material during lyophilization. The bacilli suspended in a water solution were slightly more stable than those in the serum solution during lyophilization, but the differences are not statistically significant.

On the other hand, the results obtained in the measurement of viable *M. leprae* after storage of the lyophilized samples indicate that the viability of *M. leprae* definitely was maintained for 4 years at 4°C. Comparing the viability of bacilli in distilled water with FCS to that of bacilli in water without serum, it clearly was demonstrated that *M. leprae* suspended in a solution without serum were much more labile than those in a serum solution stored for 4 years at 4°C. The lyophilized ampule with serum, containing 1.8×10^5 bacilli as an inoculum, yielded one thousand times more bacilli at harvest [$2.9 (\pm 0.36) \times 10^8$] compared with that of bacilli without serum [$2.9 (\pm 1.35) \times 10^5$]. On the other hand, no increase and an approximate tenfold increase were observed in the ampules with serum and without serum containing 1.8×10^4 and 1.8×10^3, respectively (Table 1).

Lyophilization of *M. leprae* suspended in skim milk-water. In lyophilization of *M. leprae* suspended in a solution of 10% skim milk-water, it is noted (Table 2) that the number of viable cells of *M. leprae* was reduced slightly during lyophilization, and that the viability of the lyophilized bacilli was comparable to that of the initial samples after 2 years' storage at 4°C.

DISCUSSION

Until 1969, *M. lepraemurium* had been maintained by animal passage from mouse to mouse or rat to rat because of its being an obligate intracellular mycobacterium. One of us ([8]) started the experiments for investigating the preservation of *M. lepraemurium* by means of lyophilization in 1951. *In vitro* cultivation of *M. lepraemurium* on a solid medium and in a liquid medium had been achieved independently by Ogawa and Motomura in 1969 ([10]) and by Nakamura in 1972 ([7]), respectively. Since then, the *M. lepraemurium* strains have been maintained by the *in vitro* cultivation method. Although recently cryopreservation has been applied to *M. leprae* ([1, 13]), strains of *M. leprae* still have been maintained by animal passage in most laboratories because of the lack of success with *in vitro* cultivation. To avoid possible phenotypic changes in *M. leprae* during animal passage, the authors considered maintaining the *M. leprae* strains *in vitro* by means of lyophilization because of the results obtained from the experiment on *M. lepraemurium*. Although the two experiments mentioned above were carried out independently, the results obtained in a series of these experiments generally indicate that: a) The number of viable *M. leprae* was more or less reduced during lyophilization;

TABLE 1. *Effect of lyophilization on viability of* M. leprae *suspended in water with and without 10% fetal calf serum.*[a]

Samples tested for viability	Dilution of samples (inoculum size)			
	10^0 (1.8×10^6)	10^{-1} (1.8×10^5)	10^{-2} (1.8×10^4)	10^{-3} (1.8×10^3)
Pre-lyophilization	1.2×10^{10}	1.0×10^{10}	2.0×10^9	1.2×10^9
With 10% serum (N = 2, 2 feet)	1.4×10^{10}	1.2×10^{10}	2.5×10^9	2.1×10^9
Mean (± S.D.)[b]	$1.3 (\pm 0.1) \times 10^{10}$	$1.1 (\pm 0.1) \times 10^{10}$	$2.3 (\pm 0.25) \times 10^9$	$1.7 (\pm 0.45) \times 10^9$
Without serum (N = 2, 2 feet)	7.6×10^{10}	1.1×10^{10}	5.1×10^9	3.6×10^9
	1.1×10^{10}	1.2×10^{10}	6.2×10^9	2.2×10^9
Mean (± S.D.)	$4.4 (\pm 3.25) \times 10^{10}$	$1.2 (\pm 0.05) \times 10^{10}$	$5.7 (\pm 0.55) \times 10^9$	$2.9 (\pm 0.7) \times 10^9$
Just after lyophilization	NT[c]	3.4×10^6	$<3.0 \times 10^4$	$<3.0 \times 10^4$
		1.4×10^8	$<3.0 \times 10^4$	$<3.0 \times 10^4$
With 10% serum (N = 2, 4 feet)		1.4×10^8	6.7×10^5	$<3.0 \times 10^4$
		2.3×10^8	1.2×10^6	$<3.0 \times 10^4$
Mean (± S.D.)		$1.3 (\pm 0.56) \times 10^8$	$9.4 (\pm 2.65) \times 10^5$	
Without serum (N = 2, 4 feet)	NT	1.2×10^6	$<3.0 \times 10^4$	$<3.0 \times 10^4$
		6.7×10^7	1.3×10^6	$<3.1 \times 10^4$
		1.2×10^8	1.7×10^8	$<3.0 \times 10^4$
		1.7×10^8	2.0×10^8	$<3.0 \times 10^4$
Mean (± S.D.)		$9.0 (\pm 5.54) \times 10^7$	$1.9 (\pm 0.15) \times 10^8$	
4 years after lyophilization	NT	3.9×10^8	$<3.0 \times 10^4$	3.2×10^4
		1.2×10^8	$<3.0 \times 10^4$	$<3.0 \times 10^4$
With 10% serum (N = 2, 4 feet)		7.3×10^7	$<3.0 \times 10^4$	$<3.0 \times 10^4$
		5.8×10^8	$<3.0 \times 10^4$	$<3.0 \times 10^4$
Mean (± S.D.)		$2.9 (\pm 0.36) \times 10^8$		
Without serum (N = 2, 4 feet)	NT	1.5×10^5	1.7×10^5	4.4×10^4
		4.2×10^5	4.0×10^5	3.2×10^4
Mean (± S.D.)[d]		$2.9 (\pm 1.35) \times 10^5$	$2.9 (\pm 1.15) \times 10^5$	$3.8 (\pm 0.60) \times 10^4$
		3.0×10^4	$<3.0 \times 10^4$	$<3.0 \times 10^4$
		$<3.0 \times 10^4$	$<3.0 \times 10^4$	3.6×10^4

[a] Number of AFB/foot pad 1 year after inoculation; N = number of mice inoculated in each group; $<3.0 \times 10^4$ means no detection of AFB under light microscopic observation.
[b] S.D. = Standard deviation of the number of cells counted.
[c] NT = Not tested.
[d] Mean ± S.D. of positive harvests only.

a significant reduction was observed when water and serum-water were used as preservatives and a slight reduction was detected when a solution of 10% skim milk-water was employed. Therefore, the viability of *M. leprae* is remarkably influenced by the composition of the solution in which the *M. leprae* cells are suspended. b) It might be possible to preserve *M. leprae in vitro* at 4°C for a long time by means of lyophilization.

The main reason for the reduction of the number of viable cells during lyophilization might be the pre-freezing with dry ice-alcohol before drying. Similar findings have been reported previously ([5, 12]). Shepard and McRae ([14]) earlier reported that *M. leprae* in a piece of patient's skin lost viability when frozen quickly. Colston and Hilson ([1]) have reported that the loss of *M. leprae* viability was associated with the freezing process rather than with storage or thawing, and that slow freezing was less detrimental than quick freezing in liquid nitrogen. Rees ([13]) indicated that *M. leprae* could be preserved in a 10% (v/v) DMSO solution over the critical cooling range of −15°C to −60°C and the cooling rate should be < 1°C/min. Kvach, *et al.* ([3]), measuring the ATP content of purified *M. leprae,* also found a significant reduction of ATP levels after repeated freezing-thawing cycles. As far as the suspending solution of the cells for cryopreservation of *M. leprae* is concerned, Lee and Colston ([4])

TABLE 2. *Effect of lyophilization on viability of* M. leprae *suspended in water containing 10% skim milk.*[a]

Samples tested for viability	Dilution of samples (inoculum size)		
	10^{0}	10^{-1}	10^{-2}
	(9.0×10^{6})	(9.0×10^{5})	(9.0×10^{4})
Pre-lyophilization (N = 3, 6 feet)	2.2×10^{9}	1.3×10^{9}	1.8×10^{8}
	2.2×10^{9}	8.0×10^{8}	1.8×10^{8}
	1.7×10^{9}	1.7×10^{9}	1.6×10^{8}
	3.9×10^{9}	4.2×10^{9}	2.1×10^{8}
	8.4×10^{8}	died, 10 m.	6.5×10^{8}
	1.0×10^{9}		5.8×10^{8}
Mean (± S.D.)	$2.0 (\pm 0.78) \times 10^{9}$	$2.0 (\pm 0.95) \times 10^{9}$	$3.3 (\pm 2.03) \times 10^{8}$
Just after lyophilization (N = 3, 6 feet)	4.1×10^{8}	1.6×10^{7}	5.9×10^{6}
	8.6×10^{8}	1.7×10^{7}	2.8×10^{6}
	1.7×10^{9}	3.6×10^{8}	1.9×10^{7}
	2.1×10^{9}	4.4×10^{8}	1.9×10^{7}
	1.0×10^{9}	9.5×10^{8}	2.9×10^{7}
	2.4×10^{9}	7.4×10^{8}	2.1×10^{7}
Mean (± S.D.)	$1.4 (\pm 0.55) \times 10^{9}$	$4.2 (\pm 3.38) \times 10^{8}$	$1.6 (\pm 0.86) \times 10^{7}$
2 years after lyophilization (N = 2, 4 feet)	1.7×10^{9}	1.8×10^{9}	3.3×10^{8}
	1.2×10^{9}	8.2×10^{8}	2.4×10^{8}
Mean (± S.D.)	$1.5 (\pm 0.25) \times 10^{9}$		
	1.0×10^{8} [b]	5.8×10^{8}	2.1×10^{8}
	9.5×10^{7}	2.7×10^{8}	7.7×10^{7}
Mean (± S.D.)	$9.8 (\pm 0.25) \times 10^{7}$	$8.6 (\pm 4.37) \times 10^{8}$	$2.1 (\pm 0.71) \times 10^{8}$

[a] Number of AFB/foot pad 1 year after inoculation; N = number of mice inoculated in each group.
[b] Mice were killed 8 months after inoculation.

have reported that the marked difference in the decay rate of *M. leprae* measured with the intracellular contents of ATP depended on the composition of the solution. Recently, Portaels, *et al.* ([11]) have reported on a study of the effects of freezing and thawing on the viability of *M. leprae* in which they demonstrated that the loss and reduction of *M. leprae* viability were greatly influenced by different frequencies of freezing-thawing cycles, as quantitatively determined by mouse foot pad titration. They also indicated that the reason for the loss and reduction of viability of *M. leprae* during these processes might be due to the fragility of *M. leprae* itself as observed by electron microscopy. The electron microscopy study showed that the main ultrastructural alterations induced in *M. leprae* by freezing and thawing was the change in the cell membrane profile from asymmetric to symmetric.

From our results presented here it also could be presumed that the *M. leprae* cells might be remarkably fragile because the viability of *M. leprae* is clearly affected by freezing and drying and by the presence or absence of some kind of cell-protecting agents (such as serum or skim milk) in the solution in which the cells are suspended. David and Rastogi's report ([2]) also supports this speculation on the fragility of *M. leprae* cells from another viewpoint, i.e., they demonstrated that, as judged from the electron microscopic observations, the purified bacterial cells were of different fragility in the following increasing order: *M. tuberculosis, M. smegmatis,* and *M. leprae.*

As mentioned above, the two experiments presented here were carried out separately and at different times. Therefore, the inoculum sizes and viabilities of *M. leprae* were not identical, resulting in the discrepant data obtained. However, the evidence provided by these experiments, of 2 and 4 years' observation, that skim milk was much more effective than serum for protecting the viability of *M. leprae* in the course of lyophilization and storage surely might contribute to the preservation of *M. leprae in vitro.*

Future comparative experiments of the effects of skim milk, serum, and cryopreservation on the preservation of the viability of *M. leprae in vitro* using the same starting material should be carried out. In addition, the morphological changes in the *M. leprae* cells suspended in water, serum-water, and skim milk-water after freezing and thawing should be compared with each other by electron microscopy.

SUMMARY

Although the viability of *Mycobacterium leprae* suspended in distilled water with or without 10% fetal calf serum was reduced approximately 10^{-2} to 10^{-4} from that of the starting material during the process of lyophilization, bacilli capable of multiplication in nude mouse foot pads were found in the lyophilized samples stored for 4 years at 4°C. The multiplication rate of the lyophilized bacilli which were suspended in 10% serum-water was much higher than that of the bacilli suspended in water only. On the other hand, no reduction of the viability of *M. leprae* suspended in 10% skim milk-water was demonstrated during the process of lyophilization as well as storage for 2 years at 4°C. From the results obtained here, it could be suggested that *M. leprae* might be preserved *in vitro* by means of lyophilization. In particular, the viability of lyophilized *M. leprae* was extremely stable during cryopreservation when the bacilli were suspended in 10% skim milk-water. Therefore, the composition of the solution for suspending the bacilli is definitely critical for the maintenance of *M. leprae* viability by means of lyophilization.

RESUMEN

Aunque la viabilidad del *Mycobacterium leprae* suspendido en agua destilada con o sin 10% de suero fetal de ternera se redujo durante el proceso de liofilización aproximadamente 10^{-2} a 10^{-4} en relación al material inicial, todavía se encontraron bacilos capaces de multiplicarse en la almohadilla plantar del ratón en las muestras liofilizadas mantenidas durante 4 años a 4°C. La velocidad de multiplicación de los bacilos liofilizados suspendidos en agua con 10% de suero fue mucho mayor que aquella de los bacilos suspendidos sólo en agua. Por otro lado, no se observó reducción en la viabilidad del *M. leprae* suspendido en agua con 10% de leche descremada durante el proceso de liofilización después de su almacenamiento durante 2 años a 4°C. Los resultados sugieren que el *M. leprae* podría preservarse *in vitro* por medio de la liofilización. En particular, la viabilidad del *M. leprae* liofilizado fue extremadamente estable durante la criopreservación cuando los bacilos estuvieron suspendidos en agua con 10% de leche descremada. Es claro que la composición de la solución para suspender al bacilo es crítica para mantener la viabilidad del *M. leprae* por medio de la liofilización.

RÉSUMÉ

Bien que la viabilité de *Mycobacterium leprae* mis en suspension dans de l'eau distillée avec ou sans 10% de serum de foetus de veau était réduite à environ 10^{-2} à 10^{-4} par rapport au matériel de départ durant le processus de lyophilisation, des bacilles capables de se multiplier dans le coussinet plantaire de la souris nue ont été trouvés dans des lyophilisats stockés pendant 4 ans à 4°C. Le taux de multiplication des bacilles lyophilisés qui étaient suspendus dans un mélange d'eau et 10% de serum était beaucoup plus élevé que celui des bacilles suspendus dans de l'eau seule. D'autre part, aucune réduction de la viabilité de *M. leprae* mis en suspension dans un mélange d'eau et de 10% de lait écrémé n'a été démontrée ni durant le processus de lyophilisation ni après stockage pendant 2 ans à 4°C. Ces résultats pourraient suggérer que *M. leprae* pourrait être conservé *in vitro* par lyophilisation. En particulier, la viabilité des *M. leprae* lyophilisés était extrêmement stable durant la cryopréservation lorsque les bacilles étaient mis en suspension dans un mélange d'eau et de 10% de lait écrémé. La composition de la suspension dans laquelle les bacilles sont mis en suspension est donc cruciale pour le maintien de la viabilité de *M. leprae* par lyophilisation.

Acknowledgment. The authors are grateful to the Sasakawa Memorial Health Foundation for its financial support, and to the staff of the Research Institute for Microbial Disease, Osaka University, for help with the lyophilization procedure.

REFERENCES

1. Colston, M. J. and Hilson, R. F. The effect of freezing and storage in liquid nitrogen on the viability and growth of *Mycobacterium leprae.* J. Med. Microbiol. **12** (1979) 137–142.
2. David, H. L. and Rastogi, N. Partial characterization of the cell walls of *Mycobacterium leprae.* Curr. Microbiol. **9** (1983) 269–274.
3. Kvach, J. T., Neubert, T. A., Palomino, J. C. and Heine, H. S. Adenosine triphosphate content of *Mycobacterium leprae* isolated from armadillo tissue by percoll buoyant density centrifugation. Int. J. Lepr. **54** (1986) 1–10.
4. Lee, Y. N. and Colston, M. J. Measurement of ATP generation and decay in *Mycobacterium leprae in vitro.* J. Gen. Microbiol. **131** (1985) 3331–3337.

5. LEVY, L. The effect of freezing and storage at −60°C on the viability of *Mycobacterium leprae*. Cryobiology **8** (1971) 574–576.
6. MATSUOKA, M., KOHSAKA, K. and DAYANGHIRANG, J. A. Characterization of *Mycobacterium leprae* Thai-53 strain. (Abstract) Int. J. Lepr. **60** (1992) 722.
7. NAKAMURA, M. Multiplication of *Mycobacterium lepraemurium* in cell-free medium containing α-ketoglutaric acid and cytochrome c. J. Gen. Microbiol. **73** (1972) 193–195.
8. NAKAMURA, M. Survival of *Mycobacterium lepraemurium in vitro* for 30 years by lyophilization. Int. J. Lepr. **53** (1985) 52–55.
9. NAKAMURA, M. and KOHSAKA, K. Effect of lyophilization on viability of *Mycobacterium leprae* multiplied in nude mouse. Acta Leprol. **7** (1989) 36–38.
10. OGAWA, T. and MOTOMURA, K. Studies on *Mycobacterium lepraemurium* 1. Attempts to cultivate *M. lepraemurium*. Lepro **38** (1969) 246–254.
11. PORTAELS, F., FISSETTE, K., RIDDER, K. D., MACEDO, P. M., MUYNCK, A. D. and SILVA, M. M. T. Effects of freezing and thawing on the viability and the ultrastructure of *in vivo* grown mycobacteria. Int. J. Lepr. **56** (1988) 580–587.
12. PRABHAKARAN, K., HARRIS, E. B. and KIRCHHEIMER, W. K. Survival of *Mycobacterium leprae* in tissue kept frozen at −80°C. Microbios. Lett. **1** (1976) 193–195.
13. REES, R. J. W. The microbiology of leprosy. In: *Leprosy*. Hastings, R. C., ed. Edinburgh: Churchill Livingstone, 1985, p. 39.
14. SHEPARD, C. C. and MCRAE, D. H. *Mycobacterium leprae*: viability at 0°C, 30°C and during freezing. Int. J. Lepr. **33** (1965) 316–323.
15. SHEPARD, C. C. and MCRAE, D. H. A method for counting acid-fast bacteria. Int. J. Lepr. **36** (1968) 78–82.