PRESERVATION OF MYC. LEPRÆMURIUM BY MEANS OF LYOPHILIZATION II REPORT

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A part of the serial experiments on the preservation of *Myc. lepraemurium* by means of lyophilization was reported in previous paper (1). The results obtained demonstrated that the infectious activity of *Mycobacterium lepraemurium* was still evident after two years and five months over of the preservation *in vitro* by lyophilization, and that this period of maintenance of infectious activity of the bacilli was longer than that of the data reported by Shitama (2) and Lew (3).

In the present paper, an additional experiment on the infectious activity of the bacilli which were preserved for five years and five months over in the form of lyophilization is reported.

MATERIALS AND METHODS

Bacterial strain and procedure in general was described in the previous report (1). Each lyophilized ample which was stored in the refrigerator for five years and five months over was opened, and added one ml of distilled water. 0.5 ml of each resuspended material was inoculated subcutaneously at the groin site of normal rats, these rats were killed after 170 days of inoculation of the materials, and leprosy changes at the site of inoculation and in the organs and glands of the inoculated rats were bacteriologically and histologically examined. Furthermore, the forms and staining properties of the bacilli were observed promptly after resuspension, by Ziehl-Neelsen staining and the electron micrographs.

RESULTS

Conditions of lyophilization. The conditions of dried and melted bacterial suspensions in the case of four different solutions, and stainable properties of the bacilli after dissolution are summerized in Table 1.

The finding of the lyophilization in the case of the saline solution and serum water were pulverulently, while in the case of Kirchner's medium was somewhat moist, and of glycerine water was like as wheat-gluten, that is, remained moist. When these dried materials were resuspended in distilled water, however, homogeneous suspensions were

obtained. The forms and staining properties of the bacilli in the resuspended tubes were a few differences indicated in Table 1, among four kinds of the solution.

The properties of the bacilli were sustained as similarly as the original bacterial form, and were ungranuleous, straight, and good stainable, however, the stainability of the bacilli in the case of glycerine solution was somewhat weaker than that of the original. The findings under electron microscope were also very likely as that under light microscope mentioned above. However, as observed in photo 4, a change of the bacterial cell suggesting that autolysis of the bacterial surface occurred, was recognized in the case of glycerine water.

TABLE 1

The relationship between conditions of lyophilization and properties of the bacilli

Medium	Time of hour of lyophilization	Drying condition	Dissolution	Staining quality
Saline solution	2	pulverulent	homogeneous	Good, ungranuleous but with a few big brown bodies, typical form
10% Serum water	2	pulverulent	homogeneous	typical form with good stainable properties
4% Glycerine water	3	wheat-gluten like	homogeneous	typical form with bad stainable properties
Kirchner's medium containing 10% serum	3	somewhat moist	homogeneous	Good, with a few big brown granules

Photo 1. Myc. lepraemurium suspended in the saline solution.

Photo 2. Myc. lepraemurium suspended in the serum water.

Photo 3. Myc. lepraemurium suspended in Kirchner medium.

Photo 4. *Myc. lepraemurium* suspended in the glycerine water.

TABLE 2

Determination of infectious activity of lyophilized bacilli by animal experiments

Time of storage at 4C	Date of inocul. into rats	Medium	Days of Survival after infect.	Number of rats	Bacteriological and histological results in infected rats					Infectious activity
					Site of inoculation	Groinal gland	Iliac gland	Axillary gland	Organs	
5 yrs, 5 ms, and 5 days	1, Oct., 1956	Saline	171	3	- (-)	ooo(###)	o(-)	o(-)	(-)	+
					## (∞)	ooo(###)	oo(+)	o(+)	(-)	
					+ (+)	oo(∞)	oo(+)	ooo(+)	(-)	
		Glycerine water	170	2	- (-)	o(-)	o(-)	oo(-)	(-)	-
					+ (-)	o(-)	o(-)	oo(-)	(-)	
		Serum water	141	2	+ (###)	oo(+)	o(+)	oo(-)	(-)	+
			170		## (###)	oo(###)	o(-)	oo(-)	(-)	
		Kirchner's medium	170	3	- (+)	o(###)	o(-)	oo(-)	(-)	+
					## (∞)	oo(+)	o(-)	oo(+)	(-)	
					- (-)	o(++)	o(-)	oo(+)	(-)	

Animal experiments. The leprosy infiltration was produced at the site of inoculation of the materials in the saline, serum, and Kirchner's solution, or even if the infiltration had not been recognized, the great amount of the bacilli and the leprosy cells which produced by the bacilli were observed in the stained preparation of the regional glands. On the contrary, the leprosy changes at the site of inoculation of the material in the glycerine water were not determined, though the leprosy like infiltration was found in one case of the rats tested. These results were shown in Table 2.

It is obvious, therefore, that the infectious activities of the bacilli lyophilized in the suspensions with saline solution, serum water, and Kirchner's medium still remained after five years and five months of the storage *in vitro*, while the activity of the bacilli in the case of the suspension with the glycerine water was already lost.

DISCUSSION

As discussed in the previous report (1), the research on the preservation of *Mycobacterium lepraemurium in vitro*, for example by means of lyophilization, might be very important and significant, in order to learn if the bacilli could be preserved *in vitro* for a long period or not since the cultivation method of the bacilli has not yet been succeeded and then they have been maintained only by inoculating into rats or mice up to the present day, and in order to study an approach to the clarification of the biological and chemical properties of the bacilli concerning the activity under various influences of drying and freezing. Yaoi, Tagaya, and Yoshino (4) reported that the activity of *H. pertussis* I-phase suspended in water containing 50% inactivated serum by means of lyophilization was maintained for a year and four months, and that, the kinds of the suspended media influenced on the activity of the bacilli during preservation *in vitro* by drying and freezing. Pirtle (5) reported also, that the ability of *Dipl. pneumoniae* Type 1 to produce capsules and its virulence for mice decrease rapidly when a virulent culture is serially transferred in blood medium or when lyophilized, and then the infected mice were putted into 200 by 25mm Pyrex tubes which were fitted with rubber stoppers, and placed at -20 C. The fact that the numbers of viable bacilli of BCG decrease for the preservation by lyophilization is very known. It may be presumed, therefore, that the metabolism of the bacilli has continued at low grade even during drying and freezing of the bacilli. Though it may be thought that the stability of *Myc. lepraemurium* is not as labile as that of *H. pertussis* I. phase and *Dipl. pneumoniae*, it will be not able to declare that a sort of the medium in which *Myc. lepraemurium* is suspended does not influence upon the activity of the bacilli during preservation for a long period.

In this paper, the data obtained demonstrate that the saline solution is most suitable

for maintenance of the infectious activity of the bacilli by lyophilization among the solutions tested which suspended the material, and that the activity of the bacilli in the case of glycerine water has lost. In the previous report on the preservation of the bacilli for 2 years and five months also, an analogous tendency was observed, that is, the infectious ability of the bacilli in the glycerine water was worst, and the proof of the infectious activity of the bacilli was barely determined after the inoculation to the second generation into rats. The factors that influenced on the activity of the bacilli seems to be attributed to lack of dryness of the solution suspended the materials during storage.

It may be not decided at present whether the change of the bacterial form in the glycerine water observed by electron microscope is connected with the activity of the bacilli or not.

SUMMARY

Myc. lepraemurium lyophilized in the saline solution, serum water, Kirchner's medium, and glycerine water were stored in a refrigerator for about five years and five months and then the infectious activity of the bacilli were determined by inoculation of the materials into normal white rats. The results obtained indicated that the infectious activity of the bacilli in these solutions with the exception of glycerine water had still maintained.

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