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Survival of *Mycobacterium lepraemurium in vitro* for Thirty Years by Lyophilization[1]

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It was previously demonstrated that the virulence of *Mycobacterium lepraemurium* could be maintained *in vitro* for ten years ([3]) and 16 years ([4]) by means of lyophilization. The present paper describes additional studies indicating that the infectious activity of the bacilli could be preserved for 30 years by lyophilization, and that the solution for suspending the bacilli is definitely critical for maintenance of the activity.

MATERIALS AND METHODS

M. lepraemurium. The Fukuoka strain (R-38) of *M. lepraemurium,* originally isolated by Urabe and Yoshimura ([6]) in 1938 in Fukuoka, Japan, was used. The lyophilized material was from the same sample as described in the previous reports ([3,4]). A subcutaneous leproma was homogenized in a sterile mortar and suspended in sterile physiological saline to make a 20% suspension. The suspension was divided into two aliquots and these were added, respectively, to equal volumes of physiological saline and 10% inactivated bovine serum-water. Thus, both the bacillary suspension and the media for dilution were diluted to twice the original volume.

Lyophilization procedure. One ml of each sample was added to individual ampules and immediately frozen in a dry ice-acetone mixture. The samples were then dried for approximately 5 hr using a rotary pump, sealed, and stored in a refrigerator at 4°C. At that time, the degree of vacuum was approximately 10^{-3} mm Hg, as estimated by the extinguishing of light in a Geissler's tube.

Virulence test. The ampules that had been stored in a refrigerator for 30 years after lyophilization were opened, and the dried sample in each ampule was suspended in 2 ml of sterile distilled water. Three-tenths ml of each suspension was inoculated subcutaneously into normal mice (C3H strain). A small aliquot was saved for electron microscopy; electron micrographs shadowed with chrome were taken with a type HV 12-AS electron microscope (Hitachi). The mice were sacrificed at appropriate periods after inoculation, and the persisting virulence of the lyophilized bacilli was determined by bacteriological observations of smears from the subcutaneous tissues of the mice.

RESULTS

Bacterial morphology. Lyophilization of *M. lepraemurium* was performed on 4 February 1953. The ampules, stored in a refrigerator, were opened on 7 February 1983. Morphological characteristics of the lyophilized bacilli observed via electron microscopy are shown in Figure 1. Among the bacilli in saline suspension, very few solid forms having a typical band structure were found (a, b), while all of the bacilli in 10% bovine serum-water suspension were non-solid (c, d).

Determination of virulence. Bacteriological examinations of tissue smears were performed 259 days after inoculation. The results shown in The Table indicate that the virulence of the bacilli lyophilized in 10% bovine serum-water was maintained for 30 years, whereas it appeared that the infectious ability of the bacilli in saline had been completely lost. However, a few bacilli were found in the smear prepared from the subcutaneous tissue inoculated with saline materials as shown in Figure 2. In order to confirm the viability of these bacilli, the subcutaneous tissue homogenate was inoculated into healthy C3H mice. After 118 days, autopsies were performed, but the results could not unequivocally confirm virulence of the saline sample. In contrast, a homogenate prepared from the subcutaneous tissue of the mice inoculated with the 10% bovine serum-water sample showed heavy growth of *M. lepraemurium.* This ho-

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THE TABLE. *Determination of virulence of lyophilized* M. lepraemurium *after 30 years' storage.*

Medium for lyophilization	First generation of mice					Second passage in mice		
	No. tested	Bacteriologic results				No. tested	Bacteriologic results in smears of inoculated tissues	Determination of virulence
		Inoculated site	Lung	Liver	Spleen			
Physiological saline	1	+[a]	−[b]	−	−	1	+	−
	2	+	−	−	−	2	+	−
	3	+	−	−	−	3	+	−
	4	+	−	−	−	4	+	−
	5	+	−	−	−	5	+	−
	6	+	−	−	−	6	+	−
	7	+	−	−	−			
	8	+	−	−	−			
	9	+	−	−	−			
	10	+	−	−	−			
	11	+	−	−	−			
10% bovine serum-water	1	++++[c]	+	+	+	1	++++	+
	2	++++	+	+	+	2	++++	+
	3	++++	+	+	+	3	++++	+
	4	++++	+	+	+	4	++++	+
	5	++++	+	+	+			
	6	++++	+	+	+			
	7	++++	+	+	+			
	8	++++	+	+	+			
	9	++++	+	+	+			
	10	++++	+	+	+			
	11	++++	+	+	+			

[a] + = a few bacilli per microscopic field.
[b] − = no bacilli in whole microscopic field.
[c] ++++ = numerous bacilli with globi per microscopic field.

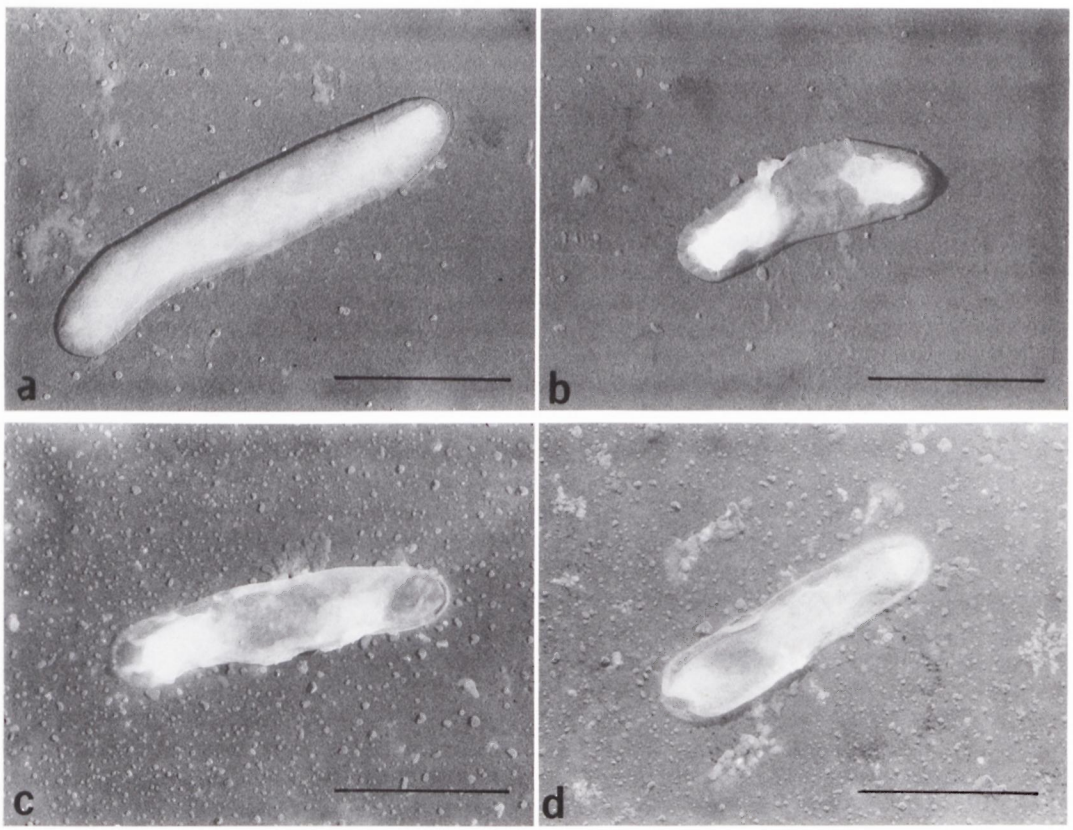

FIG. 1. *M. lepraemurium*, 30 years after lyophilization, suspended in saline (a, b) and in 10% bovine serum-water (c, d). Chrome shadowed. Bar represents 1 μm.

mogenate produced a typical leproma in secondarily inoculated C3H mice.

DISCUSSION

It is well known that lyophilization is quite useful for maintaining viability of bacteria. However, there are no detailed data indicating maintenance of viable cells of bacteria for more than 20 years *in vitro*. The author has previously demonstrated that the virulence of *M. lepraemurium* could be maintained *in vitro* for ten years ([3]) and 16

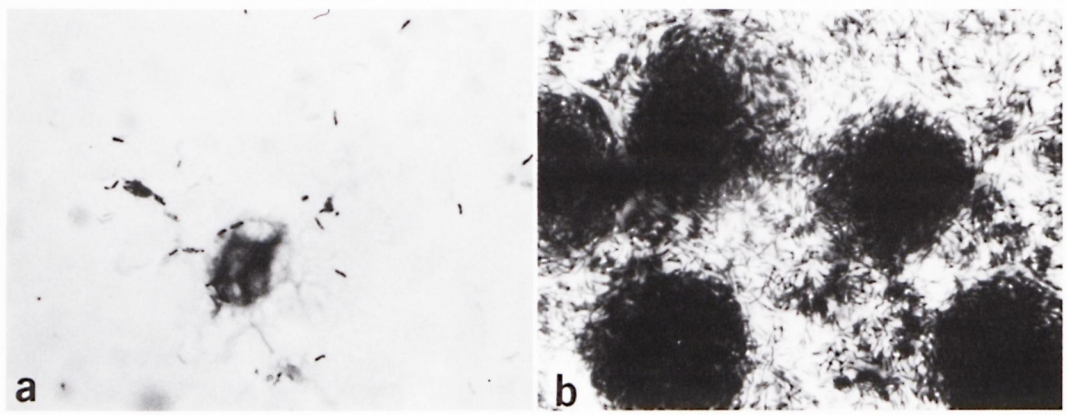

FIG. 2. *M. lepraemurium* in subcutaneous tissues of C3H mice inoculated with bacilli lyophilized in saline (a) and in 10% bovine serum-water (b).

years ([4]) by lyophilization. In the report on the ten years' maintenance, four suspending solutions, i.e., physiological saline, 2% glycerine water, 5% bovine serum-water, and Kirchner medium, were employed. All of the solutions maintained virulence, but the physiological saline and the bovine serum-water were superior to 2% glycerine water and to the Kirchner medium. The report on 16 years' storage suggested that bovine serum-water was slightly more suitable than physiological saline.

The present paper demonstrates that the virulence of *M. lepraemurium in vitro* for 30 years was completely maintained when the bacilli were suspended in bovine serum-water, whereas virtually a complete loss was observed when the bacilli were suspended in saline. Electron micrographs revealed a low percentage of solid cells in the suspension with saline and no solid forms in the bovine serum-water. This suggests that there is no significant relationship between the morphological findings and the viability of *M. lepraemurium* in the case of lyophilization.

From the results obtained here, it could be presumed that the virulence of *M. leprae* might be maintained *in vitro* by lyophilization. At present, it is possible for *M. leprae* to be transmitted and maintained from mouse to mouse ([2, 5]) or from armadillo to armadillo ([1]). However, the possibility of phenotypic changes in *M. leprae* during these animal passages might be considerable. Therefore, the lyophilization procedure is recommended for avoiding such changes.

SUMMARY

It was demonstrated that the virulence of *Mycobacterium lepraemurium* could be maintained *in vitro* for 30 years when the bacilli from the infected subcutaneous mouse tissue were suspended in 10% bovine serum-water, frozen, dried, and stored in a refrigerator. However, it was noted that a complete loss of virulence occurred when the bacilli were suspended in saline. Thus, the selection of the suspending solution is of the utmost importance in maintaining bacterial virulence by lyophilization.

RESUMEN

Se demostró que la virulencia del *Mycobacterium lepraemurium* puede mantenerse *in vitro* cuando menos por 30 años cuando los bacilos (aislados de tejido subcutáneo de ratón) se suspenden en suero bovino al 10% en agua, se liofilizan y se mantienen en refrigeración. Sin embargo, se encontró una pérdida total de la virulencia cuando los bacilos se suspendieron en salina. Así, la composición de la solución de suspensión es de gran importancia cuando se requiere mantener la virulencia micobacteriana después de la liofilización.

RÉSUMÉ

On démontre dans cette étude que la virulence de *Mycobacterium lepraemurium* pourrait être maintenue *in vitro* pendant 30 ans, lorsque les tissus sous-cutanés infectés obtenus chez la souris sont suspendus dans une solution aqueuse de sérum bovin à 10%, congelée, soumise à la dessiccation et entreposée dans un réfrigérateur. On a cependant noté une perte complète de la virulence lorsque les bacilles étaient suspendus dans une suspension saline. Il paraît dès lors extrêmement important de bien choisir la solution de suspension, si l'on veut préserver la virulence des bactéries au cours de la lyophilisation.

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