THE TISSUE SITES MOST FAVORABLE FOR THE DEVELOPMENT OF MURINE LEPROSY IN RATS AND MICE

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The acquisition of precise information regarding which tissues and organs of the susceptible animals are most favorable for the growth of *Mycobacterium leprae murium* would accelerate experimental work with that infection in several directions. Specifically, the sites in which multiplication takes place most rapidly would provide the greatest numbers of bacilli in a given time, and presumably the highest proportions of viable bacilli, for cultivation and biochemical studies. The use of those sites might expedite checking the viability of bacilli subjected to various experimental conditions *in vitro*. Available data on the histology, physiology and enzyme content of such tissues might suggest leads for *in vitro* cultivation work.

This paper presents data on the growth and accumulation of the Hawaiian strain of the murine leprosy bacillus in various tissues of moderately resistant rats and of fairly susceptible mice. The results reveal that the disease in moderately resistant rats differs from that usually described and, in certain respects, more closely resembles the human disease.

METHODS

Known numbers of bacilli from clarified supernatants ([12]) of suspensions obtained from experimentally induced lepromata were inoculated into two or more sites in each animal. To facilitate subsequent identification of the sites, one drop of India ink (1:10) was added to each cubic centimeter of inoculum just prior to injection. Refrigerated and fresh materials were used in different experiments, as will be noted.

The resistance of the inbred line of Wistar rats and the greater susceptibility of the inbred line of "Swiss" mice have been characterized in the earlier report referred to. Only animals surviving the period of observation are included in the various groups. Since the procedure permitted comparison of the developments in several tissues of each animal used, large groups of animals were not required to distinguish with certainty the least favorable from the most favorable tissue sites.

The mice and rats providing the data in Tables 2 and 3 were inoculated simultaneously, each site receiving 125 million bacilli from cutaneous

leproma slices which had been refrigerated in 50 per cent glycerol for 54 days and prepared as a clarified 5 per cent tissue emulsion. The bacilli inoculated into the rats of Table 4 were obtained from a freshly biopsied cutaneous leproma. One portion of the clarified suspension was diluted in N/15 NaOH and concentrated in the angle centrifuge at high speed for 15 minutes. The total time from the addition of alkali to neutralization was one hour.

Results were assessed by one or more of the following methods: (*a*) by estimation of the volume of subcutaneous and intracutaneous lesions during life of the animals by the paraffin-ball method ([12]), or by weighing the microscopic lesions or whole organs at autopsy; and (*b*) by rating the relative numbers of bacilli in impression films from the cut surfaces of inoculated sites, or by counting the numbers of bacilli in tissue homogenates at known dilutions.

The data on the approximate numbers of bacilli on slides prepared from the cut surfaces of tissue were recorded by a series of plus-values ranging from 0 to 5, as shown in Table 1. Since it is impossible to comprehend on an arithemetical scale the extreme spread between the numbers of bacteria in early or minimal lesions and those in established lepromatous tissues, the plus-values did not permit appropriate presentation of the results. In order to overcome this difficulty, the plus-ratings were compared with actual counts obtained from the corresponding tissue homogenates. These observations indicated that the number of organisms observed per field or per square in impression films should be multiplied approximately 20 times in order to provide estimates in line with those obtained by actual count in homogenates. An average number was, therefore, assigned for the range of numbers corresponding to each plus value observed in the impression films, and this number was multiplied by 33.4

TABLE 1.—*Conversion of plus values from impression films into appropriate order of magnitude for estimating the concentration of bacilli in lesions.*

Plus values	Range of bacterial numbers observed in films	(1) Average number assigned	(2) Average number of bacilli per field	(3) Approximate number of bacilli per gram (billions)	Log of numbers
0	No bacilli in 10 minutes	0	0	0	
1+	< one per field, positive in 10 minutes	0.1	0.1	0.003	6.48
2+	1–29 per field	15.0	15	0.502	8.70
3+	1–5 per square [a]	3.0	90	3	9.48
4+	5–50 per square	28.0	840	28	10.45
5+	50–innumerable/square	100.0	3,000	100	11.00

[a] The microscopic field is divided into small squares, 30 of which would occupy the entire field.

million to provide an estimate of the number of bacilli per gram of tissue. The factor of 33.4 million is derived from the fact that each microorganism seen in films prepared for standard counting (a standard volume of suspension on a standard area of glass, dried, gelatin coated and formalinized prior to straining) represents 1.67 million bacilli per cubic centimeters of suspension. This factor is 20 x 1.67. The results of both the estimates from impression films and the counted homogenates are expressed in billions. The logs of these numbers ars shown.

In the absence of facilities for studying the pathology of lesions in sections, it was found that the differing types of cell response in the different tissues could be recognized remarkably well from impression smears provided the following precautions were observed:

(1) Preparation of multiple impressions from each tissue without lateral movement, in order to avoid disrupting cells;

(2) Heat fixation at 100°C for 3 minutes;

(3) Staining in carbol-fuchsin at 31°C for 10 minutes; and

(4) Simultaneous decolorization and counterstaining for five minutes in Gabbett's 1:5 (equivalent to 0.2% methylene blue in 5% sulfuric acid by volume).

MULTIPLICATION OF BACILLI IN VARIOUS ORGANS

Before presenting the data on bacterial concentrations in the various tissues, we shall summarize the pertinent literature on infection of each site, state the reasons why such tissues appeared of interest in this work, and comment on the practical problem of studying infection in these sites. In order that the reader may understand the necessity for a different method of tabulating the results in certain of the infected tissues it is necessary also to describe a few distinctive features of the relationships between the bacilli and the cells which are seen in impression films prepared from the various organs infected.

ANTERIOR CHAMBER OF THE EYE

Guilliny and Montestruc (9) concluded that Marchoux's strain of the murine leprosy bacillus was able to proliferate in the anterior chamber of the eye of rats at a rate comparable with the growth of tubercle bacilli. Nevertheless, a period approaching 11 months was required before any infected eyes were reported to have doubled in volume (5).

There are technical difficulties involved in the use of this organ as a routine inoculation site for quantitative purposes. The anterior chamber will accept but small amounts of inoculum, even when the aqueous humor is aspirated with a small volume of suspension in a 0.25 cc. syringe. It is necessary to take great care to avoid loss of fluid at the point of needle insertion, and it is necessary to calculate the actual number of bacilli delivered after the two fluids have been mixed. For these reasons inoculation of the anterior chamber was confined to rats.

To make quantitative estimations of the accumulation of bacilli within the eye, entire organs were enucleated at autopsy, weighed, cooked and homogenized, and the bacilli then enumerated. In order to study the rela-

tionships between cells and bacilli, fresh eyes were bisected in the sagittal plane with a razor blade; the fluids which escaped were collected and spotted on clean slides for study of the liberated cells and bacilli, while the two hemispheres were used to make contact impression films.

In both the liberated fluids and the impression films from those rats which developed a progressive infection, the bacilli could be classified fairly sharply into two types with respect to staining: (1) red bacilli and (2) purple or bluish bacilli. The majority of the bacilli found within monocytes and histiocytes stained red. These red bacilli often appeared to be variable in quality, many being in poor condition, particularly when occuring in the smaller monocytes, which were arranged in tight clusters or were undergoing transformation to giant cells. The purple or bluish bacilli, on the other hand, were solid in appearance and deeply stained. They were usually seen outside of cells and were often long or branching. Those found in the margins of the cytoplasm of large, stuffed, ghost-like histiocytes were markedly superior to the more centrally located red bacilli. They occasionally exhibited a tendency to radial arrangement, as though attempting to form free-growing microcolonies after disintegration of the host cells.

Most of the cells in the eye fluids were lymphocytes and small monocytes. Though the bacilli occurred only in cells regarded as monocytes, and only in a modest proportion of those cells, both types of cells were found in clusters around infected monocytes. From the quality and the numbers of cells of these two types it would appear that there was a continual influx of them into the infected anterior chambers. The smaller monocytes often possessed a reddish or purplish cytoplasm after staining. This color was in accordance with the staining reaction of the bacilli being digested. It was felt, therefore, that the generally poor development of leprous infection in the anterior chambers of these rats was related to the high incidence of these cells, to their phagocytic activity, and to their clumping tendencies. In the fluids of the more resistant rats the bacilli had disappeared completely and the cell reaction had subsided at the time of autopsy. Further comment on the relation between active cell response and resistance appears in the discussion.

A low proportion of cells was regarded as histiocytes. Almost without exception these cells contained bacilli in moderate to large numbers. The nuclei were small and pale, and the cytoplasm had lost its normally basophilic character. These cells unquestionably were seriously damaged by the bacilli which they

contained. It is our impression that they became a nidus in which the microorganisms flourished as purple-staining bacilli which often protruded beyond the cytoplasm boundaries of the dying cells.

BRAIN

Marchoux, Chorine and Koechlin [18] found that intracerebral inoculation of rats produced a widespread meningitis, and that within the brain the bacilli tended to fill only the perivascular cells. Sellards and Pinkerton [22], who reported that 18 of 22 mice died after 3½ to 5½ months with extensive lesions of the brain and with metastases to the spleen and less often to the liver, found that in rats there was marked multiplication of bacilli in the cord and spinal nerves after 9 months. Lowe [16] could find only a few bacilli in the brains of rats dying of generalized infection. Fite [7] observed no brain lesions following intravenous infection of 8 rats. In the foregoing work, only the observations of Sellard and Pinkerton afford a suggestion that this route of inoculation might produce a rapidly fatal disease.

It is generally recognized that the most reliable measurement of end-points in animal experimentation can be secured by determining deaths versus survivals. Bertrand, Bablet and Bloch [2] demonstrated the rapidity and precision with which human, bovine and avian tubercle bacilli can be differentiated by intracerebral inoculation of rabbits. Smithburn [23] showed that comparisons of the virulence of tubercle bacilli in guinea-pigs could be made more accurately by intracerebral than by other routes of inoculation. Pierce, Dubos and Middlebrook [20] have found more recently that intracerebral inoculation of tubercle bacilli into the *dba* strain of mice resulted in uniformly rapid deaths. Although at autopsy the brains of these animals were found to be heavily loaded with acid-fast bacteria there were no neurological manifestations, and it was concluded that death was due to dissemination of the disease to the lungs.

In view of the importance of finding a method of producing death as a reproducible end-point for experimentation in murine leprosy, further exploration of the intracerebral route appeared desirable. During the present study a series of tissue culture experiments was conducted with fragments of infected brain. The material for this work was obtained by systematic removal of the infected hemispheres of young rats and mice over a period of several weeks following inoculation. These brain samples revealed that the proportions of infected cells and the numbers of bacilli in the brains of young rats and mice declined rather steadily, until it seemed that further experiments would be unprofitable.

The next material seen was that studied at the time of autopsy of the animals concerned in this report. At that time the impression films, much to our astonishment, revealed in nearly every instance that the infected lobes of the brain contained increased numbers of microorganisms.

The findings in impression films from the brain differed from those in any other organ. Only a low proportion of the cells contained bacilli but, almost without exception, each infected cell was heavily loaded or "stuffed" with bacilli. For this reason we could not estimate the concentration of bacilli as readily as in the films from other tissues. The data, therefore, are summarized both on the basis of the incidence of such stuffed cells and on the concentrations which it was thought would be representative if the bacilli were liberated from such cells and distributed at random throughout the microscopic fields observed. The quality of the bacilli in the stuffed cells was uniformly excellent. It is not unikely that these infected cells correspond to the heavily loaded perivascular cells described by Marchoux *et al.* (18), and that the noninfected cells were those of the brain parenchyma.

PERITONEAL CAVITY

Stefansky (24) failed to obtain experimental transmission of the natural murine disease to other rats by intraperitoneal inoculation, but reported the significant observation that certain of the recipients had caseous pearls in the omentum after 44 days (see discussion). Lowe (17) mentions six investigations, including his own, in which intraperitoneal injection of bacilli resulted in the production of large omental or mesenteric lepromata. In Lowe's experience (16) these lesions developed more rapidly after intracardial than after intraperitoneal injection. Sellards and Pinkerton (22) noted that in some instances rats escaped infection after intraperitoneal inoculation, and on one occasion they lost the Walker strain of bacilli on this account. Their mice were more susceptible: 15 of 17 showed miliary lesions of the spleen and liver after six months, and frequently the omentum was studded with lepromata. Hadler and Mauri (10) have recently published a detailed study of intraperitoneal infection, in which it was concluded that this route of inoculation produces both a local and a generalized disease which progresses with no tendency to spontaneous regression.

While at Culion the senior author (J. H. H.) observed that tremendous intraperitoneal lepromata resulted from the intra-abdominal injection of the California strain of murine bacilli into the lot of rats then being maintained by Dr. H. W. Wade.[1] In explanatory experiments to ascertain the fate of the bacilli in the cells of the peritoneal fluid, 10 cc. of citrated balanced salt solution was injected intra-abdominally into one infected rat on successive occasions, and the aspirated fluids were employed as a source of cells for *in vitro* cultures. In the fluid obtained on the fourth day following infection the bacilli were found to be equally distributed between granular leucocytes and mononuclear phagocytes, occurring in 31 and 26

[1] This strain was obtained by Dr. Wade about 1933 through the courtesy of Dr. J. C. Geiger, Director of Health, San Francisco, and is believed to have been the Walker strain used by Sellards and Pinkerton and by Badger and Fite.

per cent, respectively, of the two classes of cells. After 11 days the concentration of cells in the exudate had fallen and only fractional percentages of the mononuclear cells contained bacilli. After 70 days there was unmistakable evidence that the bacilli were proliferating. Bacilli were found in 2 per cent of the granular leucocytes and in 19 per cent of the mononuclear cells, most of which appeared to be greatly enlarged histocytes with numerous bacilli. This animal had produced several grams of mesenteric leproma when autopsied after 18 weeks.

In the family of rats now being used, on the contrary, it has not been possible to induce intraperitoneal lepromata. The results of repeated inoculations of large numbers of bacilli over a period of three years have been consistently negative. It has made no difference whether the inocula were clarified supernatants of leproma suspensions or large coarse particles of lepromata, or whether the inoculations were repeated, or made with oil as an adjuvant, or made in rats maintained on the corn diet which Dubos and Pierce (6) found to enhance the susceptibility of mice to tubercle bacilli.

Certain of the animals in each of a series of inoculated groups have been autopsied from time to time, while others have been held for periods of about 10 months. Animals which received large doses of bacilli have presented caseous or calcified pearls and nodes in the omentum, similar to those produced in rats which have received large numbers of saprophytic mycobacteria (14), and to those described by Stefansky in his attempts to transmit murine leprosy to laboratory rats.

The most important result of such intraperitoneal inoculations has been the establishment of a latent infection in certain lymph nodes, particularly the mediastinal, mesenteric and axillary nodes, in descending order with respect to the degree of involvement.[2]

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[2] We are indebted to Dr. A. C. Mauri for having drawn our attention to certain interesting features of the lymph node infection. In the rats now used by us it has not been easy to demonstrate the long-persisting latent infection in lymph nodes following intraperitoneal inoculation. Direct search with the oil immersion objective does not, in most instances, reveal bacilli even after prolonged examination, whereas scanning large areas of impression film (which have been coated with immersion oil) with the low power objective reveals occasional clusters of epithelioid or giant cells with rosy cytoplasm. The oil immersion objective serves to confirm the presence of small numbers of bacilli in these characteristic cell groups. This type of relationship persists for month after month, with constant destruction of bacilli—as evidenced by the rosy cytoplasm of the cells—at a rate which appears comparable with their growth rate. A similar picture is found in nodes related to skin sites which remain latent for long periods of time. On the other hand, in lymph nodes receiving drainage from palpable

SUBCUTIS

This classical route of inoculation has been used so universally that a few comments will suffice. It appears to have been a general experience that the primary lesions in rats can be depended upon to appear at the site of inoculation rather than at any other point, and that dissemination from the inoculation sites to lymph nodes (7) and general dissemination (16) is slow. In susceptible mice, however, there is more tendency to dissemination (22, 7). It has also been our experience that in certain of the mice the extent of generalizations is much greater than might be anticipated from the size of the local lesions.

CUTIS

Lowe (16) produced marked skin lesions and glandular involvement by intradermal inoculation. However, Badger and Fite (1) observed that the Hawaiian strain had a greater predilection for subcutaneous tissue than for the overlying skin.

The infection of both rats and mice by the intracutaneous route was undertaken in our work with the thought that the more superficial location of the inoculum might facilitate earlier recognition of minimal lesions. Regardless of whether the inoculations were subcutaneous or intracutaneous, the resulting lesions were always on the under surface of the dermis; and consequently the intradermal inoculations did not facilitate the interim preparation of slides from scraped incisions as had been hoped. Although the actual method of inoculation will be indicated in each experiment, the resulting lesions will hereafter be termed simply "skin" sites or "skin" lesions.

Due to the toughness of the tissues in which these dermal lesions arose, impression film from a cut surface always showed a high proportion of bacilli outside of the ruptured cells. For this reason no attempt is made to describe the cell-parasite relationship in either subcutaneous or intracutaneous sites. The evaluation of bacterial concentrations in latent or questionable skin sites was possible only because of the reliability with which these areas were defined by the ink in the inoculum. The bacilli from the skin sites or nodules in rats occurred at lower concentrations than in mice, and they appeared to be of poorer quality.

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cutaneous lesions or other sites of active multiplication of bacilli, the simple groups of epithelioid cells are not maintained as uniformly as in the nodes described above. With continued drainage and accumulation of large numbers of bacilli, the cell clusters eventually give way to an accumulation of stuffed cells with damaged cytoplasm and decreased ability to destroy bacilli.

TESTIS

In spite of the remarkable tendency of human leprosy bacilli to proliferate and accumulate in the testes of lepromatous males, little interest has been taken in the experimental inoculation of this organ in the rat or mouse. Lowe (16) reported that the testes in his rats became involved late in the course of generalized infections, and at a time when the ovaries, fallopian tubes and uterus also showed massive involvement. Sellards and Pinkerton (22) reported tremendous numbers of bacilli in the epididymis of infected mice, while Pinkerton and Sellards (21) reported and illustrated infection of the testicular tubules following intrasplenic injection of bacilli. Fite (7) removed testes from two animals one year after inoculation and stated that these organs were a solid leprous mass with few tubules remaining.

The testes of the rat, like those of other mammals, have a remarkable mechanism for controlling their internal temperature. According to Imig (15) the temperature of the rat testes, as measured by thermocouples, tends to be 33.4°C. in environmental temperatures as low as 9°C., 34° under usual circumstances, and only 34.7° when the room temperature rises to 32.5° (90°F).

The testes offer a pair of exposed parenchymatous organs into which relatively large volumes of inoculum may be injected with ease. As the infection develops the tubules are gradually replaced with granulomatous tissue until they disappear completely, but it is only after prodigious numbers of bacilli have accumulated that there is any increase in the size of the organ. The progress of the infection must be recognized, therefore, by the preparation of impression smears or homogenates from biopsied organs rather than by palpation.[3] Since testicular lesions were removed from rats at various intervals as a source of experimental material, most of the estimates of bacterial concentrations in this report are based on actual counts in homogenates. These counts in some instances are somewhat below the true total concentrations, because they were made from supernates which had been clarified by slow-speed centrifugation.

NUMBERS OF BACILLI PRODUCED IN THE TISSUES UNDER CONSIDERATION

INTRACEREBRAL VERSUS SUBCUTANEOUS INOCULATION

Data which afford a comparison between the numbers of bacilli produced in the brains and in the subcutaneous tissues

[3] Dr. Wade has suggested the possibility of sampling the progress of testicular injections by aspiration with a small-gauge needle.

TABLE 2.—*Intracerebral (right lobe) versus subcutaneous inoculation of 125 million glycerinated bacilli into rats and mice.*

Animal			Intracerebral				Subcutaneous *c*		
No.	Age in weeks	Weeks before autopsy	Weight in grams	Infected cells *a*	Bacilli per gram *b*	Total bacilli	Leproma (grams)	Bacilli *b* per gram	Total bacilli
Rat									
1	2	26		5% st	0.5		0	0.5	
2	2	26		0%	0.0		0	0.5	
3	2	26		2% st	0.003		0	3.0	
4	2	26		1% st	0.003		0	0.5	
Mouse									
1	3	1		<1% st	<0.003		0	0.0	
2	3	8		<1% st	<0.003		0	3.0	
3	3	8		0%	0.0		0	3.0	
4	3	26	0.2	2% st	0.5	0.1	1.5	100.0	150
5	3	26	0.2	3% st	0.5	0.1	2.0	100.0	200
6	7	26	0.2	5% ST	3.0	0.6	2.2	100.0	220

a Bacilli per gram. All numbers in this and subsequent tables are expressed in billions
b Bacilli per cell: ST = very many ("stuffed"); st = many, but less heavily loaded.
c Two subcutaneous sites in each rat, averaged; one subcutaneous site in each mouse.

of rats and mice inoculated with 125 millions of glycerinated bacilli per site are assembled in Table 2. To simplify presentation, the concentrations of bacilli and the weights of lepromata in the two subcutaneous sites in each animal have been averaged. It may be seen that although the bacilli were present in the skin sites of the four rats in concentrations around one-half to three billion per gram of tissue, they had not produced palpable lepromata in any of the rats by the end of six months. Low proportions of cells containing moderate numbers of bacilli were found in the brains of only three of the four rats after six months, and the concentrations of bacilli tended to be lower than those in the skin. Since no weight in grams can be assigned to the cutaneous lesions in the rats, it is impossible to compare the total numbers of bacilli produced in the two tissues.

In mice, on the contrary, these bacilli had reached concentrations of approximately three billion per gram of subcutaneous tissue after eight weeks, and they produced palpable lepromata after 13 to 18 weeks. The superiority of the skin over the brain was revealed not only by the data in Table 2, but also by the fact that the presumably small numbers of bacilli deposited along the needle track to the brain produced 0.3 gram of subcutaneous lepromata in each of mice Nos. 5 and 6. Each of these unexpected nodules provided approximately 30 billion bacilli, or 50 times the estimated total of 0.6 billion in the brain of the most susceptible mouse, No. 6. The estimates of the numbers of bacilli produced by comparable inocula in the brain and the skin indicate that on the average the subcutaneous tissues produced approximately 700 times more bacilli than did the brains of the same mice.

INTRATESTICULAR VERSUS SUBCUTANEOUS, INTRACEREBRAL AND INTRAOCULAR INOCULATION

Four of the rats in Table 3 received the stated dose of glycerinated bacilli into each of two testes, two subcutaneous sites and the right lobe of the brain, while two were inoculated in the anterior chamber of the right eye rather than in the brain. The data obtained from rats Nos. 5-8 indicate that the brain was a poorer site than the skin while the results in rats Nos. 9 and 10 suggest that the anterior chamber of the eye was likewise inferior to the subcutaneous tissue for the production of bacilli.

It may be noted, however, that the concentration of bacilli in the testes of rat No. 5 was three billion after only 8 weeks, and that this concentration surpassed what was attained in any other

TABLE 3.—*Testicular versus subcutaneous and intracerebral or intraocular inoculation of 125 billion glycerinated bacilli.*[a]

Animal		Weeks before biopsy	Testes			Weeks before autopsy	Subcutis		Brain		Anterior Chamber	
No.	Age in weeks		Weight in grams	Bacilli per gram *b*	Total bacilli *c*		Leproma (grams)	Bacilli per gram *b*	Infected cells	Bacilli per gram *b*	Leproma (grams)	Bacilli per gram *b*
Rat												
5	12	8	1.0	3	3	26	0	0.5	0	0?		
6	12	14	1.1	32	35	26	0	0.003	0	0?		
7	12	21	1.0	230	230	26	0	0.003	0	0?		
8	12	26	1.4	265	371	26	0	0.5	1%st	0.003		
9	12	24	0.9	250	225	26	0	0.5			0	0.5
10	12	26	1.1	325	357	26	0	0.5			0	0
Mouse												
7	7	26	0.05	700	35	26	0.03	28	5%ST	3		

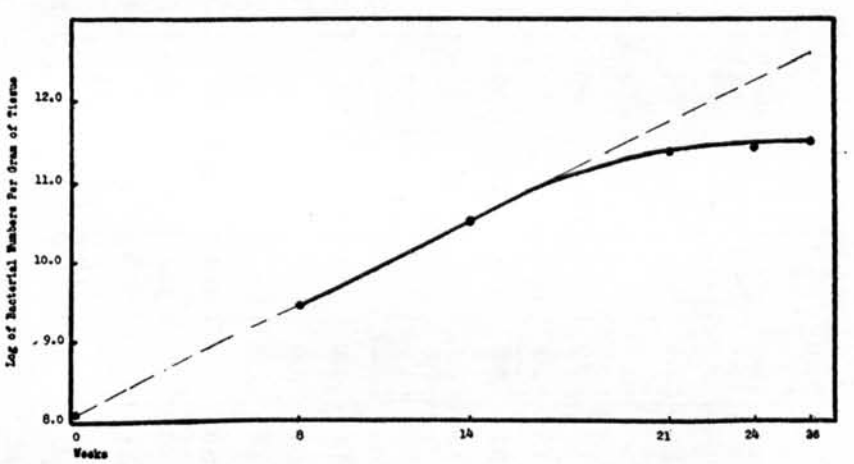
a Testes were biopsied at the intervals shown, while all lesions in other tissues were allowed to develop for 26 weeks. Two subcutaneous and 2 testicular sites in each animal; averaged results are shown.

b Bacilli per gram.

c Total bacilli in lesions.

tissues in this group of six rats during a period of 26 weeks. Furthermore, in organs weighing 1 gm. this concentration indicates even more rapid growth than in the skin of the more susceptible mice during the same interval. The estimates of three billion in the mice (see Table 2) represents tissue concentrations in the absence of visible lepromata. By the end of 14 weeks the concentration of bacilli in the testes of rat No. 6 was equivalent to that in the average rapidly developing subcutaneous nodule in this family of rats. The concentrations of bacilli in the testes, however, continued to increase until they had reached nearly 10 times the concentrations which could be demonstrated in the average subcutaneous leproma.

These data, plotted in Text-fig. 1, afford a rough idea of the rate at which murine leprosy bacilli may accumulate in a favorable organ. The logs of the numbers of bacilli inoculated and of those present at eight weeks and at 14 weeks fall on a straight line. During the period through 14 weeks the numbers of bacilli apparently doubled each 12.2 days. Thereafter the rate of multiplication declined.



TEXT-FIG. 1. Rate of accumulation of bacilli in the testes of rats Nos. 5-10, Table 3. The broken line indicates the anticipated concentration of bacilli if their numbers were to double each 12.2 days throughout the period of the experiment.

Although mouse No. 7 is for the moment a single example, it is included in Table 3 because it illustrates in the more susceptible species the superiority of the skin over the brain, and of the testis over the skin. The astronomical concentration of 700 billion bacilli per gram of testis is submitted as the most

TABLE 4.—*Testicular versus subcutaneous, intraocular and intracerebral inoculation of fresh and NaOH-washed bacilli into rats.*

Number of bacilli inoculated per site	Rat No.	Weeks before autopsy	Testes			Subcutaneous			Anterior chamber			Brain		
			Weight in grams	Bacilli per gram	Total bacilli	Leproma (grams)	Bacilli per gram	Total bacilli	Eye (g)	Bacilli per gram	Total bacilli	Rt. lobe (g)	Bacilli per gram	Total bacilli
2.8	11	22	1.0	148	148	1.8	28	50.4						
fresh	12	22	Cas *a*			1.2	28	33.6						
	13	22	1.2	500	600	3.0	50	150.0						
	14	22	7.8	240	1892	1.1	28	30.8						
Averages:			3.3	296	880	1.7	33.5	66.2						
Ratios: Weights			1.9			1								
Conc. bacilli				8.8			1							
Total bacilli					13.3			1						
14	15	30	1.1	100	110	0.05	28	1.40	0.1	0	0.0	0.8	0.003	0.0024
NaOH	16	30	0.7	100	70	0.02	28	0.56	0.1	0	0.0	0.6	0.5	0.30
washed	17	30	4.3	420	1806	2.5	50	125.0	0.2	43	8.6	0.6	5.8	3.48
	18	30	2.0	400	800	0.01 *b*	28	0.28	0.2	22	4.4	0.6	0.5	0.30
Averages:			2.0	255	696	0.65	33.5	31.8	.15	16	3.3	0.6	1.7	1.0
Ratios: Weights			3.1			1			.23			.92		
Conc. bacilli				7.6			1			.48			.05	
Total bacilli					21.9			1			.103			.03

a Caseous, contaminated.

b Weight assigned; lesion not visible.

remarkable concentration of mycobacteria which has yet been reported in any animal or human tissue.

This mouse was a litter mate of mouse No. 6 in Table 2 and exhibited larger cutaneous lesions than the latter at 18 weeks. The cutaneous lesions in mouse No. 7 regressed thereafter, and the average weight of the two lesions after 26 weeks was 0.03 gm. as shown in Table 3. Both lesions then had small ulcers, and they contained low ratios of bacilli to cells. With respect to the total numbers of bacilli produced in tissues of mice, it must be noted that the testes do not enlarge when heavily infected, and that they produce a total number of 35 to 40 billion bacilli whereas the larger skin lepromas may produce around 200 billion bacilli before they ulcerate.

In Table 4 are assembled the data from rats inoculated subcutaneously and intratesticularly with 2.8 billions of fresh bacilli, and in all four tissue sites with five times that number of NaOH-washed bacilli. It may be noted that the fresh bacilli were consistently capable of producing cutaneous lepromata in each of four rats within less than 22 weeks, and that in these lepromata the bacilli attained an average concentration of 33.5 billion per gram of tissue. The growth and accumulation of bacilli in the testes was, however, greatly superior, since the bacilli reached 9 times greater concentrations and the total numbers produced were 13 times greater. Although the NaOH-washed bacilli were used at five times greater concentration, they had produced, after 30 weeks, a palpable leproma in only one of four rats. In the testes the bacilli on the average had reached 7.5 times greater concentrations and the total crop was 22 times the number of bacilli produced by subcutaneous inoculation. Furthermore, the increased weights of the testes in four of the eight rats indicates that maximal concentrations of bacilli had existed in these organs for a long time prior to biopsy.

In the anterior chamber of the eye in the two more resistant rats (Nos. 15 and 16) inoculated with NaOH-washed suspension it was not possible to find bacilli, while the right eye in rats Nos. 17 and 18 had doubled in weight. Counts in the homogenates of these two eyes revealed concentrations of bacilli comparable to those in the skin lepromata. Although bacilli were found in the brains of these four rats more consistently than in the eyes, concentrations which could be counted in 10 per cent tissue homogenates occurred only in the most susceptible rat, No. 17. From the average ratios shown in the bottom section of Table 4, it would appear that the eyes tended to produce about 10 per cent

TABLE 5.—*Subcutaneous versus intracutaneous inoculation in rats.*

Rat				Subcutaneous			Intracutaneous		
Bacilli inoculated per site	No.	Age in weeks	Weeks before autopsy	Leproma (grams)	Bacilli per gram	Total bacilli	Leproma (grams)	Bacilli per gram	Total bacilli
14	19	12	30	0.05	28 *a*	1.40	0.01 *b*	28 *a*	0.28
NaOH	20	12	30	0.02	28 *a*	0.56	0.01 *b*	28 *a*	0.28
Washed	21	12	30	2.5	50 *a*	125.00	0.6	50 *a*	30.00
Averages:				0.86	35.4	42.3	0.21	25.6	10.2
2.8	22	12	22	1.1 1.0	35 *c* 35	38.5 35.0	0.3 0.1	22 *c* 22	6.6 2.2
fresh	23	12	22	1.8 1.8	50 50	90.0 90.0	1.2 0.9	30 30	36.0 27.0
	24	12	22	1.4 0.9	21 21	39.2 25.2	0.7 0.6	16 16	11.2 9.6
	25	12	22	2.6 3.6	50 50	130.0 180.0	0.3 0.1	28 28	8.4 2.8
Averages:				1.78	39	78.5	0.53	24	13.0
Ratios: Weights				1			0.3		
Conc. bacilli					1			0.6	
Total bacilli						1			0.17

a Microscopic estimates from impression films.

b In the absence of a visible leproma, this weight was assigned to permit calculation.

c Data for rats Nos. 22-25 are from homgenates of the two subcutaneous and the two intracutaneous lesions in each rat.

of the numbers of bacilli that developed after subcutaneous inoculation, and the brains about 3 per cent of the numbers developing in the subcutaneous lesions.

SUBCUTANEOUS VERSUS INTRACUTANEOUS INOCULATION

Data which compare the results of subcutaneous versus intracutaneous inoculation of fresh and NaOH-washed bacilli are summarized in Table 5. Rats Nos. 19-21 each received 14 billions of washed bacilli in a single subcutaneous and a single intracutaneous site. One of the three rats developed a palpable subcutaneous leproma within 30 weeks, while each of the other two presented small subcutaneous lepromata at autopsy. One intracutaneous lesion was palpable, while the corresponding sites in the other two rats were identified only by the carbon deposit. However, the concentrations of bacilli in these two sites corresponded to the numbers which characterized lepromata. The intracutaneous sites apparently produced about 25 per cent as many bacilli as the subcutaneous sites.

Rats Nos. 22-25 each received 2.8 billions of fresh bacilli in two subcutaneous sites. The weight of each leproma and the concentrations of the bacilli found in homogenates of the two subcutaneous and the two intracutaneous lesions from each rat after 22 weeks are shown. It may be seen that the weight of lepromata in subcutaneous sites in each instance exceeded that of the contralateral intracutaneous sites. On the average the intracutaneous sites yielded only 30 per cent of the leproma weight, contained only 60 per cent of the bacillary concentration, and produced *in toto* only 17 per cent as many bacilli as the subcutaneous sites.

DISCUSSION

The results of the present work provide the basis for a quantitative concept of the growth and accumulation of murine leprosy bacilli in different tissues of two animal species. However, there is no simple method of determining to what extent the apparent proliferation rates in each tissue may be influenced by the drainage of bacilli from lesion sites or by the effectiveness of phagocytic destruction. Therefore, the data do not indicate the actual or potential growth rate of murine bacilli, and must be regarded simply as a measure of the numbers of bacilli which may be produced and accumulated in the several tissues studied.

In terms of the rate of bacterial growth and accumulation, murine leprosy evolves more completely in a few months than

does lepromatous human leprosy in many years. The two diseases differ not only in the absence, in rodents, of "complete leprosy" (including involvement of peripheral nerves) but also in the fact that murine leprosy as reported by most investigators develops with great rapidity in the peritoneal cavity and the internal organs. Granting the great unlikelihood that the mixed or "complete" form of leprosy can be reproduced in rats or mice, it is of interest that the disease in the moderately resistant Wistar family of rats differs from the classical experimental murine leprosy in certain important respects, and that in these respects it more closely resembles the human disease.

The failure to produce progressive infection of the omentum, mesenteries, lymph nodes, liver or spleen following intra-abdominal injection is an example of the shift from the murine toward the human lepromatous type of syndrome. In these tissues the bacilli can at best maintain a latent infection following intraperitoneal inoculation. Furthermore, even when multiple lepromata in the skin and testes have discharged large numbers of bacilli into the lymphatics for several months, only an occasional rat of this strain will show macroscopic lesions or high concentrations of bacilli in the viscera. This relatively successful control of visceral infection cannot be attributed to an inability of the Hawaiian bacilli to proliferate at the body temperature of the rat, because Badger and Fite have described this strain as more virulent than either the Florida or the California strains when inoculated intraperitoneally into the rats. The Hawaiian strain is also able to disseminate successfully in the less resistant mice being maintained in this laboratory. The failure to produce a progressive intraperitoneal or lymphatic infection and the modification of the disease syndrome are attributed, therefore, to the resistance of the rats.[4]

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[4] It has often been stated that Stefansky's failure to transmit murine leprosy to laboratory rats was due to the short period during which he maintained the rats prior to autopsy. The observations of the senior author in the Culion rats, as well as much data in the literature, suggest that Stefansky's bacilli should have multiplied unmistakably in the peritoneal cavity during the period of his observations, had the rats been highly susceptible. On the basis of his description of the omental response in the rats which he employed, and of our experience in the present family of rats, it may be concluded that he dealt with resistant animals. It is thought unlikely that intraperitoneal infections might have succeeded even after prolonged latency. Certain pathological features described by Gavrilov, Dubois and Fester (8) in connection with their difficulties in the transmission of murine leprosy indicate that they also dealt with a high level of resistance of certain of the rats inoculated.

A second outstanding feature of the human disease is its tendency toward latency. The ability of the rats to restrain the infection in this state for approximately one year, whenever glycerinated or alkali-washed bacilli were inoculated into or beneath the skin (13) provides an interesting example of the delicate balance between latency and effective accumulation in leprous infection. This phenomenon must be attributed to the relative resistance of the cutaneous tissues in the rats, since these modified bacilli produced lesions in the skin of the more susceptible mice in much shorter periods of time. Furthermore, there appears to have been no important lag prior to growth in the testes of the same rats, and even less in the testes of the mice. From this and a previous study it would appear that measurable degrees of latency can be induced experimentally in these rats: (*a*) by use of bacilli derived from mice; (*b*) by including blood-cell debris in the inoculum; (*c*) by the natural or acquired resistance of the rats (12); and (*d*) by slight modifications in the quality of the bacilli. This latency can also be influenced by the resistance of the tissues into which the bacilli are inoculated. If these factors can be demonstrated to influence the fulminating infection of murine leprosy, it is conceivable that their importance is greatly magnified in the case of the human leprosy bacillus and the far more resistant host species, man.

From the data now accumulated on the concentrations of bacilli required to produce recognizable lesions in man, in the rat, and in the mouse, it would appear that the simplest concept which takes into account the varied levels and manifestations of resistance or susceptibility in leprosy is the ratio of cells or tissue to bacilli. In human tuberculoid lesions this ratio may be extremely high. In the average lepromatous case each gram of nodule tends to contain around 2 billions of bacilli (11); a leproma from an exceptionally susceptible individual contained 7 billions per gram. The subcutaneous lesions of the rats in this study contained from 16 to 50 billions, while lepromata in mice contained 70 to 100 billions per gram.

Furthermore, the different degrees of response to lepromin can be interpreted on the same basis. The recognized cutaneous lesions in humans who react positively to lepromin represent considerable accumulations of cells in the presence of very few demonstrable bacilli. These persons respond with increased cell infiltration and a small granuloma when inoculated with less than 10 millions of bacilli (the approximate number in 0.1 cc. of lepromin prepared from a nodule containing 2 billions of bacilli

per gram). Bacteriologically positive cases tend to show a lower incidence of positive lepromin reactions. Lepromatous individuals usually fail to respond to the numbers of bacilli provided in the lepromin dose. From existing data on the concentrations of bacilli in nodules (21), it appears that lepromatous individuals may require the general order of a billion bacilli to cause augumentation of tissue.

With respect to the concentrations of bacilli produced in the several tissue of the rodents studied, these tissues may be arranged in the following ascending order of excellence: peritoneal cavity, brain, eye, cutis, subcutis, and testis. In all the sites compared in the two animal species, the bacilli reached higher concentrations in the mice, which were susceptible, than in the rats, which were more resistant. Within the tissues of a single animal species there is also exhibited an orderly relationship between the extent of new cell accumulation and the resistance of the infected tissues. The lesions in the anterior chambers of the rats on the average revealed low concentrations of bacilli and a preponderance of noninfected cells. The more susceptible dermal tissues permitted slow bacterial accumulation to the general order of 16 billions of bacilli per gram before tissue volume increased perceptibly. In the highly susceptible testes concentrations of several hundred billions of bacilli per gram were reached long before the organs increased in size.

The inferiority of the intracutaneous route by comparison with the subcutaneous one in the present work is thought to have been due to two factors: (*a*) the extreme density and toughness of rat skin inhibited progress of the infection until a locus had been established on the undersurface of the skin, just as after subcutaneous inoculation; and (*b*) by this time the resistance of rats inoculated in multiple sites had been increased to the point where normal development of lesions was definitely restrained. Other evidence of the role of immunization has been presented earlier (12). In the present work, the small delayed lesions resulting from intracutaneous inoculation always caseated or ulcerated at the same time as the larger lesions initiated by subcutaneous inoculation. This phenomenon affords another illustration of the influence of increased resistance during lepromatous infection.

The fact that the testis is the favored site for the development of the murine leprosy bacillus cannot be explained solely by the lower temperature of this organ. The anterior chamber of the eye is also a low-temperature region, and it is the only body site to

which testicular tissue has been transplanted without interfering with spermatogenesis (25). At the moment there is also no basis for concluding that the bacilli find superior nutritional conditions in the testis. There is, however, evidence that the factor of resistance may play a more important role in the skin than in the testis. Pearce and Brown (19) found that the vigorous granulomatous reaction of the skin and subcutis to their tumor —originally cutaneous—was a manifestation of active resistance which they were able to circumvent by intratesticular inoculation. Cheever and Janeway (4) mention two instances of simultaneous inoculation by intracutaneous and intratesticular routes; no intracutaneous tumor developed in one rabbit while three skin tumors regressed in the other, yet in both rabbits the tumors developed in the testes. More recently Casey, Myers and Drysdale (13) have shown that New Zealand white rabbits were highly resistant to subcutaneous inooculation (53% regressions and 14% mortality) but were susceptible to intratesticular inoculation (12% regressions and 63% mortality). The growth and metastasis of the tumor following subcutaneous inoculation was inhibited by a developing immunity, while there was no evidence that developing resistance could inhibit the growth of testicular tumors.

SUMMARY AND CONCLUSIONS

1. Murine bacilli were inoculated into several tissue sites in rats and mice. With respect to lesion production and the numbers of bacilli recovered, the tissues studied may be arranged as follows: brain < eye < cutis < subcutis < testis. The testis was the most favorable organ for the development of the bacilli of murine leprosy.

2. Greater concentrations of bacilli were produced in the respective organs of the susceptible mice than in those of the more resistant rats.

3. The disease syndrome in the moderately resistant rats used differed in several respects from that in the mice, or in rats as described by other workers. The infection did not disseminate readily from skin or testicular lepromas; progressive visceral or lymphatic infection could not be produced by intraperitoneal inoculation; subcutaneous inoculation of bacilli which had been modified by refrigeration in glycerol or had been washed in dilute NaOH resulted only in the production of latent lesions. This shift from the classical murine toward the human lepromatous type of syndrome suggests that the experimental

disease could be exploited more profitably than in the past if appropriately selected animals were used in experiments intended to shed light on human leprosy.

4. The data now available on the ratio of cells or tissue to bacilli in leprous lesions of man, the rat, and the mouse indicate that this ratio is a universal expresion of the varied levels of resistance and susceptibility.

REFERENCES

1. BADGER, L. F. and FITE, G. L. Leprosy: Variations in the virulence of strains of rat leprosy. Nat. Inst. Health Bull. No. 173, 1940, pp. 77-83.
2. BERTRAND, I., BABLET, J. and BLOCH, F. Sur l'inoculation intracérébrale expérimentale envisagée comme moyen d'indentification des différents types de bacilles de Koch. Ann. Inst. Pasteur **59** (1937) 231-258.
3. CASEY, A. E., MEYERS, L., and DRYSDALE, G. R. Intratesticular versus subcutaneous transplantation of Brown-Pearce tumor in New Zealand whites. Proc. Soc. Exper. Biol. & Med. **69** (1949) 576-579.
4. CHEEVER, F. S. and JANEWAY, C. A. Immunity induced against the Brown-Pearce carcinoma. Cancer Res. **1** (1941) 23-27.
5. CHORINE, V., GUILLINY, R. and MONTESTRUC, E. Inoculation de bacilles de Stéfansky dans la chambre antérieure de l'oeil du rat. Compt. rend. Soc. Biol. **116** (1934) 1261-1262.
6. DUBOS, R. J. and PIERCE, C. The effect of diet on experimental tuberculosis of mice. American Rev. Tuberc. **57** (1948) 287-293.
7. FITE, G. L. II. Leprosy: The pathology of experimental rat leprosy. Nat. Inst. Health Bull. No. 173, 1940, pp. 45-76.
8. GAVRILOW, W., DUBOIS, A. and MME. FESTER. L'infection expérimentale du rat par le bacille de Stefanski observée en Belgique. Internat. J. Leprosy **6** (1938) 515-527.
9. GUILLINY, R. and MONTESTRUC, E. Étude sur la lèpre oculaire murine. Bull. Soc. Path. exot. **26** (1933) 901-905.
10. HADLER, W. A. and MAURI, A. C. Lepra murína: Estudo patogenico-evolutivo no rato inocúlado por via peritoneal. Rev. brasileira Leprol. **16** (1948) 137-163; *reprinted,* THE JOURNAL **18** (1950) 67-79.
11. HANKS, J. H. A note on the numbers of bacilli which may occur in leprous nodules. Internat. J. Leprosy **13** (1945) 25-26.
12. HANKS, J. H. Three factors which may influence the transmission of leprosy. Internat. J. Leprosy **18** (1950) 33-48.
13. HANKS, J. H. To be published.
14. HANKS, J. H. and SOULE, M. H. An attempt to confirm an "*in vitro* cultivation of rat leprosy organism." J. Infect. Dis. **85** (1949) 225-229.
15. IMIG, C. L. Personal communication, 1949.
16. LOWE, J. Studies in rat leprosy. Indian J. Med. Res. **22** (1934) 187-198.

17. LOWE, J. Rat leprosy; a critical review of the literature. Internat. J. Leprosy **5** (1937) 311-328 and 463-482.

18. MARCHOUX, E., CHORINE, V. and KOECHLIN, E. Le bacille de la lèpre et le système nerveux. Ann. Inst. Pasteur **59** (1937) 549-576.

19. PEARCE, L. and BROWN, W. H. Studies based on a malignant tumor of the rabbit. II. Primary transplantation and elimination of a co-existing syphilitic infection. J. Exper. Med. **37** (1923) 631-645.

20. PIERCE, C., DUBOS, R. J. and MIDDLEBROOK, G. Infection of mice with mammalian tubercle bacilli grown in Tween-albumin liquid medium. J. Exper. Med. **86** (1947) 159-174. Also DUBOS, R. J. Personal communication, 1949.

21. PINKERTON, H. and SELLARDS, A. W. Histological and cytological studies of murine leprosy. American J. Path. **14** (1938) 435-441.

22. SELLARDS, A. W. and PINKERTON, H. The behavior of murine and human leprosy in foreign hosts. American J. Path. **14** (1938) 421-434.

23. SMITHBURN, K. C. The standardization of longevity against dose in experimental tuberculosis by intracerebral inoculation. J. Exper. Med. **64** (1936) 771-790.

24. STEFANSKY, W. K. Eine lepraähnliche Erkrankung der Haut und der Lymphdrüsen bei Wanderratten. Centralbl. f. Bakt., Orig. **33** (1903) 481-487.

25. TURNER, C. D. Intra-ocular homotransplantation of prepuberal testes in the rat. American J. Anat. **63** (1938) 101-149.