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Effects of Freezing and Thawing on the Viability and the Ultrastructure of *in Vivo* Grown Mycobacteria[1]

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Storage in a frozen state is a convenient and efficient procedure for the maintenance of the viability of a number of bacteria including mycobacteria ([3]). In the case of *Mycobacterium leprae* the need for frozen samples is increased because the leprosy bacillus is not cultivable *in vitro*. Since the finding that huge numbers of *M. leprae* cells can be obtained from experimentally infected armadillos ([4]), it has become a common practice to use *M. leprae*-infected armadillo tissues frozen at −70°C for studies on *M. leprae*.

Few studies have been devoted to the quantitative assessment of *M. leprae* viability after freezing and thawing, and usually such studies have analyzed the effects of a single freezing-thawing cycle ([1, 8, 15]).

In the present study, we quantitatively evaluated the loss of viability of *M. leprae* in experimentally and naturally infected armadillo tissues by mouse foot pad titrations after one and up to four freezing-thawing cycles. In addition, we used electron microscopy to determine the mechanism of the lethal action of freezing-thawing on *M. leprae* and on *in vivo* grown cultivable mycobacteria (*M. lepraemurium* and *M. avium*).

MATERIALS AND METHODS

***M. leprae*-infected tissues.** A total of 86 samples were analyzed. Twenty-five samples (liver, spleen, lymph nodes) came from armadillos experimentally infected with *M. leprae*. Their origins are indicated in Table 1. The experimentally infected tissues were placed in a freezer at −70°C directly after autopsy of the animals. Frozen tissues were transported on dry ice to Antwerp, where they were again placed at −70°C until used.

Sixty-one samples (liver, spleen, lymph nodes and lepromas) came from naturally infected armadillos which were captured and autopsied in the state of Louisiana, U.S.A., in collaboration with Dr. G. P. Walsh (Armed Forces Institute of Pathology, Washington, D.C., U.S.A.). The tissues were collected under aseptic conditions as described previously ([13]). They were placed at −20°C for 1 or 2 days and then transported on dry ice to Antwerp, where they were maintained in a freezer at −70°C until used.

At the time of the investigation, the tissues were thawed at room temperature (RT). After thawing, some pieces of tissue were subjected to further freezing-thawing (FT) cycles (up to four), and were analyzed after each FT cycle.

For electron-microscopy studies, we used tissues from some of the naturally infected armadillos captured in Louisiana. Pieces of axillary lymph nodes from armadillos 1177 and 1210, along with a skin leproma and the spleen from armadillo 1210, were fixed immediately after collection. The pieces of axillary lymph node and spleen from armadillo 1210, a cervical lymph node and the spleen from armadillo 1177, and the spleen from armadillo 1102 were fixed after one cycle of FT (−70°C/RT).

The samples were fixed by a procedure recently found to preserve the asymmetric profile of normal *M. leprae* cells (Silva, *et al.*, unpublished data) (1% OsO_4 in Palade's veronal acetate buffer, pH 6.0, supplemented with 10 mM $Ca2+$ for 16–24 hr, then a wash in several changes of 50 mM cacodylate buffer supplemented with 10 mM $Ca2+$, pH 7.0, then fixation with glutaral-

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dehyde-formaldehyde-calcium for 24 hr as previously described by Silva and Macedo[24]). After a wash in water, the samples were postfixed in aqueous 1% uranyl acetate for 1 hr. All fixations were carried out at room temperature. Further processing for electron microscopy was carried out with ethanol dehydration and Epon embedding. Ultrathin sections were cut with an LKB Ultratome III and contrasted with lead citrate ([30]) for 5 min. Observations were done with Siemens Elmiskop IA or 102 electron microscope.

Contamination of *M. leprae*-infected tissues by cultivable mycobacteria. Cultivable mycobacteria were isolated from *M. leprae*-infected armadillo tissues according to Portaels, *et al.* ([11, 12]).

Determination of *M. leprae* viability by mouse foot pad technique. For each armadillo sample, the percentage of viable bacilli after different numbers of FT cycles was determined by titration in the mouse foot pad ([17]). Mice (strain NMRI) were inoculated in both hindfoot pads with serial dilutions of the suspensions so that groups of eight mice received inocula of 5×10^3, 5×10^2, 50, and 5 bacilli per foot pad. Foot pads were harvested after 12 months, and the bacilli were counted as described previously ([18, 19]).

M. leprae were said to have multiplied when the harvests yielded at least 10^5 acid-fast bacilli (AFB) from a foot pad. The results were analyzed by determining the most probable number (MPN) of viable bacilli ([29]) in the largest inoculum ([2]).

***M. lepraemurium*- and *M. avium*-infected tissues.** *M. lepraemurium* (Douglas strain) and *M. avium* (strain ATCC 25291) were inoculated (2×10^8 bacilli) by the intraperitoneal route in C57BL/6 mice (males, 4–6 weeks old), and the livers were collected after 4–5 months. The samples, fresh or after one FT (−70°C/RT), were processed for electron microscopy as described above for tissues from armadillos.

Statistical analysis. The comparison of the average proportion of viable mycobacteria in the naturally and experimentally infected tissues, and between the different FT cycles, has been based on the median as the basic descriptor. The statistical inference has been done through the median-based procedure of McGill, *et al.* ([9]), corroborated by the Mann-Whitney test. Given the skewed distribution, the data were gaussianized; the fourth root transformation was found to be the best normalizing procedure; subsequently a t test was carried out, corroborating once more the results obtained through the median-based procedure.

The reduction in viability was calculated by dividing the difference in viability between consecutive FT cycles by the viability level of the reference FT cycle. The differences in reductions were tested by the Pearson χ^2 test of association.

TABLE 1. *Origin of 25 samples of armadillo tissues experimentally infected with* M. leprae.

Tissue	No. samples	Source[a]
Liver	1	NIMR
	4	MRI
	6	CDC
	2	AFIP
	1	IP
	1	GWL
Spleen	4	MRI
Lymph nodes	6	MRI

[a] NIMR = National Institute for Medical Research, London, U.K.; MRI = Medical Research Institute, Melbourne, Florida, U.S.A.; CDC = Centers for Disease Control, Atlanta, Georgia, U.S.A.; AFIP = Armed Forces Institute of Pathology, Washington, D.C., U.S.A.; IP = Institut Pasteur, Cayenne, French Guyana; GWL = Gillis W. Long Hansen's Disease Center, Carville, Louisiana, U.S.A.

RESULTS

Viability assessment of *M. leprae* in mouse foot pads

Effects of successive FT cycles of the same tissues from naturally infected armadillos. The percentages of viable bacilli obtained by mouse foot pad titration, after successive FT of the same tissue, are indicated in Table 2. For all tested samples, except liver 1096, there was an important reduction in viability after the second FT cycle (65.4%–97.4%). Figure 1 shows the very marked reduction between FT cycles 1 and 2 (average median reduction was 75%) and FT cycles 2 and 3. After three FT cycles, the median reduction in viability was 100% but three samples (S22, S23, and S26) still showed some viability (Table 2).

Effects of FT cycles of different tissues from experimentally and naturally infected

TABLE 2. *Viability of* M. leprae *and cultivable mycobacteria after successive freezing-thawing cycles of the same tissue.*

Tissue no.	FT	Sample no.	*in Vitro* culture	% Viable	Reduction in viability
1096 Liver	1	S3	+	0.090	
	2	S17	+	0.156	0%
	3	S30	+	0.000	100%
	4	S73	+	0.000	100%
1102 Liver	1	S9	+	0.280	
	2	NT[a]			
	3	S72		0.000	100%
	4	S69	+	0.000	100%
1102 Spleen	1	L660	+	0.220	
	2	S7	+	0.040	81.8%
	3	S25	−	0.000	100%
	1	L661	+	0.136	
	2	S8	+	0.016	88.5%
	3	S26	−	0.004	97.1%
1112 Spleen	1	L659	+	0.156	
	2	S1	+	0.004	97.4%
	3	S22	+	0.004	97.4%
	1	L657	+	0.260	
	2	S2	+	0.090	65.4%
	3	S23	+	0.004	98.5%
1112 Cervical lymph node	1	L697	+	0.080	
	2	S24	+	0.026	67.5%
1177 Axillary lymph node	1	S52	+	0.136	
	2	NT			
	3	S102	+	0.000	100%
1177 Inguinal lymph node	1	S58	+	0.009	
	2	NT			
	3	S104	+	0.000	100%
1177 Liver	1	A350	+	1.900	
	2	NT			
	3	S105	−	0.000	100%

[a] NT = not tested.

armadillos. The effects of different FT cycles on the viability of *M. leprae* in different tissues are indicated in Table 3 and are shown graphically in a boxplot display in Figure 2. At each FT cycle, there were no significant differences between the viability proportions of the experimentally and naturally infected armadillos. The proportions of viable bacilli in the experimentally infected armadillos are not significantly different between FT cycles 1 and 2 ($p = 0.09$) but are highly significantly different between cycles and 1 and 3 ($p = 0.001$). In the naturally infected armadillos, the differences between cycles 1 and 2 were not significant ($p = 0.17$), but those between cycles 1 and 3 were highly significant ($p = 0.0001$). No multiplication of *M. leprae* was observed in the two samples subjected to four FT cycles.

Viability of cultivable mycobacteria

Table 2 shows that mycobacteria were cultivated from all samples submitted to one and two FT cycles. After three FT cycles, *in vitro* cultures were obtained from seven samples while two samples (S25 and S26) remained negative. Mycobacteria were still cultivated from the two samples (S73 and S69) submitted to four FT cycles.

Ultrastructural observations

The more conspicuous ultrastructural alteration found in *M. leprae* cells subjected to one FT cycle occurred in the cytoplasmic membrane (Table 4). The percentage of bacilli with membranes and of bacilli with normal, asymmetric membranes decreased in frozen samples as compared to samples

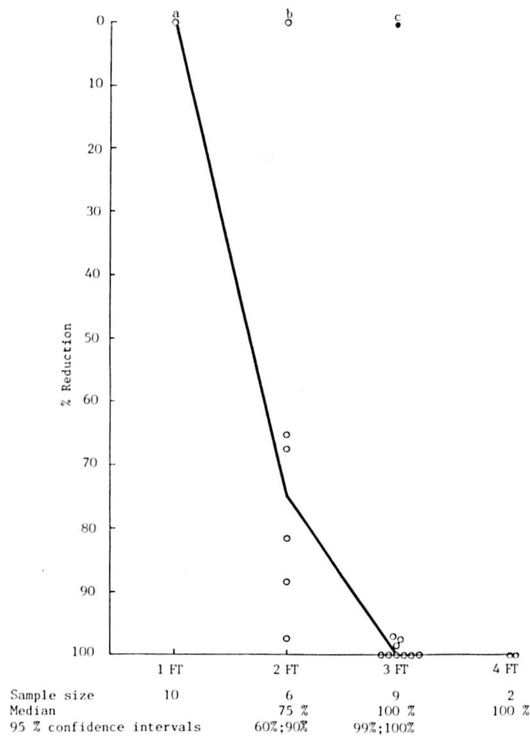

FIG. 1. Reduction in viability of *M. leprae* after successive freezing-thawing (FT) cycles of the same tissue. (a = first FT cycle = reference level; b = one outlier (no. 1096 liver with 0% reduction); c = all sample values ranging between 97% and 100%).

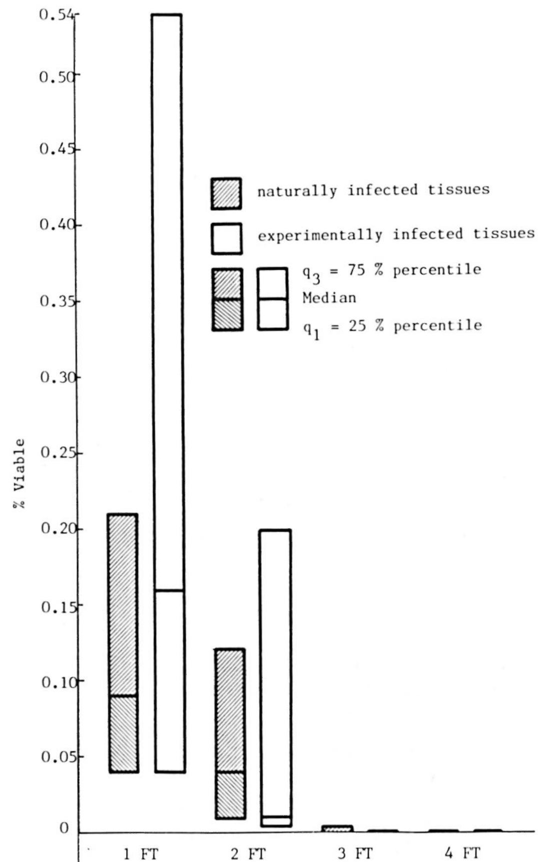

FIG. 2. Viable bacilli in naturally and experimentally infected tissues after 1 and up to 4 FT cycles.

fixed immediately after collection ($p < 0.0001$). It is interesting that 84.8% (57.6% out of 67.9%) of the symmetric membranes had gross discontinuities (Table 4) and 56.8% of the bacilli with discontinuous membranes still had ribosomes.

M. lepraemurium and *M. avium* showed similar membrane alterations when subjected to one FT cycle ($p = 0.44$). However, these alterations were significantly less extensive than in *M. leprae* ($p < 0.0001$).

DISCUSSION

A decrease in viability of *M. leprae* suspensions after one cycle of freezing and thawing was observed by several authors who used different techniques for viability assessment, such as multiplication in mouse foot pads ([1, 7, 8, 10, 15, 18]) or the measurement of the ATP content ([5, 6]). Most of these studies were not quantitative. However, Colston and Hilson ([1]), using the mouse foot pad technique, demonstrated that there was a reduction of viability of 88% to 97.8% when fresh *M. leprae* suspensions (prepared in Bacto TB broth with 7.5% DMSO) were subjected to slow freezing to −70°C and then stored in liquid nitrogen. A decrease of 30%–40% in the ATP content of *M. leprae* was found by Lee and Colston ([6]) after freezing and thawing. Kvach, *et al.* ([5]), measuring the ATP content of purified *M. leprae* bacteria, also found a significant reduction of ATP levels after repeated FT cycles. All of these studies were performed on bacterial suspensions while, in general, the *M. leprae*-infected armadillo samples supplied to laboratories are frozen pieces of tissues.

Shepard and McRae ([18]) mentioned that *M. leprae* in a piece of a patient's skin lost viability when quickly frozen.

For technical reasons, it was impossible for us to evaluate the viability of *M. leprae* in fresh armadillo tissues using the mouse

TABLE 3. *Effect of freezing-thawing (FT) cycles on viability of* M. leprae *in different tissues from experimentally and naturally infected armadillos.*

FT cycle	*M. leprae*-infected armadillos						p values
	Experimentally			Naturally			
	No.	Median[a]	Loss of viability[b]	No.	Median[a]	Loss of viability[b]	
1	7	0.16%		41	0.09%		NS[c]
2	4	0.01%	94%	9	0.04%	56%	NS
3	3	0.00%	100%	7	0.00%	100%	NS
4	9	0.00%	100%	2	0.00%	100%	NS

[a] Median percentage of viable bacilli.
[b] Percent reduction in median viability.
[c] NS = not significant ($p > 0.05$) (comparison between experimentally and naturally infected tissues).

foot pad technique but based on the results of Colston and Hilson ([1]), we estimate that the loss in viability was ±1 logarithm.

Table 3 indicates that the median percentage of viable *M. leprae* cells after one FT cycle for experimentally or naturally infected armadillo tissues was not significantly different (0.16% vs 0.09%). These low values are due in part to the fact that the tissues were already frozen once. The observation that experimentally and naturally infected tissues can give comparable results suggests that the etiologic agent of the natural infection does not differ from *M. leprae* as far as the susceptibility to FT cycles is concerned. This is in agreement with other studies showing that the natural infection is, in fact, caused by an agent indistinguishable from *M. leprae* ([31]).

Our results with *M. leprae*-infected armadillo tissues demonstrate a loss of viability between 65.4% and 97.4% (Table 2) when pieces of frozen tissues were subjected to a second FT cycle, with a mean median reduction of 94% for experimentally infected tissues and 56% for naturally infected tissues (Table 3).

Our results also show that after three FT cycles, the great majority of *M. leprae* bacilli were unable to multiply in the mouse foot pad. It is interesting that cultivable mycobacteria present in some armadillo tissues were still viable after four FT cycles, a treatment that always inhibits the multiplication of *M. leprae* in the mouse foot pad (Table 2). Although these results seem to indicate that cultivable mycobacteria are more resistant than *M. leprae* to the damaging effects of FT, it is important to take into account that, by necessity, the methods used for assessing the viability of *M. leprae* (mouse foot pad titration) and cultivable mycobacteria (culture *in vitro*) are not comparable. Pattyn ([10]) has already mentioned that the fragility of *M. leprae* to freezing distinguishes this organism considerably from other mycobacteria. The resistance of cultivable mycobacteria to repeated FT cycles has already been demonstrated by Kim and Kubica ([3]) who obtained 100% preservation of the viability after several FT cycles.

The higher sensitivity of *M. leprae* to FT as compared to two cultivable mycobacteria (*M. lepraemurium* and *M. avium*) grown *in vivo* was also demonstrated by the ultrastructural data. The electron-microscopy study showed that the main ultrastructural alteration induced in *M. leprae* by FT is the change in the membrane profile from asymmetric to symmetric. This alteration was previously shown to represent membrane damage in several gram-positive bacteria, including acid-fast bacteria ([20-28]). Another indication for the occurrence of membrane damage in frozen-thawed *M. leprae* cells is the presence of many discontinuous membranes. A rather surprising observation was the presence in frozen-thawed samples of a high proportion of bacilli with ribosomes in spite of having discontinuous, symmetric membranes. It has been previously found that membrane damage due to bacterial degradation *in vitro* or *in vivo* is usually accompanied by ribosome disappearance ([16, 22, 26, 28]). The electron-microscopy data suggest that the cytoplasmic membrane is an important target for the lethal action of

TABLE 4. *Ultrastructure of membranes in* M. leprae, M. lepraemurium, *and* M. avium *from infected tissues.*

	% Asymmetric membranes	% Symmetric membranes		% No membrane	Total counted
		Total[a]	Discontinuous		
M. leprae					
Im fix[b]	86.4	7.1[e]	(6.8)	6.5	382
1 FT[c]	16.5[d]	67.9[f]	(57.6)	15.6[g]	311
M. lepraemurium					
Im fix	88.2	6.2[e]	(5.0)	5.6	339
1 FT	55.1[d]	35.1[f]	(5.9)	9.8	205
M. avium					
Im fix	90.9	7.5[e]	(6.7)	1.6	252
1 FT	60.0[d]	37.1[f]	(5.1)	2.9	175

[a] Percent continuous and discontinuous membranes.
[b] IM fix = immediate fixation.
[c] FT = freezing-thawing cycle.
[d] Differences between Im fix and 1 FT highly significant ($p < 0.0001$) for *M. leprae, M. lepraemurium,* and *M. avium.*
[e] Differences between Im fix for *M. leprae, M. lepraemurium,* and *M. avium* not significant ($p > 0.05$).
[f] Differences between 1 FT of *M. leprae* and *M. lepraemurium* and also *M. leprae* and *M. avium* highly significant ($p < 0.001$).
[g] Differences between Im fix and 1 FT highly significant ($p < 0.001$) for *M. leprae.*

FT in mycobacteria. In this respect, *M. leprae* is more sensitive than the two other mycobacteria studied. It is interesting that the membrane of the leprosy bacillus was found also to be more susceptible to the damaging action of aldehyde fixatives as compared to the membranes of several cultivable mycobacteria ([13], and Silva, *et al.,* unpublished data).

In conclusion, our results show that the widespread practice of using *M. leprae*-infected armadillo samples that have been repeatedly thawed and refrozen should be avoided when viable *M. leprae* cells are needed for microbiological or immunological studies. Also the history of each sample regarding the number of FT cycles must be precisely recorded in order to anticipate the quality of the samples in terms of viability of the leprosy bacilli.

SUMMARY

The influence of different frequencies of freezing-thawing cycles on the viability of *in vivo* grown mycobacteria was investigated. Pieces of armadillo tissues naturally or experimentally infected with *Mycobacterium leprae* were analyzed. The viability of *M. leprae* was determined by mouse foot pad titration. The viability of cultivable mycobacteria, sometimes present in armadillo tissues, was determined by culture. Electron-microscopic studies were performed on fresh or frozen-thawed armadillo tissues with natural leprosy and on livers of C57BL/6 mice experimentally infected with *M. avium* or *M. lepraemurium.* We found that the percentage of viable *M. leprae* bacilli is identical for naturally infected and experimentally infected tissues, frozen and thawed once.

When the tissues were subjected to a second freezing-thawing cycle, a considerable loss of viability was observed (65%–97%). A third freezing-thawing cycle was lethal for most of the *M. leprae* cells, and after four freezing-thawing cycles no viable bacilli were found. The cultivable mycobacteria present in some armadillo tissues were found to be more resistant than *M. leprae* to freezing-thawing since these mycobacteria could still be cultivated after four freezing-thawing cycles. The results of the electron-microscopy study support the conclusion that *M. leprae* is more sensitive to freezing-thawing than the cultivable mycobacteria and show that the cytoplasmic membrane appears to be the target for the lethal action of freezing-thawing on mycobacterial cells.

These results emphasize the importance of avoiding repeated thawing and refreezing of *M. leprae*-infected tissues when viable *M.*

leprae cells need to be studied. Also, the history of each sample regarding the number of freezing-thawing cycles must be precisely recorded in order to anticipate the quality of the samples in terms of viability of the leprosy bacilli.

RESUMEN

Se investigó el efecto de diferentes ciclos de congelación-descongelación sobre la viabilidad de micobacterias crecidas "*in vivo*". Se analizaron piezas de tejido de armadillo infectados natural- o experimentalmente con *Mycobacterium leprae*. La viabilidad del *M. leprae* se determinó por titulación en la almohadilla plantar del ratón. La viabilidad de micobacterias cultivables, presentes algunas veces en los tejidos de armadillo, se determinó por cultivo. Los estudios al microscopio electrónico se hicieron con tejidos frescos (o descongelados) de armadillos con lepra natural y con hígados de ratones C57BL/6 infectados experimentalmente con *M. avium* o con *M. lepraemurium*. Se encontró que el porcentaje de bacilos *M. leprae* viables fué idéntico en los tejidos (congelados y descongelados una sola vez) de los animales infectados naturalmente o experimentalmente. Cuando los tejidos se sometieron a un segundo ciclo de congelación y descongelación, se observó una considerable pérdida de viabilidad (65%–97%). Un tercer ciclo de congelación y descongelación fue letal para la mayoría de las células de *M. leprae* y después de un cuarto ciclo no se encontraron bacterias viables. Las micobacterias cultivables presentes en algunos tejidos de armadillo fueron más resistentes a la congelación y descongelación que el *M. leprae* puesto que tales micobacterias pudieron cultivarse aún después de 4 ciclos de congelación y descongelación. Los resultados del estudio al microscopio electrónico apoyan la conclusión de que el *M. leprae* es más sensible a la congelación y descongelación que las micobacterias cultivables y muestran que la membrana citoplásmica parece ser el blanco del efecto letal de la congelación y descongelación.

Estos resultados enfatizan la importancia de evitar la congelación y descongelación repetida de los tejidos infectados con *M. leprae* cuando se necesita estudiar a los bacilos vivos. También, debe anotarse con precisión la historia de cada muestra en cuanto al número de veces que se ha congelado y descongelado con el fin de anticipar la calidad de las muestras en cuanto a la viabilidad del bacilo de la lepra.

RÉSUMÉ

On a étudié l'influence de différentes fréquences des cycles congélation-décongélation, sur la viabilité de mycobactéries poussées *in vivo*. Des fragments de tissus de tatou, qui avaient été infectés naturellement ou expérimentalement par *Mycobacterium leprae* ont été analysés. La viabilité de *M. leprae* a été déterminée par la titration du nombre de bacilles par la méthode du coussinet plantaire de la souris. La viabilité des mycobactéries cultivables, qui sont parfois mises en évidence dans les tissus de tatou, a été déterminée par la culture. Des études au microscope électronique ont été pratiquées d'une part sur des tissus frais de tatous qui avaient été naturellement infectés, soumis à des cycles de congélation-décongélation, et d'autre part sur des foies de souris C57BL/6 infectées expérimentalement par *M. avium* ou par *M. lepraemurium*. On a observé que le pourcentage de bacilles de la lèpre viables était identique dans les tissus d'animaux naturellement infectés, et chez ceux qui avaient été infectés expérimentalement, si ces tissus n'étaient congelés et dégelés qu'une seule fois. Lorsque les tissus sont soumis à un second cycle de congélation-décongélation, on observe une perte considérable de la fiabilité, allant de 65 à 97 %. Un troisième cycle est létal pour la plupart des cellules de *M. leprae*. Après 4 cycles, il n'est plus possible de trouver aucun bacille viable. Les mycobactéries cultivables qui étaient présentes dans certains tissus de tatous, étaient plus résistantes que *M. leprae* à la congélation-décongélation, car ces mycobactéries pouvaient encore être cultivées après 4 cycles. Des résultats obtenus par microscopie électronique renforcent la conclusion, à savoir que *M. leprae* est plus sensible à la congélation-décongélation que les mycobactéries cultivables. Ils montrent également que la membrane cytoplasmique semble être la cible de l'action létale de la congélation-décongélation dans les cellules mycobactériennes. Ces résultats soulignent l'importance d'éviter des congélations-décongélations répétées des tissus infectés par *M. leprae*, lorsque il s'agit d'étudier des cellules viables. De plus, il est nécessaire de tenir une comptabilité précise du nombre de cycles de congélation-décongélation, afin de connaître à l'avance la qualité des échantillons en termes de fiabilité des bacilles de la lèpre.

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