THE INFLUENCE OF TEMPERATURE AND TISSUE PARTICLES ON THE VIABILITY OF *M. LEPRAEMURIUM*[1]

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It has been assumed that mycobacteria from the lesions of murine leprosy, if kept at 4°C in an aqueous solution containing 40 per cent glycerol, will remain viable for 21 months. It is said, however, that if they are kept at 37°C, the viability falls off rapidly, no organisms remaining alive after 12 days. [12] Although inactivating treatment decreases the viability [8] the effect of temperature seems to predominate.

The presence of tissue particles in the bacillary suspension is also to be considered. It has been stated that crude suspensions of *M. lepraemurium*, containing tissue particles, if kept at 4°C, remain viable only three weeks [13], whereas if they are kept at the temperature of solid carbon dioxide, viability is preserved until the 42nd week.[1] On the other hand, tissue-free suspensions kept at 4°C remain viable for more than 6 months according to Goulding [3], or even 2 years. [14, 15]

A close relation exists between the size of inoculum of *M. lepraemurium* and the mean of survival time for rats [6] and for mice. [7] Statistical analyses of this correlation show that it is linear; i.e., the rate of evolution of murine leprosy is a linear function of the dose of viable bacilli injected. On this basis the mean survival time of rats inoculated with a standard dose of *M. lepraemurium* could be used to estimate their viability after subjection to various experimental conditions. The influence of temperature and tissue particles on the viability of *M. lepraemurium*, as studied on this basis, is the subject of the present paper.

MATERIALS AND METHODS

Rats of both sexes, weighing 80-100 gm. at the beginning of the experiment, were inoculated intraperitoneally with a suspension of *M. lepraemurium*, in such a way that each animal received 3.0 mgm. of bacilli. Two kinds of inoculum were used: (1) a gross suspension containing a mixture of tissue particles and bacilli, and (2) a tissue-free bacillary suspension prepared by a slight modification of the technic of Hanks (8). The latter suspension contained carefully washed bacterial cells only, as far as could be determined by accurate control tests.

The inoculum was kept at different temperatures for several different lengths of time, before injection, as shown in Table 1. A second group of rats, injected intraperitoneally with 30 mgm. of heat-killed bacilli (120°C for 1 hour), was used as a control.

After inoculation the animals were kept until their death; they supplied the data used to estimate the mean length of survival for each animal group. Material for pathologic study was obtained at necropsy. Histologic study was made of specimens of liver, spleen, lymph nodes, lungs, and omentum, fixed in 10 per cent formalin solution, embedded in paraffin, and stained by the hematoxylin-eosin method and the Ziehl-Neelsen procedure as modified by Faraco (2).

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TABLE 1. *Rats inoculated intraperitoneally with 30 mgm. of* M. lepraemurium.

Group	Number of animals	Inoculum		
		Tissue particles	Temperature	Length of treatment in days
1	15	+	4°C	0 (control)
2	15	+	4°C	8
3	15	+	4°C	15
4	15	+	4°C	30
5	15	+	4°C	60
6	15	+	4°C	90
7	15	+	4°C	150
8	15	free	−(control)	0 (control)
9	15	free	4°C	30
10	15	free	4°C	60
11	15	free	4°C	90
12	15	free	4°C	150
13	15	free	−15°C	30
14	15	free	−15°C	60
15	15	free	−15°C	90
16	15	free	−15°C	150
17	15	+	120°C	1 hour

RESULTS

Pathologic study—Table 2 summarizes data on the severity of murine leprosy lesions, as shown macroscopically and histologically in inoculated rats kept under different conditions. As far as could be determined on the basis of intensity of the disease process, no change in viability of the bacilli in the crude suspension of *M. lepraemurium* was evident until the eighth day. After that, the viability decreased progressively, as inferred from the lesser degree of development of lesions in comparison with controls, and from the appearance of some lesions that showed the structural signs characteristic of regressing lesions of murine leprosy. [4, 5]

As regressive lesions appear only if the bacilli injected are already dead [5], the results shown in Table 2 indicate that the viability of organisms in crude suspensions of *M. lepraemurium* kept at 4°C for more than 60 days had declined to zero. Under the conditions named, murine leprosy does not develop.

In contrast, bacilli from tissue-free suspensions kept at the same temperature for 60 days, showed no change in viability, as indicated by pathologic study. Viability decreased slowly, however, and some regressive lesions could be seen, in rats inoculated with tissue-free bacillary suspensions that had been kept at 4°C for 90 days.

No change in the viability of bacilli in tissue-free suspensions kept at −15°C for 150 days was evident on pathologic examination.

Survival study.—Table 3 tabulates results, in terms of mean length of survival, in animals inoculated with suspensions of *M. lepraemurium*

TABLE 2. *Macroscopic and histologic severity of lesions in rats inoculated with bacillary suspensions kept at 4°C and at —15°C. Average intensity in spleen, liver, lymph nodes, and omentum 3-6 months after inoculation.*

Treatment of inoculum		Tissue particles	Macroscopic lesions	Microscopic lesions	
Temperature	Length of treatment in days			Evolutive lesions	Regressive lesions
4°C	0 (control)	+	++++	++++	−
4°C	8	+	++++	++++	−
4°C	15	+	+++	++	++
4°C	30	+	++	+	+++
4°C	60	+	−	±	++
4°C	120	+	−	−	++
4°C	0 (control)	−	++++	++++	−
4°C	30	−	++++	++++	−
4°C	60	−	+++	++++	−
4°C	90	−	++	±	+
4°C	150	−	+	+	++
−15°C	30	−	++++	++++	−
−15°C	60	−	++++	++++	−
−15°C	90	−	++++	++++	−
−15°C	150	−	+++	++++	−
Control inoculated with heat killed bacilli[a]			−	−	++

[a]Sixtieth day after inoculation.

that had been kept at 4°C or −15°C for varying lengths of time. Statistically, significant differences are evident among the means of survival time, which depend on the temperature and length of time at which the bacilli were kept. For each temperature the regression of bacillary viability on time length appears, on statistical analysis, to be linear. On the other hand, comparison of the three coefficients of regression for the three sets of experimental conditions employed, showed significant differences among them. This finding shows that the slope of the regression line probably varies under different experimental conditions, indicating that the proportion of nonviable bacilli as a function of length of time depends on the conditions to which the mycobacteria are subjected. In the case of a bacillary suspension kept at 4°C, viability decreases less rapidly when the suspension is tissue-free. The viability of bacilli kept at −15°C is well preserved for 150 days.

DISCUSSION

The results of this study indicate that tissue particles have a harmful effect on the viability of *M. lepraemurium*. This finding is in agreement with the report of Hanks and Gray [10], who called attention to the influence of extracellular inhibitors on the viability of *M. lepraemurium*. Moreover, it has been found that liver extract and blood serum inhibit the respiration of *M. lepraemurium* [11], an effect suggesting that certain enzyme inhibitors may be responsible for the harmful action of tissue particles.

TABLE 3. *Mean of survival time of rats inoculated intraperitoneally with* M. lepraemurium *suspension kept under different conditions. Regression of viability of* M. lepraemurium *on timelength.*

Days of maintenance of bacilli	Mean of animal survival in days	
	Data obtained	Theoretical data
Bacilli kept at 4°C, mixed with tissue particles		
0	237.7 ± 3.8	241.8
8	300.2 ± 4.4	292.8
15	338.0 ± 6.0	342.2
30	>550	—
Coefficient of regression of viability: b = 188.4 days		
Tissue-free bacillary suspension kept at 4°C		
0	275.6 ± 7.3	251.3
30	275.5 ± 8.1	320.3
60	418.0 ± 12.9	389.2
90	405.2 ± 25.7	423.7
150	485.8 ± 24.1	475.5
Coefficient of regression of viability: b = 68.98 days		
Tisue-free bacilliary suspension kept at −15°C		
0	275.6 ± 7.3	258.6
30	265.4 ± 6.5	291.3
60	319.2 ± 7.7	324.0
90	351.4 ± 12.9	340.3
150	367.2 ± 13.1	364.9
Coefficient of regression of viability: b = 32.70 days		

Nevertheless, it is to be noted that tissue-free suspensions of washed bacilli also lose viability progressively in the course of time. Our study shows that animal survival yields results more suitable for conclusions than pathologic studies of the evolution of lesions. Survival studies indicate death of mycobacteria, at an earlier time, and show that the regression of viability of bacilli on time is linear. Using the ability of tissue-free bacillary suspensions of *M. lepraemurium* to reduce methylene blue, as an index of their viability, Planet *et al.* [16] found that the viability of bacilli decreased after they had been kept for 10 days at 4°C. This finding shows that loss of viability occurs early, as survival results in our studies also indicate.

On the basis of the line of regression, it is possible to predict the viability of a suspension of *M. lepraemurium*, kept under standard experimental conditions, in terms of time. On the other hand, since the length of survival of rats inoculated with *M. lepraemurium* is a function of inoculum size [6], the proportion of viable cells in a fixed weight of bacilli may be estimated on the basis of the mean of survival time.

SUMMARY

The influence of temperature and tissue particles on the viability of *M. lepraemurium* in bacillary suspensions, kept at 4°C and at −15°C up to 150 days, was studied in rats. The animals were inoculated intraperitoneally with 3.0 mgm. of bacilli that had been subjected to varying conditions for various lengths of time. Results were determined, in the animals inoculated, on the basis of (1) pathologic study of the evolution of lesions of rat leprosy, and (2) the mean of survival time.

Pathologic study indicated that bacillary suspensions containing tissue particles, kept at 4°C, show no changes in viability during the first eight days; after that, viability decreases progressively, reaching zero after 60 days. Tissue-free suspensions kept at 4°C retain their viability without change until the 60th day; subsequently viability decreases slowly until the 150th day. Tissue-free suspensions kept at −15°C show no viability change within 150 days.

Survival studies showed that the regression of bacillary viability on length of time was linear under the conditions of the experiment. The coefficient of regression varied significantly with each experimental condition, a fact indicating that the proportion of viable bacilli as a function of time depends on the experimental conditions to which the mycobacteria are subjected. Bacillary suspensions kept at 4°C lose viability less rapidly when tissue-free. When maintained at −15°C, tissue-free suspensions of bacilli show no change in viability for 150 days. On the basis of survival time it is possible (1) to estimate the proportion of viable bacilli in an unknown suspension in which the weight of bacilli is known, and (2) to predict the rate of decrease in viability in a suspension when the conditions to which it has been subjected are known.

RESUMEN

Fué estudiada en ratas la influencia de la temperatura y partículas de tejidos en la viabilidad del *M. lepraemurium* en suspensiones bacilares guardadas a 4°C y a −15°C hasta 150 dias. Los animales fueron inoculados intraperitonealmente con 3.0 mgm. de bacilos que fueron sometidos a variadas condiciones por variados períodos de tiempo. En los animales inoculados, los resultados fueron determinados sobre la base de (1) estudios patológicos sobre la evolución de las lesiones en la lepra de las ratas y (2) el término medio de tiempo de sobrevida.

Los estudios patológicos indicaron que las suspensiones bacilares conteniendo particulas de tejido, guardadas a 4°C no mostraron cambios en la viabilidad durante los primeros 8 días; despues de eso, la viabilidad decrece progressivamente, alcanzando a cero despues de 60 dias. Suspensiones libres de tejido guardadas a 4°C retienen su viabilidad sin cambios hasta el dia 60; subsecuentemente la viabilidad decrece lentamente hasta el dia 150. Suspensiones libres de tejidos guardadas a −15°C no mostraron cambios en la viabilidad dentro de los 150 dias.

Los estudios de sobrevida mostraron que la regresión de la viabilidad bacilar a lo largo del tiempo fué linear bajo las condiciones de este experimento. El coeficiente de regresión varió significativamente con cada condición experimental, un hecho indicador de que la proporción viable bacilar, como una función del tiempo depende de las condiciones experimentales a las que se somete a la bacteria. Las suspensiones bacilares

guardadas a 4°C pierden la viabilidad menos rápidamente cuando están libres de tejido. Cuando mantenidas a −15°C, las suspensiones de bacilos tejidos-libre no mostraron cambios en viabilidad por 150 dias. Sobre la base de tiempo de sobrevida es posible (1) estimar la proporción de bacilos viables en una supensión desconocida en la cual el peso del bacilo es conocido y (2) predecir el nivel de disminución de viabilidad en una suspension, cuando las condiciones a la cual han sido sometidas es conocida.

RÉSUMÉ

L'influence de la température et des particules de tissus sur la viabilité de *M. lepraemurium* en suspensions bacillaires maintenues à 4°C et à −15°C durant 150 jours a été étudiée chez des rats. Les animaux ont été injectés par voie intra-péritonéales à raison de 3.0 mg de bacilles qui avaient été soumis à diverses conditions pour des périodes de temps variables. Chez les animaux inoculés, les résultats ont été déterminés (1) sur la base de l'étude pathologique de l'évolution des lésions de la lèpre du rat, et (2) d'aprés la moyenne du temps de survie.

Une étude pathologique a montré que les suspensions bacillaires contenant des particules tissulaires et maintenues à 4°C ne subissent pas de modifications quant à leur viabilité durant les premiers huit jours; aprés cette période la viabilité décroît progressivement pour atteindre zéro aprés 60 jours. Des suspensions libres de tissus maintenues à 4°C conservent leur viabilité inchangée jusqu'au 60ème jour; ultérieurement la viabilité décroît lentement jusqu'au 150ème jour. Des suspensions libres de tissus maintenues à −15°C ne témoignent pas de modifications de la viabilité durant 150 jours.

Des études sur la survie ont montré que la régression de la viabilité bacillaire par rapport à la durée était linéaire dans les conditions où ont été menées cette expérience. Le coefficient de régression varie de façon significative avec chaque condition expérimentale, ce qui indique que la proportion de bacilles viables en fonction du temps dépend des conditions expérimentales auxquelles les mycobactéries sont soumises. Des suspensions bacillaires maintenues à 4°C perdent leur viabilité moins rapidement que des suspensions libres de tissu. Lorsqu'elles sont maintenues à −15°C, les suspensions bacillaires libres de tissu ne témoignent pas d'altérations de la viabilité pendant 150 jours. Sur la base du temps de survie, il est possible (1) d'estimer la proportion de bacilles viables dans une suspension inconnue dans laquelle le poids de bacilles est connu, et (2) de prédire le taux de réduction de la viabilité dans une suspension quand les conditions auxquelles celle-ci a été soumise sont connues.

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