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MYCOBACTERIUM LEPRAE: VIABILITY AT 0°C, 31°C, AND DURING FREEZING[1]

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The methods for maintaining the viability of *Mycobacterium leprae in vitro* are of obvious importance to those working in experimental leprosy. The problem is especially critical in this disease because many research laboratories, including ours, are located at a distance from important endemic areas.

Earlier experience with foot pad inoculation of mice (4) had shown that *M. leprae* in skin biopsies, or in centrifuged nasal washings in Hanks' balanced salt solution (BSS) containing 0.1 per cent bovine albumin (BA), did not lose viability quickly at 0°C (wet ice in a thermos bottle). In the very first isolation attempt in mouse foot pads in this laboratory there was a shipment delay that resulted in a lapse of about 24 hours from collection of bacilli to inoculation of mice. Yet the isolation was successful. In a later experience involving a shipment delay with material sent from Manila, leprosy bacilli survived 100 hours at 0°C without apparent deterioration. Also infectious foot pad suspensions have been shipped successfully at 0°C by air to English and Belgian laboratories. One shipment was delayed en route, and was received after 7 days; no ice remained, but viable bacilli were found (2).

MATERIALS AND METHODS

The methods have been published for the inoculation of mice by the foot pad route and observing the resultant infection (4, 5), for counting acid-fast bacteria (AFB) (6), and for estimating the proportion of solidly staining bacilli (8). Our general approach was to judge viability of the bacilli inoculated by the time taken for them to grow to countable concentrations. A group of mice was inoculated in the foot pads with a suspension of bacilli, and at one-month intervals a mouse was taken and histologic sections prepared from the inoculated foot. After inoculation of 5×10^3 AFB or less, the bacilli are difficult to find in the sections, until they have multiplied *in vivo*. Hence, when significant numbers of AFB appeared, it was taken as a signal that mycobacterial multiplication had taken place, a batch of mice was killed, and the AFB counted in suspensions made from the infected foot pad tissues. The results are expressed as the number of AFB harvested per foot pad and also as the "generation time," or average rate of multiplication between inoculation and harvest. The "generation time," being a single number, permits easy comparison of the results. When no AFB were seen during the counting procedure, the number corresponding to one AFB was calculated, and the harvest expressed as less than this number. As previously described, all suspensions were cultured on Loewenstein-Jensen medium, 25 per cent blood-agar, and liquid and solid 7H9 media. They were incubated at 33°C for 4 months and inspected at predetermined intervals. No cultivable mycobacteria were isolated.

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TABLE 1.—*Survival of* M. leprae *at 0°C.*[a]

Material inoculated	Time stored at 0°C. (days)	Inoculation		Mycobacterial findings in sections (month)[d]			Harvest[e]		Generation time, G[f] days
		AFB[b]	S[c]/(N+S) (%)	0	+	++, +++, and ++++	Time (days)	AFB	
1. Mouse passage (fifth). Usual tissue suspension in BSS + 0.1% BA.	0	5.0×10^3	82	1-3		4	153	4.9×10^5	23.1
	4	Do.	50	1-3		4	147	1.7×10^5	29.0
	7	Do.	58	1,3-5	2	6	234	1.9×10^6	27.3
	14	Do.	46	1-3,6		4	162	2.1×10^5	24.9
	29	Do.	44	1-9		10	332	6.4×10^5	47.1
	54	Do.	12[g]	1-16				$<4.4 \times 10^3$	
2. Nasal washing after usual centrifugal concentration and NaOH treatment. Stored in BSS + 0.1% BA.	0	5.0×10^3	8	1,2,5	3,4	6	210	1.9×10^6	24.5
	5	Do.		1-5		6	212	5.2×10^5	31.6
	9	Do.		1-5		6	208	3.8×10^5	33.4
	20	Do.		1-6	7		253	5.1×10^5	37.8
	40	Do.		1-12			364	$<4.7 \times 10^3$	

[a] Suspensions of bacilli were diluted so that 0.03 ml. inoculum would contain 5×10^3 AFB and stored in tubes in thermos bottles containing crushed ice.

[b] Number of bacilli in inoculum based on original count before storage.

[c] S/(N + S)=proportion of solidly staining bacilli in inoculum (8).

[d] At monthly intervals mice were sacrificed, histologic sections of the inoculated feet were examined, and the size of the area containing AFB or associated infiltrate was graded. The entry in the first line signifies that no AFB were seen in the sections from months 1-3, and that significant numbers of AFB were present in those from month 4. 0 = no AFB; + pertains to less than 30 cells; ++, +++, and ++++ to more extensive involvement.

[e] At the time after inoculation indicated, four mice were sacrificed, ttheir inoculated foot pads pooled and minced, suspensions prepared in tissue disintegrator, and the AFB counted.

[f] The average rate of growth from inoculation to harvest. $G = t/\log_2 (H/F)$, where t is the time in days, H is the number of AFB harvested, and F the number of AFB inoculated.

[g] Many of the "solid" bacilli in this inoculum did not stain brightly.

RESULTS

Viability at 0°C (Table 1).—In the first experiment, carried out with a suspension of mouse passage bacilli stored in BSS with 0.1 per cent BA, there was no significant change in viability until after 14 days. With inocula stored for 0, 4, 7, and 14 days, respectively, AFB appeared in histologic sections at about the same time, and the harvests of AFB were about the same number. After 29 days' storage there was a pronounced delay in the appearance of bacilli in the sections. After 54 days' storage there was no indication of remaining viability.

The decrease in the proportion of solidly staining bacilli lagged considerably behind the loss of viability under these conditions. Other work has indicated that in the patient and in the infected animal the loss of solid staining in *M. leprae* may also lag behind the loss of viability ([8]).

The second experiment in Table 1, carried out with a suspension of *M. leprae* from the nasal washings of a patient with lepromatous disease, gave confirmatory results. After 5 and 9 days' storage there was not much change, but after 20 days there was apparently partial loss of viability. After 40 days there was complete loss of viability.

Viability at 31°C (Table 2).—A mouse passage suspension of *M. leprae* was centrifuged and resuspended in a menstruum consisting of 0.218 M sucrose, 0.00376 M KH_2PO_4, 0.0071 M K_2HPO_4, and 0.0049 M sodium glutamate (SPG) ([1]), with 1 per cent BA and 25 units penicillin/ml. The suspension was incubated in 0.2 ml. volumes in 15 mm. tubes with well-fitting screw caps at 31°C for 7 and 14 days. Smears failed to reveal contaminating nonacid-fast bacteria in any tubes. At 14 days the contents of the tubes were pooled, the tubes washed through with small volumes of distilled water, and the washings added to the pool to make it up to the volume before incubation. Preparations were then made for counts, and mice were inoculated. A set of tubes was put up similarly in medium based in 7H9 (Difco)[2] ([3]). It consisted of 6.3 volumes of 7H9 broth base, 0.7 volume Dubos oleic acid-albumin, 1 volume 5 per cent BA, 2 volumes 1 M sucrose, with penicillin added to 25 units/ml. A contaminating corynebacterium was seen in smears from all tubes, but the contents were pooled and inoculated nevertheless.

The counts of AFB on the incubated suspension were somewhat unreliable because of the presence of 0.2 M sucrose, but they showed the persistence of AFB of good staining quality. The results of the inoculation of mice proved that viability had been maintained for 14 days without much change. The harvests of Table 2 were passed into more groups of mice in the usual manner and found to behave in a manner entirely typical of *M. leprae*.

Viability losses associated with freezing and storage at −60°C (Table 3).—Storage at low temperatures has been used to preserve

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[2]. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U. S. Department of Health, Education, and Welfare.

TABLE 2.—*Survival of* M. leprae *at 31°C.*[a]

Treatment of bacillary suspension	AFB/ml	S/(N+S) (%)	Inoculation of mice: AFB inoculated	Mycobacterial findings in sections (month): 0	+	++, +++, and ++++	Harvest: Time (days)	AFB	Generation time: G	G_s[b]
I. Mouse passage (sixth). Usual tissue suspension in Hanks + 0.1% BA. Stored overnight at 0°C.	1.7×10^5	38	6.1×10^3	1-2, 4-7	3	8	285	3.6×10^6	31.0	26.9
II. Portions of I centrifuged and sediment resuspended to volume in SPG with 1% BA. and 25 u. penicillin/ml. Incubated at 31°C.										
A. 7 days	1.9×10^5	48								
B. 14 days	6.5×10^4	28	2.0×10^2	1-9		10	272	2.0×10^5	51.1	22.9
III. Same as II but resuspended to volume in 70% 7H9 with oleic acid-albumin, 0.2 M sucrose, an additional 0.5% BA, and 25u. penicillin/ml. Incubated at 31°C.										
A. 7 days	1.3×10^5	18								
B. 14 days	1.6×10^5	18	4.6×10^3	1-7		8	272	3.6×10^5	44.3	31.1

[a] A suspension of *M. leprae* treated as described on the left was inoculated into mouse foot pads. See Table 1 for definitions.
[b] $G_s = t/\log, (H/F_s)$, where F_s is the number of solidly staining AFB inoculated (8).

viruses, rickettsiae, mammalian cells, and bacteria including mycobacteria. It would be of very great help if *M. leprae* also could be preserved in this way. In the first experiment recorded in Table 3 bacilli in a piece of patient's skin lost viability when quick-frozen. In the second experiment a suspension of *M. leprae* in mouse passage was quick-frozen in 0.2 M sucrose and 0.1 per cent BA; there was severe but not complete loss of viability. In the third experiment the slow-freezing methods used for the preservation of mammalian cells were tried, and various additives were compared. The conditions of slow-freezing were those used successfully in this laboratory for the preservation of mammalian and amphibian tissue culture cells when glycerol or dimethyl sulfoxide is added. There was extensive loss of viability, least with glycerol and most with dimethyl sulfoxide.

In the fourth experiment slow-freezing was not helpful in the presence of BA in BSS (4b and 4c *vs.* 4d and 4e). Raising the concentration of BA from 0.1 to 3.5 per cent was not helpful either (4b and 4d *vs.* 4c and 4e). Sucrose (0.2 M), when added to 0.1 per cent BA in BSS, was somewhat beneficial (4d *vs.* 4f). Glycerol in a concentration of 10 per cent was distinctly favorable (4d *vs.* 4g), perhaps more so when the material was stored overnight at 4°C before being frozen (4g *vs.* 4h). The results with glycerol were better in experiment 4 than in experiment 3. In the fourth experiment mycobacteria did not appear in sections of groups b, d, and e until 7 months. Second harvests that were then carried out, on days 266 and 267, gave the following results for groups 4b through 4h, respectively: 3.1×10^6 AFB (28.6 days/generation), 2.1×10^6 (30.6), 7.0×10^5 (37.4), 2.6×10^6 (29.5), 3.1×10^6 (28.8), 5.6×10^6 (26.4), and 3.5×10^6 (28.1). Thus viable leprosy bacilli had clearly been present in the samples frozen in the absence of glycerol, although in small numbers.

Estimate of the degree of loss of viable M. leprae *during freezing.*— The experimental design would not differentiate between (a) decrease in number of viable bacilli and (b) partial damage to many viable bacilli resulting in a prolonged lag phase (see [7] and [8] for growth curves of *M. leprae* in mouse foot pads). If it is assumed that alternative (a) is correct, that the lag phase remained constant, and the bacillary generation time during the logarithmic phase was 12.5 days [7],

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Footnotes to Table 3

[a] In each experiment infectious material was inoculated before and after freezing. See Table 1 for definitions.

[b] B = skin biopsy from lepromatous patient; P5 = passage material in fifth passage; P3 = passage material in third passage.

[c] Q = quick-frozen in dry ice-ethanol bath; thawed by brief immersion in water not over 37°C; OS = overnight storage at 4°C, then slow-frozen by placement in cardboard container at —60°C; thawed as above: S = slow-frozen by placement in cardboard container at —60°C; thawed as above.

[d] Number of AFB in inoculum based on count before freezing, except in experiment 1.

[e] DMSO = dimethyl sulfoxide.

TABLE 3.—*Effect of freezing to −60°C on viability of* M. leprae.[a]

Experiment	Starting material[b]	Freezing medium	Freezing method[c]	Time stored at −60°C (days)	AFB inoculated[d]	Mycobacterial findings in sections (month)			First harvest		
						0	+	++, +++, and ++++	Time (days)	AFB	Generation time, G (days)
1a	B	Not frozen			1.0×10^3	1-8		9,10	329	8.8×10^5	33.5
b		Part of specimen frozen intact	Q	21	1.1×10^3	1-14			Harvest not done		
2a	P5	Not frozen			1.4×10^4	1-5,9	6	7,8	252	8.0×10^6	27.5
b		0.2 M sucrose +0.17 BA in BSS	Q	71	1.1×10^4	1-10		11	356	2.4×10^6	46.6
3a	P5	Not frozen			5.0×10^3	1-3,5	4		167	1.5×10^6	20.3
b		0.5% BA in 40% tryptose +60% BSS	OS	15	5.0×10^3	1-6			189	1.1×10^5	42.2
c		10% glycerol + 0.57% BA + 40% tryptose + 50% BSS	OS	15	5.0×10^3	1-5		6	189	1.3×10^5	40.3
d		35% sorbitol + 0.5% BA in BSS	OS	15	5.0×10^3	1-6			189	1.0×10^5	43.0
e		10% DMSO[e] + 0.5% BA + 40% tryptose + 50% BSS	OS	15	5.0×10^3	1-6			189	1.9×10^4	99.0
4a	P3	Not frozen			5.0×10^3			4	154	8.9×10^5	20.6
b		0.1% BA in BSS	Q	31	5.0×10^3	3-6		7	143	2.6×10^4	60.7
c		3.5% BA in BSS	Q	31	5.0×10^3	3-7			143	1.0×10^4	134.
d		0.1% BA in BSS	S	31	5.0×10^3	3-6		7	143	$<3 \times 10^4$	
e		3.5% BA in BSS	S	31	5.0×10^3	3-6		7	146	$<3 \times 10^4$	
f		0.2 M sucrose + 0.1% BA in BSS	S	31	5.0×10^3	3-7			146	5.2×10^4	43.1
g		10% glycerol + 0.1% BA in BSS	S	31	5.0×10^3	3,6		4,5,7	146	2.9×10^5	24.7
h		10% glycerol + 0.1% BA in BSS	OS	31	5.0×10^3	3	6	4,5,7	147	4.2×10^5	23.0

it can be estimated that the decrease in numbers of viable bacilli in experiment 2b (Table 3) was on the order of 500-fold. Similarly the loss in 3b, 3c, and 3d would be about 50-fold. The loss in 1b may not have been any greater; the results indicate that control inoculum did not contain many viable bacilli. The loss in 4g and 4h would be only about 5-fold. This last amount of loss approaches a workable method of preserving *M. leprae*, and experiments are in progress to see if the results can be confirmed and if the conditions can be improved.

DISCUSSION

In crushed ice *M. leprae* maintained nearly full viability for about 2 weeks. This is enough time to allow for collection of specimens in most endemic areas, their transportation to international airports, and their shipment by air to laboratories in other countries. For example, we recently shipped skin biopsy specimens from a remote air strip in the highlands of New Guinea to Atlanta, with re-icing en route. The time elapsed from collection of specimens until inoculation of mice was 4 days, and multiplication of the leprosy bacilli in the mice took place in the usual manner.

The loss in bacillary viability on freezing has been a great handicap. The results with glycerol are distinctly encouraging, and our current laboratory practice is to slow-freeze unused portions of infectious suspensions in 10 per cent glycerol after overnight storage at 4°C. This is regarded only as a possible safeguard against losing strains of bacilli through a disastrous loss of mice. In order to maintain strains consistently and to have infectious material of good quality regularly available for future experiments, we feel it is still necessary to maintain a number of strains in continuous passage.

The experiments at 31°C were carried out as a first step in cultivation attempts. Several workers had predicted on theoretic grounds that this usually intracellular bacillus would die rapidly in the extracellular state. Fortunately our experimental finding was no loss of viability at 31°C for the period tested. Hence it appears possible to proceed directly to test various media for growth-promotion.

SUMMARY

1. At 0°C (in crushed ice) suspensions of *M. leprae* in 0.1 per cent bovine albumin balanced salt solution maintained their viability (as measured by their ability to multiply in mouse foot pads) with little change for about 2 weeks. There was a distinct loss in viability after 3 to 4 weeks.

2. At 31°C in bacteriologic media containing about 1 per cent bovine albumin and 0.2 M sucrose, *M. leprae* maintained viability with little change for 2 weeks.

3. Freezing and storage at −60°C caused serious losses in viability under most conditions. However, in the presence of 10 per cent glycerol, losses of viability were sometimes only moderate (estimated as about 5-fold).

RESUMEN

1. Las suspensiones de *M. leprae* en 0.1 por ciento de albúmina bovina en solución salina balanceada a 0°C (en hielo machacado) mantuvieron su viabilidad con poco cambio por alrededor de 2 semanas (viabilidad medida por la habilidad de multiplicarse en la planta del pie del raton). Hubo una clara perdida de viabilidad despues de 3 a 4 semanas.

2. El *M. leprae* a 31°C en un medio bacteriológico conteniendo alrededor de 1 por ciento de albúmina bovina y 0.2 M sucrosa, mantuvo su viabilidad con poco cambio por 2 semanas.

3. Congelamiento y deposito a —60°C causaron serias pérdidas de la viabilidad bajo la mayoría de las condiciones. Sin embargo, en presencia de glicerol al 10 por ciento, la pérdida de la viabilidad fué algunas veces solamente moderada (estimada como alrededor de 5 pliegos).

RÉSUMÉ

1. A zéro degré centigrade, dans de la glace pilée, des suspensions de *M. leprae* dans une solution d'un mélange équilibré d'électrolytes additionné d'albumine bovine à une concentration de 1% conservent sans grand changement, durant environ deux semaines, leur vitalité telle qu'elle est mesurée d'après la capacité de ces bacilles à se multiplier dans la sole plantaire de la souris. Il s'est produit une perte notable de vitalité après 3 à 4 semaines.

2. A 31°C, dans un milieu physiologique contenant environ 1% d'albumine bovine et 0.2 M de sucrose, *M. leprae* a conservé sa vitalité sans grand changement durant 2 semaines.

3. La congélation et l'entreposage à −60°C a entraîné une perte marquée de la vitalité dans la plupart des circonstances. En présence de 10% de glycérol, toutefois, la perte de vitalité n'a été de temps à autres que légère (une réduction d'environ un à cinq d'après ce qui l'on a estimé).

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