THE INFECTIOUSNESS OF MURINE LEPROSY BACILLI AFTER EXPOSURE TO DIFFERENT CONDITIONS *IN VITRO*

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Studies on murine leprosy bacilli were undertaken in this laboratory for the specific purpose of reinoculating animals with microorganisms which had been exposed to different experimental conditions. It was proposed in this way (*a*) to define those circumstances which are either favorable or unfavorable to survival and infectiousness of these microorganisms, and (*b*) to ascertain whether substances which increase or decrease their metabolic activity serve a corresponding biological function with respect to viability or infectiousness.

Certain interesting observations on the infectiousness of murine leprosy bacilli after exposure to various conditions *in vitro* have been published by earlier workers. Since the conditions of treatment or incubation have sometimes been inadequately defined and are often misquoted, a summary of pertinent data is presented in Tables 1 and 2. From the results cited in these tables one gains the impression that the infectiousness of the bacilli declined rapidly at 37°C., and that little damage was caused by treatment with acid or alkali, by prolonged washing, or by refrigeration for extended periods of time. For this reason it must be emphasized that such experiments were not designed to measure the relative infectiousness of the treated or incubated bacilli by comparison with the original bacilli. From these and other qualitative data on experimental infection with mycobacteria which have been exposed to acids, alkalies, washing or refrigeration there seems to have arisen a widespread and erroneous impression that infectiousness is not seriously impaired by decontaminating or washing procedures. The findings by the more quantitative methods to be described in this communication will reveal that all such procedures do in fact impair infectiousness very markedly. These findings provide the first points in a series of observations which will demonstrate that the infectiousness of mycobacteria is dependent

[1] With the assistance of Miss Tobey Backerman.

upon an optimal degree of physiological activity, and that many procedures or conditions which depress viability only slightly may seriously interfere with the successful parasitism of host cells.

TABLE 1. *The survival of murine bacilli during exposure to chemical agents and during refrigeration, as reported by earlier workers.*

Type of Test		Condition of Experiment	Survival
Exposure to Chemical Agents	1932, Marchoux & Chorine (11)	2 gm. leproma ground in 1 cc 10% H_2SO_4, diluted immediately in 9 cc. water, washed 4 times in NaCl	++
		2 gm. leproma ground in 1 cc 15% antiformin, 37°C., 1 hour, washed 3 times	+
	1935, Prudhomme (13)	Suspension in 15% H_2SO_4 5 min., washed 3 times in NaCl (see Table 2)	+
	1935, Berny (4)	Suspension in 0.5% methylene blue, 24 hrs., room temperature, then washed free of color	++
	1938, Prudhomme (14)	Suspension in 15% antiformin, 37° C.0 20 min., washed 4 times in NaCl (see Table 2)	+
	1938, Prudhomme (15)	Suspension in NaCl, washed 4 times in NaCl (see Table 2)	+
	1940, Badger & Fite (2)	Suspension in 3% H_2SO_4 one hr. in centrifuge, washed 2 times in NaCl, 1 time in 5% Na_2CO_3, 2 times in NaCl.	+
Refrigeration	1931, Marchoux (10)	Leproma pulp in 40% glycerol, 17 mos. in sealed tubes	+
	1934, Chorine (6)	Same conditions, 39 and 51 mos.	+
	1944, Linhares (9)	Lepromas in 40% glycerol, 24 mos.	+
	1949, Carpenter (5)	20% leproma suspensions in deep freeze, at -20°C., 3 mos.	+

The data reported here are intended primarily (*a*) to define the relative advantages of test inoculation into different tissues of two animal species, and the choice of test animals for different experimental purposes; (*b*) to present inoculation patterns which permit reliable comparisons of infectiousness in small groups of animals; and (*c*) to provide a quantitative estimate of the influence of certain factors on the infectiousness of treated or incubated bacilli.

METHODS

Suspensions of bacilli (Hawaiian strain) were prepared by homogenizing infected tissues in glass-distilled water containing M/75 Na_2HPO_4. The glycerinated bacilli were obtained from slices of a cutaneous leproma which had been dropped into 40 per cent cold glycerol and refrigerated for 54 days at 6°. Fresh bacilli were obtained from fresh cutaneous or testicular lepromas. The 5 per cent tissue suspensions were cleared of tissue debris by centrifugation for two minutes at 200xG. Aside from

TABLE 2. *The survival of murine bacilli at incubator temperatures, as reported by earlier workers*

Date	Author	Condition of Experiment			Survival
1931	Marchoux (10)	Emulsion in NaCl solution, sealed tubes		12 days	—
1931	Marchoux et al (12)	Shiga's glycerol potato medium		12 days	—
		Wherry's glycerol egg medium		12 days	—
		Agar and rabbit blood		12 days	+
		Agar and rabbit blood		<12 days	+
1935	Prudhomme (13)	Bacilli in glycerol broth, 14 days	Final pH	Positive Lesions	
			5.5	0/3	—
			6.1–7.2	11/11	+
			7.3–7.6	0/6	—
1938	Prudhomme (14)	Bacilli with M. *phlei* pellicle on Sauton's synthetic medium	Room temp.	40 days	—
			37°	40 days	+
			"	210 days	+
1938	Prudhomme (15)	Reducing capacity of washed bacilli tested with 0-cresol indo 2-6 dichlorophenol	Fresh control bacilli		
			Incubated	12 days	+
			"	15 days	—
			Formalin	15 min.	—
			100°	15 min.	—
			Glyc. refrig.	8 mos./*a*	+
			" "	13 mos./*a*	+
1938–41	Badger & Fite (3)	Egg medium, 90 days, viability very low			+
		Miscellaneous bacteriological media, 30–40 days			+
		NaCl, 4 days, reasonably prompt lesions			+
		NaCl, 7 days, lesions more delayed			+
		NaCl, 30 days, lesions in low proportions of animals			+

/a Refrigerated in 40 per cent glycerol prior to preparing washed suspensions.

treatment with acid or alkali, further dilutions or manipulations were carried out in biological NaCl or other nontoxic electrolytes known to be suitable for cell cultures. Microscopic counts on these suspensions showed that the number of stainable microorganisms did not decrease during the intervals that the bacilli were retained *in vitro*. Details on the experimental modification of aliquots will be presented for each type of experiment reported.

The test animals were members of a susceptible subfamily of "Swiss" mice and a moderately resistant subfamily of Wistar rats. These animals have been characterized in earlier communications (7, 8). Test inoculations, except testicular, were arranged along either side of the middorsal line. One drop of India ink in each cubic centimeter of inoculum served to identify all inoculation sites. The effects of N/15 NaOH and of refriger-

ating bacilli in 40 per cent glycerol were ascertained by inoculating separate groups of animals rather than by multiple site inoculation. In the remaining experiments the comparisons of infectiousness were made by inoculating each animal in one or more sites with control aliquots of the fresh bacillary suspension, and in the remaining sites with aliquots which had been modified by experimental treatment. The inoculation patterns employed in various types of experiments are indicated in Table 3.

TABLE 3. *Inoculation patterns employed for comparing control versus experimentally modified aliquots of bacterial suspensions.*

	1 Effect of H_2SO_4 and NaOH	2 Incubation in presence of: *M. phlei* Fungi	3 Effect of electrolytes and complex substrates	4 Storage at room temperature in sucrose and glycerol
Arrangement of skin sites on the back of each animal	O O H^+ H^+ OH^- OH^-	O O&p O O&f 6 1 4 1 5 2 3 2 4 3	0 1 5 2 4 3	Suc Glyc
Testicular sites				Suc Glyc
Test animals	Rats	Rats Rats	Rats	Mice

1. O, control aliquots; $H+$, sulfuric acid; $OH-$, sodium hydroxide treated aliquots.

2. O, control aliquots; O&p, same mixed with dense suspension of *M. phlei*. Control aliquots were inoculated immediately and experimental aliquots #1-6 after the bacilli had been maintained in *M. phlei* cultures for one month.

O&f, control aliquots mixed with fungi; otherwise as above.

3. O, control aliquots inoculated immediately; experimental aliquots #1-5 inoculated after bacilli had been *in vitro* five days.

4. No control aliquots. Bacilli stored in sucrose were inoculated in the left skin sites and one testis, and glycerol bacilli on the opposite side of the animals.

The occurrence of positive lesions was ascertained by monthly palpation of the inoculated skin sites. The volume of individual lesions was estimated by means of the paraffin-ball method described earlier (7), and the results were expressed in grams. In multiple-site comparisons, the data from each animal at each reading are considered a complete unit. Since about 1 rat in 10 is more susceptible than the average, and the time required for the appearance of lesions varies in the other individuals, the

results from each animal in a group are not averaged after a fixed or standard time interval. When the results of an experiment are at hand it is apparent that a more or less uniform period of time separates the appearance of lesions caused by the control and by one or more of the experimental aliquots. In a given experiment, for example, there may be in each animal a lapse of three months between the appearance of the first positive lesion and of other lesions produced by less infectious bacilli. In such an experiment the results are obtained by summarizing the values obtained in each rat at three months after the first lesion was palpable.

EXPERIMENTAL RESULTS

The arrangement of experiments is in accordance with the headings in Tables 1 and 2. Although each type of data will be introduced as a problem in the preservation of bacilli, the influence of the testing method on the results will be in most instances the chief point of emphasis.

The influence of acids and alkali.—A clarified suspension of murine leprosy bacilli was divided into three aliquots. To one was added an equal volume of N/1 H_2SO_4, to another N/1 NaOH, and to the third an equal volume of water. After 30 minutes exposure to the acid and alkali the treated aliquots were neutralized and a corresponding amount of water was added to the third. Two rats were inoculated in each of two sites with 0.1 ml. of each of these suspensions (see pattern, Table 3). Although the untreated bacilli produced positive lesions in five months, the sites inoculated with the treated aliquots remained negative at the end of eight months. No trace of lesions could be discerned in the corresponding ink sites when the animals were examined at autopsy.

Treatment of bacilli with alkali which is much too weak to serve for decontamination of suspensions also has a deleterious effect on infectiousness. As may be seen from the data in Table 4, clarified leproma suspensions which had been exposed to N/15 NaOH for one hour at room temperature, and promptly neutralized, were markedly inferior to the control aliquot which had been treated similarly with distilled water. It may also be noted that the infectiousness of the treated bacilli was demonstrated most readily by inoculation of the most susceptible tissue —the testes—in these animals, whereas the distinction between the control and treated bacilli was more exaggerated and more clearly demonstrated by subcutaneous inoculation.

TABLE 4. *The effect of N/15 NaOH on the infectiousness of murine type bacilli, as judged by subcutaneous and intratesticular inoculation of rats.*

Treatment of bacilli	Number of rats	Route of Inoculation	Number of sites/*a*	Proportion of sites positive (months)		
				4	5	6
Fresh bacilli, water control	4	Testes	8	100		
	4	Skin	16	75	100	
N/15 NaOH	4	Testes	8	?	100	
	4	Skin	16	0	0	25/*b*

a Each rat was inoculated in both testes and four skin sites.
b One more susceptible animal among four.

Refrigeration in glycerol.—Refrigeration of leproma slices or suspensions in the presence of glycerol appears to have been the most satisfactory means of preserving murine leprosy bacilli *in vitro*. Protocols which permit a direct comparison of fresh and glycerinated bacilli are not necessary to show the marked loss of infectiousness which occurs during refrigeration in glycerol (see Table 5). Although the glycerinated bacilli pro-

TABLE 5. *A comparison of mice and of rats inoculated by two routes for demonstrating the infectiousness of glycerol-refrigerated bacilli.*

Treatment of bacilli	Animals inoculated	Sites inoculated	Proportion of sites positive (months)			
			4	6	8	12
Refrigerated in glycerol 40% for 54 days prior to preparing suspensions	Mice (4♂)	Skin (8)	100			
	Rats (10♂)	Testes (20)	100			
		Skin (20)	0	0/*a*		
	Rats (13♀)	Skin (13)	0	0	8/*b*	54

a Results were collected from this group of rats after six months, while the 13 females were maintained for one year. Extensive data fails to reveal differences between male and female rats following subcutaneous inoculation.
b One more susceptible rat among 13.

duced lesions in the testes of moderately resistant rats and in the skin of susceptible mice in less than four months, they failed to produce a single lesion in any of the 33 subcutaneous sites in 23 rats by the end of six months. Among 13 female rats which were maintained for 12 months, only 1 of unusual susceptibility developed a lesion within eight months and 6 more between

the eleventh and twelfth months. Thus, were it not for several favorable circumstances, such as the intratesticular inoculation of rats and subcutaneous inoculation of mice, and the occurrence of 1 susceptible rat among the 13 females, it could have been concluded at the end of almost one year that the glycerinated bacilli were completely noninfectious. Other observations show equally clearly that glycerinated bacilli are not satisfactory for subcutaneous infection in moderately resistant rats.

Preservation at room temperature.—Attempts to mail murine leprosy bacilli at atmospheric temperatures have been uniformly unsuccessful. One example may be cited. Fresh, thin, leproma slices which had been dropped into chilled 40 per cent glycerol and also packed in dry sucrose crystals were refrigerated for two days to permit dehydration of the tissue. Packages similar to those shipped were allowed to stand at room temperature for one month prior to testing for survival of bacilli by the inoculation of mice in both skin and testicular sites (see patterns, Table 3). The testes of mice are the most susceptible of all tissues which have been studied in this laboratory (8). Nevertheless, six months after these inoculations none of the 12 skin sites and 12 testicular sites provided any evidence of proliferation of the bacilli.

Loss of viability in the presence of M. phlei.—Prudhomme's observations on the survival of murine leprosy bacilli for seven months when incubated in the presence of *M. phlei* appeared to represent the most instructive of all experiments which have been conducted with murine leprosy bacilli *in vitro*. Four major attempts to confirm these observations produced negative results. In each experiment, large numbers of fresh bacilli were added to recently established cultures of *M. phlei:* (*a*) on Long's synthetic medium, (*b*) in a complex medium containing casein hydrolysate and glucose, and (*c*) in a simple electrolyte base lacking sodium and providing ammonium ion, citrate and Tween 80. Fresh aliquots of the leprosy bacilli alone and of leprosy bacilli mixed with dense suspensions of *M. phlei* served as control inocula. After one month of maintenance at 35°C., the experimental aliquots were inoculated into an additional series of four or six sites on the previously infected rats (patterns in Table 3) and also into new rats which did not bear control sites. The control sites inoculated with fresh bacilli produced lepromata, irrespective of the accompanying *M. phlei*. Not one of the 158 sites inoculated with the experimental aliquots pro-

duced a positive lesion in the 32 rats which survived for periods of six months to one year.

Three similar experiments with fungi as "host" cells during incubation for one month likewise produced negative results.

Influence of electrolytes and complex substrates during refrigeration.—The influence of electrolytes on the infectiousness of refrigerated bacilli was investigated by adding one volume of clarified 5 per cent supernate in M/75 Na_2HPO_4 to an equal volume of water and of the double strength salt solutions listed in Table 6. Control sites were inoculated immediately from suspensions in M/150 Na_2HPO_4. The refrigerated aliquots were inoculated after 5 days (patterns in Table 3). The two experiments were carried out six months apart, with low-quality glycerinated bacilli in one instance and with a larger number of fresh bacilli in the other.

TABLE 6. *Influence of electrolytes on infectiousness of bacilli in clarified tissue homogenates during refrigeration for 5 days at pH 7.*

Exp.	Source of bacilli	Bacilli per site	Rat no.	Weights of positive lesions produced by/*a*						Average time required to obtain results (weeks)
					Aliquots refrigerated five days in:					
				control/*b* aliquots	M/150/*b* Na_2HPO_4	BSS/*c* Ca[5]	KCl/*d* NaCl	NaCl/*e* KCl	BSS/*f* —Ca	
A	Glycerol nodule, refrigerated	200 x 10^6	1	0.2	0.2	0.5	.....	.....	.....	52
			2	1.2	1.1	0.5	0.2	.....	.....	
			3	0.5	0.3	0.2	0.1	.....	.....	
			4	+/*g*						
B	Fresh testicular leproma	1,100 x 10^6	1	0.5	....	0.05	0.1	.....	.....	17
			2	0.5	0.1	0.1	0.1	.....	.....	
			3	0.5	0.2	0.1	0.1	.....	.....	
			4	0.3	0.2	0.1	0.2	0.1	.....	
	Totals:			3.7	2.1	1.6	0.8	0.1	0.0	

a Data from each rat at two months after the control sites became positive.

b Control aliquots also in M/150 Na_2HPO_4, but injected immediately.

c BSS-Ca[5]—Balanced salt solution with calcium 5mg%.

f BSS-Ca—Same, but lacking $CaCl_2$.

d KCl/NaCl—isotonic solution, K and Na in intracellular ratio, 4:1.

e NaCl/KCl—isotonic solution, Na and K in extracellular ratio, 19:1.

g Died before experimental aliquots produced positive lesions.

The data in Table 6 reveal that bacilli which had been refrigerated for only five days were definitely less infectious than at the outset. If mental deductions were made for the two large lesions in animal No. A 2 the differences between the original and the refrigerated bacilli remain unmistakable. Three of the

storage solutions appear to be fairly comparable, and two unfavorable.

Employing similar methods with bacilli of the same sources and dates, a second series of five aliquots were refrigerated in the presence of complex substrates. The unfortunate choice of BSS-Ca as the electrolyte for diluting the albumin,[2] serum and serum ultrafiltrate illustrates the impossibility of outguessing nature when exploratory data are not available prior to animal studies. From the data in Table 7 it may be seen that none of the complex substances was measurably superior to dilute phosphate buffer (Table 6). Bovine albumin and serum diminished the toxicity of BSS-Ca, but not that of the lot of serum ultra-

TABLE 7. *Influence of complex substrates on infectiousness of bacilli during refrigeration for five days at pH 7.*

Bacilli as in experiment	Rat number	Estimated weights of positive lesions/*a*					
			Aliquots refrigerated five days				
		Control/*b* aliquots	Alb V/*c* 0.25%	Rts_5/*d*	Rts_5/*e* SF_{33}	SF_{33}/*f*	SS/*g*
			(&SF_{33})				
	1	0.1	....	0.2	....	....	0.2
A	2	1.0	....	0.5	....	....	0.2
	3	+/*h*					
	4	+/*h*					
			(—SF_{33})				
	1	1.5	1.0	0.3	0.5	0.1	1.0
B	2	0.5	0.3	0.2	0.2	0.1	0.2
	3	0.5	0.2	0.2	0.2	0.1	0.2
	4	0.4	0.2	0.3	0.2	....	0.1
(Subtotals)		(2.9)	(1.7)	(1.0)	(1.1)	(0.3)	(1.5)
Totals:		4.0	.....	1.7	.....	.....	1.9

a Data from each rat at two months after control site became positive.

b Control aliquots in M/150 Na_2HPO_4; injected immediately.

c Alb V—purified bovine serum albumin, fraction V, 0.25%, diluted in BSS-Ca.

d Rts_5—rat serum 5% diluted in BSS-Ca.

e Rts_5SF_{33}—rat serum 5%, and ox serum ultrafiltrate 33%, diluted in BSS-Ca.

f SF_{33}—ox serum ultrafiltrate 33%, diluted in BSS-Ca.

g SS—supplementing solution of Anfinsen *et al.* (1), diluted in BSS Ca[5].

h Died before experimental aliquots produced positive lesions.

[2] Bovine albumin fraction V, kindly supplied by Armour and Company, Chicago, Illinois.

filtrate which was used in Experiment A. There is no indication that the long list of nutritional and accessory factors in the SS solution were of benefit to the bacilli.

Influence of electrolytes and substrates during incubation.— Since the data in the two preceding tables provide a picture of the results obtained by multiple-site inoculation in small groups of animals, the influence of the electrolytes and the complex substrate during incubation of the bacilli for five days is summarized in Table 8. After incubation for five days the glycerinated bacilli proved to be incapable of producing a single positive lesion in 7 rats maintained for 16 months. Data were obtainable only in Experiment B with large numbers of fresh bacilli.

TABLE 8. *Influence of electrolytes and complex substrates on infectiousness of bacilli incubated for five days at 35°C at pH7.*

Experiment	Number of rats	Results with control aliquots/*a*	Results with incubated aliquots/*a*		Average perio d required to obtain results
			Solutions tested	Total weights of positive lesions	
B	4	Large lepromas after 20 weeks/*b*	M/150 Na_2HPO_4	0.2	12 mos.
			KCl/NaCl	2.7	
			NaCl/KCl	1.7	
			BSS Ca^s	0.5	
			BSS –Ca	0.2	
B	4	As above	Bov. alb. 0.25%	2.7	12 mos.
			Rat serum 5%	0.0	
			Rts$_s$ SF$_{ss}$	0.2	
			SF$_{ss}$	0.0	
			SS	0.0	

a Control aliquots in M/15 Na_2HPO_4 were inoculated immediately and the experimental aliquots after five days. See Tables 6 and 7 for description of solutions in which the bacilli were incubated.

b Lepromas produced by control aliquots were removed as they became large enough to ulcerate. In some animals they recurred and were removed a second time. Note that the interval between the results with control aliquots and with experimental aliquots is now seven months rather than two months.

It is evident that a phenomenal loss of infectiousness occurred during incubation for only five days in the most favorable solutions tested. The solutions which permitted the persistence of any reliable degree of infectiousness were limited to simple chlorides and to dilute serum albumin. It is perhaps of greater significance that the balanced salt solutions, the rat serum, the serum ultrafiltrate and the supplementing solution—standard

components of cell-culture systems—appeared to be definitely unfavorable.

Improved inoculation patterns.—In the foregoing experiments the control bacilli were always injected into the upper skin sites, while the treated aliquots were inoculated into the remaining positions. I am indebted to Dr. James A. Doull for pointing out the desirability of testing each aliquot at each level on the back. The inoculation patterns since that time have corresponded with those shown in Table 9. By shifting the position of the No. 1 (or control) aliquot forward two positions on the clock, each aliquot is tested in each anatomical region involved.

TABLE 9. *Improved patterns for comparing the infectiousness of control and of experimental aliquots by inoculation of multiple skin sites on the backs of rats.*

Comparisons between control suspension (No. 1) and																				
Three aliquots				Five aliquots						Seven aliquots										
										8/*a*	1	6	7	4	5	2	3
4	1	2	3	6	1	4	5	2	3	7	2	5	8	3	6	1	4
3	2	1	4	5	2	3	6	1	4	6	3	4	1	2	7	8	5
				4	3	2	1	6	5	5	4	3	2	1	8	7	6
(3)/*b*		(3)		(3)		(3)		(3)		(2)		(2)		(2)		(2)	

a This pattern can be used only on large rats. In all cases the uppermost sites should not be directly behind the shoulder since intrascapular fat and retroscapular lymph nodes complicate palpation.

b Minimal numbers of rats are suggested in each group, since repetition of the experiment with additional suspensions of bacilli is needed to permit generalization. Combination of experiments permits basing overall conclusions on adequate numbers of animals.

In experiments involving brief exposure of the experimental aliquots, control and experimental suspensions are inoculated simultaneously. If the experimental aliquots are to be incubated *in vitro* for less than one week, the control suspension is inoculated while fresh and the experimental aliquots when the test period has elapsed. When the control sites are established for several weeks or months prior to inoculation of the experimental aliquots, one must consider the possibility that the general resistance of the rats may have been increased, and that the results with the experimental aliquots will not be representative of their actual quality. For this reason several experiments have been made with larger numbers of animals in order that

sub-groups might be inoculated with three types of patterns: (*a*) the usual complete patterns, (*b*) five experimental aliquots only, and (*c*) one or more control sites only. An interval of five or seven days between inoculation of the control and the experimental aliquots (as in the electrolyte experiments) did not interfere with the production of lesions by experimental aliquots. When the interval was one month, the infectiousness of the experimental aliquots had fallen so low that they produced no lesions in sub-groups (*a*) or (*b*) above. The possibility of immunization by control inoculations must be reinvestigated when reasonably infectious bacilli can be maintained *in vitro* for periods of several weeks or months.

The results obtained by comparing the success of control sites followed by experimental aliquots ((*b*) above) and of control sites alone ((*c*) above) showed that the addition of the five or seven experimental aliquot injections after either one week or one month was incapable of modifying or suppressing the development of lesions in the control sites.

DISCUSSION

The observations of earlier workers and the present results obtained by subcutaneous inoculation of treated bacilli into more resistant rats are reconciled by the fact that the intratesticular inoculation of these rats or subcutaneous inoculation of the more susceptible mice permits the production of lesions by bacilli which possess a low order of infectiousness. It is apparent that the susceptibility of the test animals, or of the tissues inoculated, can determine the conclusions to be reached with regard to the infectiousness of experimentally modified bacilli. It is further evident that selection of experimental animals should be based on their known susceptibility, and that the choice between susceptibility and resistance depends on the purpose of the study. The moderately resistant rats, when inoculated subcutaneously, make a remarkably sharp distinction between fresh bacilli and those which have been experimentally modified. This degree of discrimination is of the utmost importance when comparing the infectiousness of high-quality bacilli, for example, if one wishes to ascertain whether bacilli recovered from *in vitro* experiments are the equivalent of those obtainable directly from infected tissue. It is, however, disadvantageous during the present stage of the work, when infectiousness cannot be maintained *in vitro*.

Further reports will demonstrate that the necessary distinctions can be measured more quickly in more susceptible animals.[3]

With respect to inoculation methods, the intratesticular inoculation of rats, the use of four skin sites in mice, or systems employing single sites in individual animals are ruled out by the limited number of comparisons possible in each animal. The variability among the rats and mice of the inbred families reveals that narrow limits of variation could not be expected. Furthermore, the testes can be transformed into lepromata without palpable change in the organs, and there is in a low proportion of the mice a tendency for generalization of the disease without the production of local lesions. The highest degree of economy and precision is obtained by the inoculation of multiple subcutaneous sites in rats. If the results from each rat are included in summaries or averages at a time when that animal reveals the sharpest distinctions, one accumulates a series of comparisons which are not influenced by individual differences in susceptibility. Statistical variation should be correspondingly minimized.

The results obtained by reinoculation of preserved viruses or of treated murine leprosy bacilli are often regarded as measures of viability or survival. The failure of the relatively resistant rats to develop lepromata following inoculation of as many as 10^9 treated or incubated bacilli cannot be attributed to small numbers of surviving organisms, since these animals consistently develop lepromata following the injection of 10^5 fresh bacilli. In view of the more prolonged "survival" recorded by earlier workers, and the present demonstration of infectiousness for susceptible tissues, it is highly improbable that the treated suspensions contained less than 0.01 per cent viable bacilli after treatment with acid or alkali or refrigeration for only five days. The failure of infection in the cutaneous tissues of these animals must be attributed to loss by the bacilli of certain properties required for the infection of moderately resistant tissues.

The demonstration of such rapid losses in the infectiousness of murine leprosy bacilli for moderately resistant tissues sug-

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[3] Since preparation of this manuscript, similar conclusions have been reported from a much more adequate study concerning the factors involved in measuring the virulence of typhoid bacilli in mice (Batson, H. C. Statistical methods in immunology, *J. Immunol.* **66** (1951) 737-56. Small differences in virulence could be demonstrated only in immunized or in a resistant strain of mice. "Demonstration of this difference with normal mice of susceptible strain has failed just as regularly."

gests certain interesting implications. In the first place, it is evident that the accepted practice in many laboratories of exposing bacilli to acids, alkalies or prolonged washing is not without marked deleterious effect on the most interesting property of these pathogenic agents. In the second place, if the infectiousness of murine leprosy bacilli is diminished by such mild procedures as washing in distilled water (see Text-Figure 1, ref. 7), the attribute of infectiousness for even more resistant animals must be an extremely labile one. In this connection it will be recalled that attempts to transmit leprosy to normal humans by the injection of suspensions have failed, whereas more direct accidental transmissions have been reported (7).

Discussion of the influence of electrolytes, nutrients and proteins on the preservation of murine leprosy bacilli *in vitro* will be deferred until a later occasion. Although the results may be valid measures of the infectiousness which persisted in the particular samples used, they are limited in value by the impracticability of adequate repetition and by the fact that the microorganisms were accompanied by minerals, mitochondrial debris, soluble extractives and enzymes from the tissues.

SUMMARY AND CONCLUSIONS

1. A method permitting quantitative comparisons of the infectiousness of fresh murine leprosy bacilli and of treated aliquots has been described.

2. Comparisons between fresh and acid- or alkali-treated bacilli, refrigerated bacilli, or incubated bacilli revealed that the infectious quality of the bacilli for moderately resistant tissues deteriorated markedly under each of the circumstances evaluated.

3. Although the observations of earlier workers on the preservation of murine leprosy bacilli *in vitro* could not be confirmed by subcutaneous inoculation of moderately resistant rats, they were readily confirmed by the inoculation of more susceptible tissues such as the testes of rats and the subcutis of the more susceptible mice.

4. An analysis of the results necessitates the conclusion that the property of infectiousness for resistant tissues is not dependent on viability alone, but on other more readily modified properties of the microorganisms. The implications of this conclusion have been discussed.

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