Experimental Murine Leprosy: Growth of *Mycobacterium lepraemurium* in C3H and C57/BL Mice After Footpad Inoculation

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Forty-three female C57/BL and C3H mice were inoculated with 2.7×10^6 *Mycobacterium lepraemurium* into each hind footpad. The foot thickness and the number of acid-fast bacilli in the footpad and popliteal and inguinal lymph nodes were recorded. In addition the morphological index and the mean bacillary length were determined in the footpad and in the popliteal lymph node. The bacilli multiplied in both strains during the first 4 weeks after inoculation. After that time no further increase in acid-fast bacilli was observed in the C57/BL strain; the bacilli became elongated and the morphological index decreased. These changes were preceded by a local swelling of the footpad due to the onset of an immune reaction. Thus, under the present conditions, C57/BL mice were able to resist experimental infection with *M. lepraemurium* by developing an immune response. In C3H mice no indication of an immune reaction was detected, and the bacilli continued to multiply throughout the observation period. The mouse footpad model seems to provide an excellent basis for the use of experimental murine leprosy to study immunity to mycobacterial infections. Certain aspects of the present model are discussed in relation to the mouse footpad model as used in the study of *M. leprae* infection in mice.

Human leprosy is characterized by great variation in the ability to mount and maintain a cell-mediated immune response against *Mycobacterium leprae* and thereby to control the infection. Patients with tuberculoid leprosy exhibit strong cell-mediated immunity (CMI) against *M. leprae* (2, 11, 13); they have few lesions in which few or no bacilli are found. Patients with lepromatous leprosy are devoid of CMI against the bacilli (2, 11, 13); these patients harbor many lesions which contain vast numbers of leprosy bacilli. Between the two polar types there is a continuous spectrum of intermediate forms classified as borderline leprosy (18, 23). Although CMI is believed to play a major role, the basis for the variation in host resistance against leprosy is at present poorly understood.

Efforts have been made to establish an experimental model closely reproducing the spectrum of human leprosy. Inoculation of *M. leprae* into the footpads of mice leads to a limited multiplication of the bacilli terminated by an immune reaction after several months (19, 24). The histological features of the lesion arising at the inoculation site resemble human borderline leprosy (22). In the thymectomized irradiated mouse model (21), disseminated growth of the bacilli occurs after footpad inoculation and in many respects the infection mirrors human lepromatous leprosy. However, the spectrum that may be established by combining these two models does not seem suitable for studying naturally occurring variation in host resistance.

Presently it seems necessary to search for alternative experimental models involving infection with bacteria other than *M. leprae*. Since leprosy is unique among human infections in that it develops over a period of years rather than days or weeks, time is a paramount factor which should not be ignored (22). The only bacterium known so far to have a generation time similar to that of *M. leprae* is *Mycobacterium lepraemurium* (MLM). This fact alone seems to make murine leprosy preeminent as an experimental model for human leprosy.

It has previously been demonstrated that outbred mice vary markedly in their response against MLM (4, 5), whereas mice of an inbred strain all respond in a rather uniform manner (5). Furthermore, significant differences may be observed between various inbred strains of mice (5, 16), showing that resistance to murine leprosy is determined by genetic factors of the host. The types of infection developing in C3H and C57/BL mice seem to represent polar forms of murine leprosy. After subcutaneous injection of MLM, histological examination indicated that in both strains the infection proceeded in a similar manner for the first 4 weeks. At that time a granulomatous reaction developed at the injection site in C57/BL mice (6). This reaction, which presumably is due to development of CMI against the bacilli, was strong enough in

most instances to suppress the infection and keep it localized. In C3H mice no signs of host reaction were observed, the bacilli continued to multiply, and, eventually, the animal died from disseminated infection (7).

In the present work the multiplication of MLM was studied quantitatively in C57/BL and C3H mice, and the effect of the host reaction in C57/BL on bacillary morphology was observed. To inject the bacilli into the footpad clearly represented an improvement of the experimental model as compared with earlier studies (5, 6, 7, 16).

MATERIALS AND METHODS

Experimental animals. Female mice of the inbred strains C3H/A and C57/BL/6J were obtained as specific pathogen-free animals from Gl. Bomholtgård Ltd., Denmark. They were kept in cages, 16 to 20 mice in each, fed on pellets (Norwegian Standard Stock no. 1, mice and rats), and allowed tap water ad libitum. The animals weighed 14 to 18 g at the beginning of the experiment.

Propagation of bacilli and preparation of the inoculum. MLM (Douglas strain) were propagated in the outbred mouse strain NMRI. The animals were given approximately 10^9 bacilli intraperitoneally and bacilli were harvested from the liver 16 to 20 weeks later. The bacilli were isolated from the liver by differential centrifugation as previously described (5), resuspended in 0.15 M NaCl, and left for sedimentation at $1 \times g$ for 2 h to remove large clumps of bacilli. A suitable dilution was then prepared; the bacilli were counted as described below and immediately used for injection.

Experimental inoculation and harvesting of bacilli. Using a 100-μl syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) fitted with a 27-gauge needle, 10 μl of a bacillary suspension containing 2.7 $\times 10^8$ bacilli/ml was injected into both hind footpads, resulting in a total dose of 5.4×10^6 bacilli/mouse. Forty-three animals of each strain were inoculated. Immediately after each injection, the needle path was sealed with plastic spray (Nobecutan, AB Bofors, Nobel-Pharma, Sweden) to avoid leakage of the inoculum. At various times after inoculation four mice of each strain were arbitrarily selected and killed with ether, both hind feet were cut off with scissors, and the popliteal and inguinal lymph nodes were removed under a dissection microscope. The toes and the dorsal part of one foot were then removed with scissors; the rest, including the footpad, was placed on a piece of paraffin wax (Tenax Wax, S.S. White Dental MFG Co., Philadelphia, Pa.) under the dissection microscope, and a drop of distilled water containing 0.1% bovine serum albumin and 0.02% NaN$_3$ was added. The metatarsal bones were removed and the remaining tissue was cut into small pieces with a razor blade and, in a total volume of 1 ml, transferred to a small tube by means of a Pasteur pipette. One footpad and one of the popliteal and inguinal lymph nodes were fixed and prepared for histological studies (O. A. Haugen and O. Closs, to be published). From those animals killed 100 and 226 days after inoculation, the retroperitoneal lymph nodes and, from the latter group of animals, the spleen also were removed for bacterial counting.

Counting of bacilli. The tissue was homogenized in a 2-ml Tenbroeck, all-glass tissue grinder (Bellco Glass Inc., Vineland, N.J.). Initially, the footpad tissue was homogenized in 1 ml; later it was diluted according to the expected number of bacilli. In the early harvests the popliteal and inguinal lymph nodes were pooled separately and homogenized in a total volume of 1 ml to enable the counting of the low numbers of bacilli present. From 11 weeks on, each popliteal node from the C3H mice was homogenized separately. The bacilli were counted by a slide technique (14). A small volume (0.004 ml) of the homogenate was spread over an 8-mm-diameter circle on a glass slide using a calibrated platinum loop. After fixation by gentle heating and 5 to 10 min in formol vapor, the preparations were stained by Ziehl-Neelsen's method. They were examined under oil immersion using a $\times 100$ objective and a $\times 10$ eye piece. Usually one diameter across the preparation was counted and the number of bacilli per milliliter of homogenate was computed by multiplying with the factor F = (1/loop volume) $\times$ (area of preparation/area examined) = 1.5 $\times 10^4$. If the number of bacilli on the slide was high, only eight microscope fields were counted, two at the periphery and six across the central part. Since the bacilli were often more numerous at the periphery, the counts in the peripheral and the central fields were computed separately. The counts were done on coded preparations and the code was not broken until all the preparations from one harvest had been counted. Doubling time (t_D) for the bacilli in various tissues was calculated from the formula $t_D = 1n\ 2/([1nB_t - 1nB_0]/t)$, where B denotes the amount of bacilli and t the time.

Morphological evaluation of bacilli. The morphology of the acid-fast bacilli (AFB) recovered from the footpads and popliteal lymph nodes was studied at various times after inoculation with a light microscope (Leitz Ortholux with objective P1 100/1.32 and eyepiece Periplan $\times 10$). Three categories were recognized: (i) solid-staining bacilli; (ii) granulated bacilli with a more or less distorted outline in which the cytoplasm showed an irregular pattern of clumps, vacuoles, and frequently empty segments; (iii) bacilli which could not be classified as either (i) or (ii). The morphological index (MI) was calculated as the percentage of solid-staining bacilli, usually based on examination of 200 bacilli. When bacilli were scarce, as in most of the popliteal lymph nodes, the number of bacilli examined per preparation could be as few as 40 or 50.

Length of bacilli. The length of the Ziehl-Neelsen-stained bacilli was measured with an ocular micrometer, each scale unit measuring 0.82 μm; 50 bacilli were measured per preparation. ·

Measurement of foot thickness. In collaboration with B. Amundsen, a special measuring device was constructed based on the Minikator model C-31 (C. E. Johansson AB, Eskilstuna, Sweden). This instrument is equipped with a spring which exerts an even pressure throughout the scale of measurement. The original spring was exchanged for a weaker one to avoid compression of the foot during the measurement. A holder was attached to the Minikator designed to fit around the dorsum of the foot and leaving the plantar surface accessible to the sensor of the instrument, which was moving in the horizontal plane. Calibration was carried out by inserting a cylindrical steel bar 2 mm in diameter into the foot
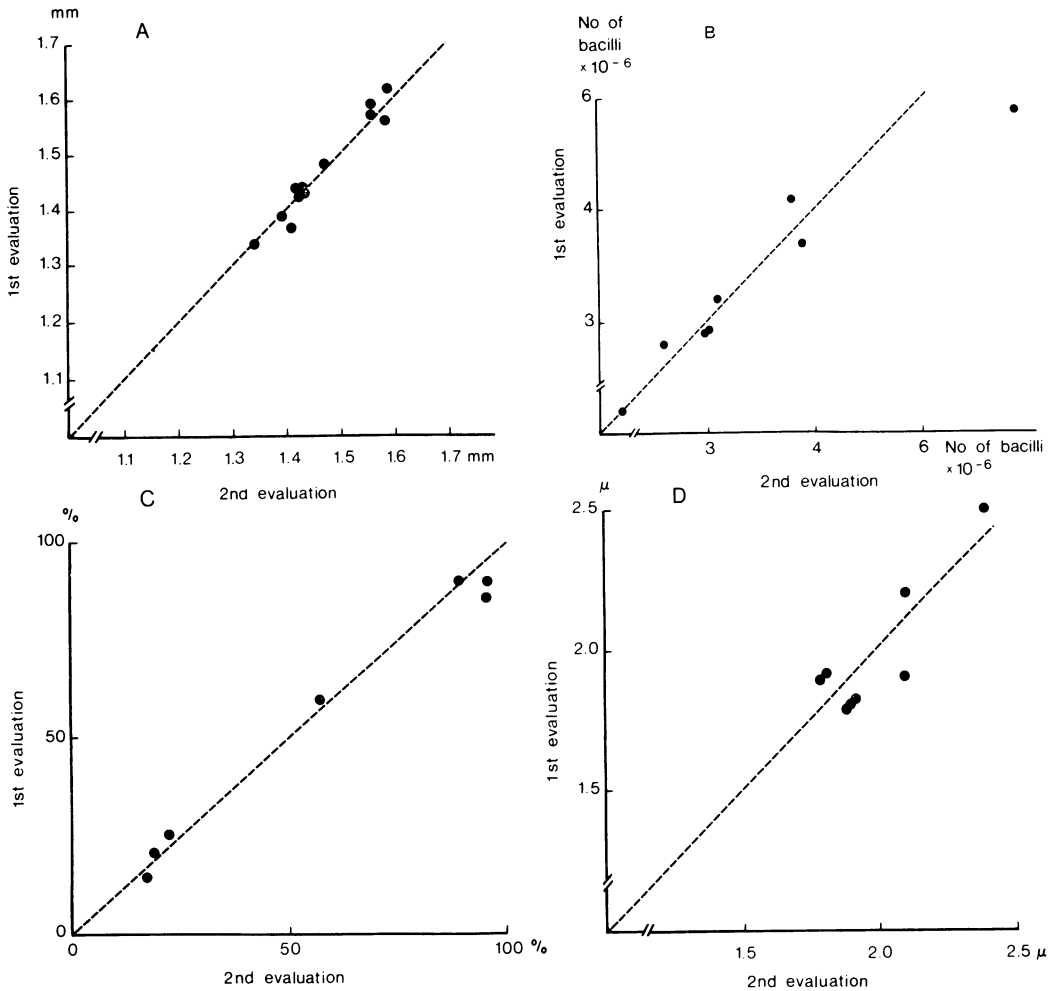
FIG. 1. *Reproducibility of experimental methods.* (A) *Foot thickness; each point represents the mean of five successive measurements.* (B) *Number of bacilli; evaluation of two different preparations made from the same homogenate. Between 200 and 400 bacilli counted per preparation.* (C) *MI; each point is based on the examination of 200 bacilli.* (D) *Length of bacilli in micrometers; each point represents the mean of 50 measurements of individual bacilli.*

holder. (More detailed information about the apparatus is available from the author). The mouse to be measured was held by an assistant and the readings of five successive measurements were recorded, removing the foot from the holder between each measurement. If the foot was not properly relaxed the process sometimes had to be repeated.

Reproducibility. To test the reproducibility of the methods used to measure foot thickness, count bacilli, determine MI, or estimate the mean bacillary length, the preparations were reexamined after several weeks by a blind technique. The results have been plotted in Fig. 1. The method error(s), i.e., the standard deviations of these parameters, were computed from the usual formula $s = \sqrt{\Sigma d^2/2n}$ (9), where d denotes the difference between duplicates and n the number of pairs compared. The standard deviations were: foot thickness, 0.014; counts of bacilli, 0.25; MI, 2.88; and mean length of bacilli, 0.08. The coefficient of variation, $v = s/\bar{x}$ (9), was found to be 0.9, 7.4, 5.4, and 3.9%, respectively. As regards the counting of bacilli,

the values are based on preparations made from the footpad. In the popliteal and inguinal lymph nodes where a very small number of bacilli were found in the early harvests, the coefficient of variation was considerably larger, i.e., 20 and 125%, respectively.

RESULTS

The local reaction to the injection 2.7×10^6 MLM in the footpads of C3H and C57/BL mice was monitored by measuring the foot thickness at weekly intervals for the first 4 weeks and every 2 or 3 weeks thereafter. The results are shown in Fig. 2. In both strains some residual swelling occurred on day 1 after inoculation but very little remained on day 2. Thereafter there appeared to be a transient period with increased swelling in both strains. After 2 weeks the foot thickness had returned to normal in the C57/BL mice. At 3.5 weeks it increased again very
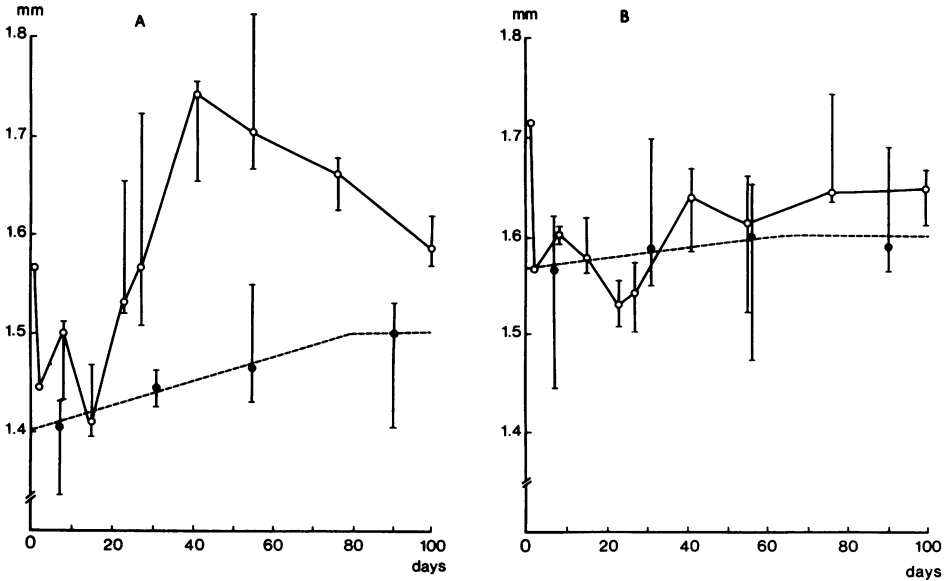
Fig. 2. *Foot thickness in normal mice after injection of 2.7 × 10⁶ MLM. (A) C57/BL mice; (B) C3H mice. Symbols: O, infected animals; ●, normal controls. The range and median of four observations are indicated.*

significantly to reach a maximum 6 to 8 weeks after inoculation, signifying that a local reaction was taking place at the inoculation site. Then it started to decrease and approached its normal size after 14 weeks. In the C3H mice there appeared to be no significant deviation from the normal trend during the period from 3 to 14 weeks after inoculation, and thus there was no indication of any local reaction in this strain.

On the average, 1.6 × 10⁶ of the 2.7 × 10⁶ bacilli injected on day 0 were recovered as AFB from the footpads of the four C57/BL animals killed after 24 and 48 h, representing a recovery of about 60%. Somewhat fewer bacilli were recovered from the C3H mice at 24 and 48 h, the mean recovery being 10⁶ AFB or 37%. As shown in Fig. 3, the number of AFB per footpad increased in both strains during the following 4 weeks. In the period from 1 to 4 weeks the multiplication rate seemed to be somewhat faster in C57/BL than in C3H mice, the calculated doubling time for bacilli in the footpad being 10.5 and 13.8 days, respectively (Table 1). In C57/BL mice a sudden change occurred in the growth of MLM 4 weeks after inoculation: the number of AFB did not increase any further, and after remaining at the same level for 4 weeks, the number dropped to about half and remained remarkably constant until the study was terminated at 32 weeks. In contrast, the growth of MLM in C3H mice continued for the whole observation period although the rate appeared to be reduced after 6 weeks when the mean doubling time increased markedly in the footpad (see Table 1).

The number of AFB found in the popliteal lymph node 1 week after inoculation represents only about 2% of the number that was found at the same time in the footpad. As shown in Fig. 4, the number of bacilli in the popliteal node followed a different pattern from that observed in the footpad. Because there was some difficulty in finding the node during the first harvest, these results have been excluded. In the popliteal lymph node there seemed to be insignificant multiplication of bacilli in both strains from 1 to 4 weeks after inoculation. Then the number of bacilli started to increase very rap-
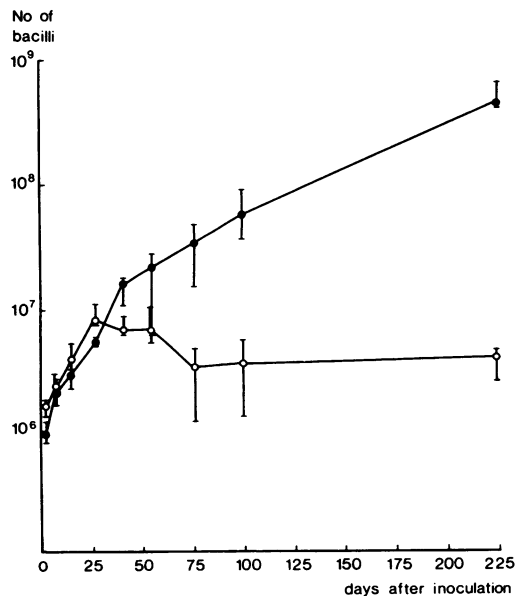

Fig. 3. *Multiplication of MLM in the footpads of mice. Symbols: ●, C3H mice; O, C57/BL mice. The range and median of four observations are shown.*

TABLE 1. *Doubling time in days for MLM in the footpad of C57/BL mice and in the footpad and various lymph nodes of C3H mice*

Time after inoculation (days)	C57/BL footpad	C3H			
		Footpad	Popliteal lymph node	Inguinal lymph node	Retroperitoneal lymph node
8–15	9.2[a]	15.8[a]			
15–27	11.1[a]	12.6[a]			
8–27	10.3[b]	13.7[b]			
27–41		8.9	7.7		
41–76		31.5	9.6		
76–100		32.4	7.7	9.6	
100–226		42.1	18.2	13.3	10.0
27–100		21.3	8.5	15.2	
8–226		27.7	13.5	15.3	

[a] $P = 0.04$ (Wilcoxon-van Eltern test).
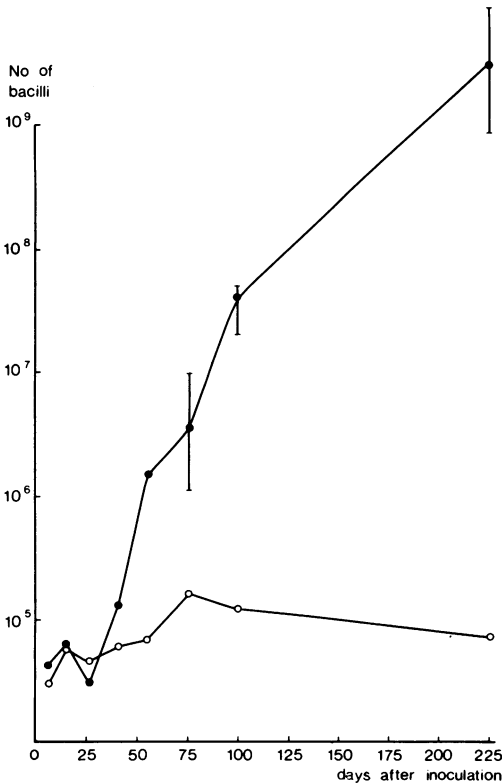[b] $P = 0.05$ (Wilcoxon test).

FIG. 4. *Multiplication of MLM in the popliteal lymph node of C3H and C57/BL mice after footpad inoculation. Pool of four lymph nodes counted except in C3H at days 76, 100 and 226, where the median and range of four individual counts are indicated. Symbols as in Fig. 3.*

idly in the C3H strain and eventually reached a number which was nearly 10 times higher than in the footpad. Eleven weeks after inoculation the two sites contained approximately the same number of bacilli. As shown in Table 1, the doubling time for MLM was considerably shorter in the popliteal lymph node than in the footpad during the period from 4 to 11 weeks. After 11 weeks the number of bacilli continued to increase at a faster rate in the node than in the footpad but the doubling time was then prolonged in both sites. Regarding the C57/BL strain there was a slight increase in the number of AFB in the popliteal node up to 11 weeks and a slight decrease after that time, but definite evidence of bacterial multiplication was never found. Both in the beginning and in the end of the observation period the amount of AFB found in the node was about 2% of that found in the footpad. During the period when the bacilli multiply most rapidly in the footpad, this percentage fell to 0.5, indicating that growth of MLM in the footpad was not paralleled by growth of the bacilli in the popliteal node.

In both strains the number of AFB found in the inguinal lymph node initially was about one-tenth of that found in the popliteal node. As shown in Fig. 5, the number of MLM remained fairly constant and approximately the same in both strains until about 11 weeks when it started to increase in the C3H strain. The mean doubling time for MLM at this site between 11 and 32 weeks was 11.0 days (see Table 1). Although the values are less exact than those for the counts in the popliteal node or the footpad, the curve for the inguinal node indicates that the multiplication starts at a later time here than in the popliteal node (see Fig. 3). Thus, although bacilli were already found in the popliteal and inguinal lymph nodes 24 h after inoculation, their multiplication was delayed for 4 weeks in the popliteal lymph node and, possibly, for as long as 11 weeks in the inguinal lymph node.

The retroperitoneal lymph nodes were examined only after 14 and 32 weeks, but the number of MLM was found to increase quite rapidly during this period, the mean doubling time

No of bacilli

days after inoculation

Fig. 5. *Multiplication of MLM in the inguinal lymph node of C3H and C57/BL mice after footpad inoculation. Pool of four lymph nodes counted. Symbols as in Fig. 3.*

being 10.0 days.

At 32 weeks, when the experiment was terminated, AFB were counted in footpad, popliteal, inguinal, and retroperitoneal lymph nodes, and spleen, to compare the full extent of the infection in the two strains at this late stage. The results (Table 2) show very pronounced differences between the number of bacilli found in C3H and C57/BL mice at all the sites examined. It should be noted that the footpad is the site where the difference between the two strains is least pronounced. Since the spleen and the retroperitoneal and inguinal lymph nodes may receive bacilli from both of the injected footpads, the counts in these organs were divided by two when calculating the overall doubling time for the amount of bacilli injected into one footpad, i.e., 2.7×10^6 MLM.

During the counting of bacilli it was observed that the morphology of MLM differed at various stages of the infection. Such morphological changes were particularly pronounced in C57/BL mice. During periods of exponential growth the MLM were typically seen as straight or slightly curved rods 1 to 3 μm long and with all parts of the cytoplasm stained. Some bacilli showed uneven staining of the cytoplasm with one or several more densely stained areas. About 4 weeks after inoculation, when the number of bacilli in the footpad reached a plateau in C57/BL mice, an increasing proportion of the bacilli became elongated and took on a granulated appearance. The staining became condensed into clumps which were separated by more or less completely empty spaces. Sometimes bacilli were seen, large portions of which appeared to be empty. The main feature of
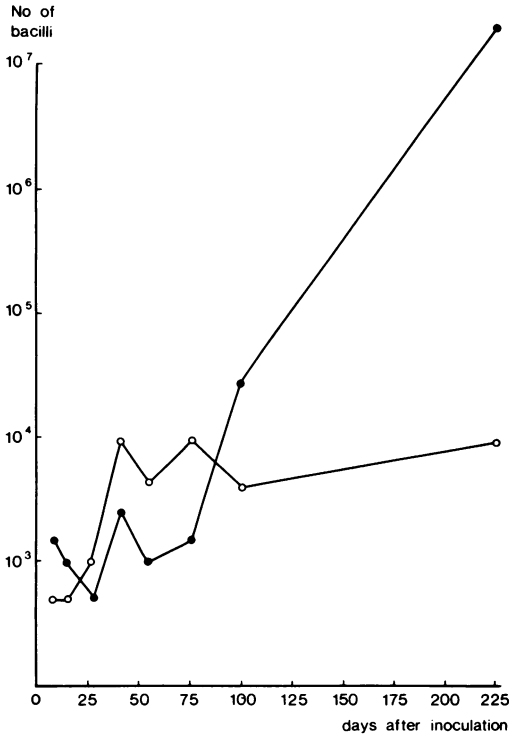
TABLE 2. *Number of bacilli in millions in various organs of C3H and C57/BL mice 226 days after footpad inoculation with MLM*

Strain	Animal	Footpad	Popliteal lymph node	Retro-peritoneal lymph node	Inguinal lymph node	Spleen	Total	t_D[a]
C3H	9–1	690	3,200	20	10	13,000	16,920	18.7
C3H	9–2	420	2,800	100	10	10,300	13,630	19.3
C3H	9–7	480	800	470	50	6,400	8,200	21.0
C3H	9–8	440	8,000	110	20	6,000	14,570	18.7
	Median	460	3,000	105	15	8,400	14,100	19.0
C57/BL	9–3	3.7	0.07	0.03	0.01	1.0	4.8	
C57/BL	9–4	4.1	0.07	0.03	0.01	0.1	4.3	
C57/BL	9–5	4.6	0.07	0.03	0.01	<0.1	4.7	
C57/BL	9–6	2.5	0.07	0.03	0.01	0.1	2.7	
	Median	3.9	0.07[b]	0.03[b]	0.01[b]	0.1	4.5	
C3H/C57/BL	Ratio	120	42,800	3,700	2,000	84,000	3,100	

[a] t_D, Overall doubling time in days for bacilli injected into one footpad.
[b] Mean of four counts of four lymph nodes pooled.

distinction between unevenly stained and granulated forms was that the demarcation between the strongly and weakly stained parts was diffuse in the former but relatively sharp in the latter, frequently revealing completely empty segments. However, the distinction was not always an easy one and a third, intermediate group was therefore introduced to enable the determination of the MI in a reproducible manner. The MI of the bacilli in the footpad of the two strains of mice during the period from 2 to 14 weeks after inoculation is given in Fig. 6. Whereas the percentage of solid-staining bacilli remained very high in C3H mice throughout the period, it started to fall after 4 weeks in C57/BL and continued to fall during the rest of the observation period.

The variation in MI in the popliteal lymph node followed a pattern different from that in the footpad. Initially, i.e., 1 and 2 weeks after

inoculation, the MI in both strains was significantly lower in the lymph node than in the footpad, 30 to 40% in the lymph node versus 85 to 90% in the footpad. As shown in Fig. 7, the MI started to decrease in C57/BL mice between 4 and 6 weeks and remained lower than in the footpad throughout the observation period. In C3H mice the MI increased markedly during the period from 6 to 14 weeks and eventually became somewhat higher than in the footpad.

The mean length of bacilli in the footpads of the two strains is shown in Fig. 8. Initially, the length was the same in both strains; at 4 weeks the bacilli in some C57/BL mice had started to elongate; at 8 weeks elongation was pronounced and at 14 weeks even more pronounced. In contrast, the mean bacillary length was nearly constant in C3H mice throughout the period from 2 to 14 weeks.

Two weeks after inoculation, bacilli in the popliteal node were longer in both strains than those found in the footpad (Fig. 8). During the following weeks the mean bacillary length tended to decrease in C3H mice while it increased in the C57/BL strain.

The relation between footpad reaction and changes in the morphology of the bacilli was studied in 4 C57/BL mice 23 and 27 days after inoculation (Table 3). Animal no. IV-7 had increased foot thickness at day 23 which was more pronounced at day 27, indicating a reaction of at least 4 days duration. At day 27 the bacilli from this animal were longer and had a lower MI than the bacilli recovered from the other animals. Animal no. IV-5 had no reaction at day 23 but a slight reaction at day 27. However, the bacilli from this animal were not different morphologically from bacilli recovered from animals without any signs of footpad reaction. These findings indicate that the reaction precedes the morphological changes which seem to develop gradually in the course of days.

DISCUSSION

The present study confirms previous observations showing that in both C3H and C57/BL mice there is a compatible phase of the infection during which the MLM may multiply freely in the macrophages (6, 7). It is now shown that during this early phase the bacilli multiply exponentially in both strains and that the growth rate appears to be somewhat faster in the C57/BL strain. In C57/BL mice the logarithmic phase ends abruptly after about 4 weeks after inoculation and there is no indication of further multiplication of MLM after that time. From 4 weeks on the morphology of the bacilli changes gradually. These changes consist in
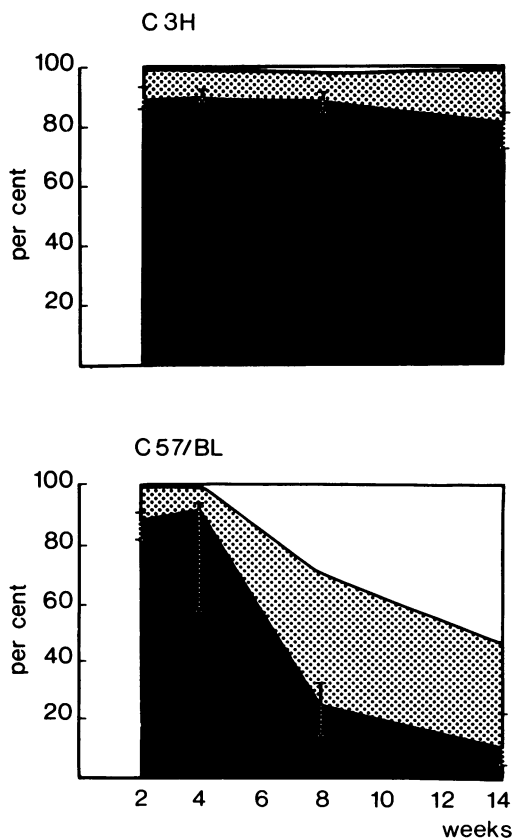

FIG. 6. *Morphological evaluation of MLM in the footpad of C57/BL and C3H mice at various times after inoculation. Black zone represents solid-staining bacilli; dotted zone intermediate forms; clear zone granulated bacilli. Zones correspond to median values. Vertical lines indicate range of four observations.*
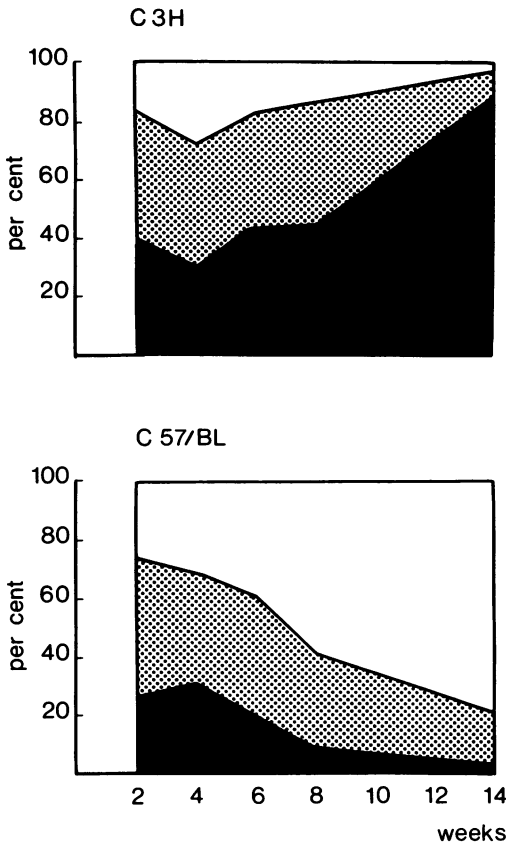
C 3H

C 57/BL

FIG. 7. *Morphological evaluation of MLM in the popliteal lymph node of C3H and C57/BL mice at various times after inoculation. Symbols as in Fig. 6.*

elongation and granulation of the bacilli, the latter indicating loss of viability (1, 20). As indicated in Table 3, the degenerative changes are preceded by a local reaction in the footpad as evidenced by an increase in foot thickness. The local reaction observed in C57/BL mice is due to marked inflammatory changes (Haugen and Closs, to be published), similar to those observed in that strain after subcutaneous inoculation of MLM, and probably represents a CMI reaction against the injected bacilli (6, 7). It is therefore conceivable that the bacilli are killed or damaged as a result of an immune reaction. This effect is strong enough to completely inhibit any further multiplication of the bacilli for at least 28 weeks. Thus, C57/BL mice seem to be able to resist experimental infection with MLM by mounting an immune response against the bacilli, provided the infecting dose is small and is given so as to produce a localized infection.

This appears to be the first report connecting degenerative changes in MLM in vivo to the

immune response of the infected animal. The destruction of MLM by the macrophages is obviously a slow and incomplete process; after being reduced to about one-half between 8 and 11 weeks, the number of bacilli remains almost the same until the termination of the experiment at 32 weeks.

Soon after inoculation the bacilli found in the popliteal lymph node of both strains were more elongated and had a lower MI than the bacilli in the footpad. This could be either the result of some selective process tending to lodge damaged bacilli in the lymph node, or indicate that lymphoid tissue of both strains has a certain ability to damage the bacilli. It also appears that the lag period before multiplication is longer in the lymph nodes than in the footpad, but because of the uncertainties connected with the counting of low numbers of bacilli, the data are not conclusive on this point. As the infection proceeded, the bacilli in the C3H lymph nodes gradually took on the same appearance as those in the footpad, whereas in C57/BL they continued to be more elongated and fragmented. This indicates that the lymphoid tissue of both strains initially may be able to inhibit multiplication of the bacilli; in C57/BL this ability is augmented by the onset of the immune response whereas in C3H it is depressed by some unknown mechanism.

In C3H mice the bacilli continued to multiply throughout the observation period. After 6 weeks the doubling time of bacilli in the footpad became more than doubled, resulting in a sharp bend on the growth curve at 6 weeks. Thus it might seem as if the C3H mice gained some resistance to the infection at that time. However, the bacilli were still able to increase rapidly in number in the popliteal, inguinal,
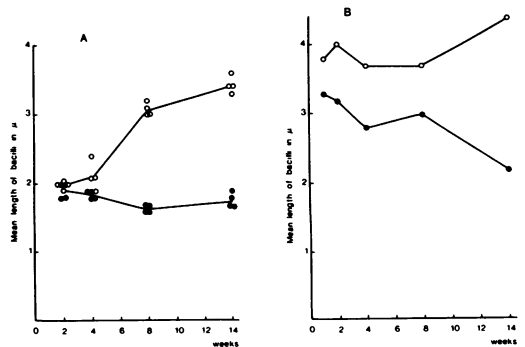

FIG. 8. *Mean length of MLM at various times after inoculation into C3H and C57/BL mice. (A) Footpad; (B) popliteal lymph node. Symbols: O, C57/BL; ●, C3H. Each point represents the mean of 50 individual measurements. Curves are drawn between medians.*

TABLE 3. *Footpad reaction, MI, and length of bacilli 4 weeks after inoculation of MLM into C57/BL mice*

Animal no.	Foot thickness (mm)		Mean length of bacilli (µm)	MI
	23 days	27 days		
IV–5	1.54	1.62	2.1	0.93
IV–6	1.53	1.51	1.9	0.94
IV–7	1.66	1.73	2.4	0.58
IV–8	1.52	1.52	2.1	0.91

and retroperitoneal lymph nodes. Histological examination of the footpad did not reveal any signs of a host reaction (Closs and Haugen, unpublished data). It is therefore unlikely that these mice develop any local or systemic immune response. The apparent reduction in multiplication rate in the footpad is probably due to overflow of bacilli into the lymphatic system, unfavorable growth conditions due to crowding of the macrophages, or both. In the lymph nodes there might be both influx and efflux of bacilli during a certain time period making the calculation of doubling time very unreliable. The best estimates of the doubling time for MLM in this strain are probably the values for the first 4 to 6 weeks in the footpad.

When the spleen and several lymph nodes were harvested after 32 weeks, only a minor proportion of the bacilli, i.e., about 7%, was found in the footpad of C3H mice. In addition to the amount of MLM recovered it is likely that at this late stage substantial amounts of bacilli were also present in other sites, e.g., liver, lungs, peripheral lymph nodes, and bone marrow. Therefore, the true proportion of the MLM remaining in the footpad at 32 weeks is probably considerably less than 7% of the total amount of bacilli. The presence of an unknown quantity of bacilli in other sites also affects the calculation of the overall doubling time for the 32-week period. Assuming that at least 10% of the bacilli were recovered during harvest, the minimum value for the median doubling time is 14.9 days whereas the maximum value (Table 2) is 19.0 days.

Quantification of the growth of MLM depends on the recovery of the bacilli from the infected animal. In turn, recovery is influenced by the distribution of the bacilli after inoculation, i.e., in the mouse footpad model by the proportion of the bacilli which are leaving the footpad after the injection and by their site of secondary lodgement. According to some investigators the mouse footpad model may be extremely difficult to use for quantification of MLM multiplication (3).

As pointed out previously (7), a limited systemic spread of bacilli appears to take place soon after local injection of MLM. Evidence of early systemic spread of bacilli has also been reported after injection of *M. leprae* into the footpads of mice (15, 22). The exact amount of the inoculum leaving the footpad shortly after injection cannot be assessed from the present data; less than 10% was found in the popliteal lymph node whereas approximately 0.1% was found in the inguinal node. However, it is obvious that the present procedure does not result in a strictly localized infection.

Assuming that little damage occurs to blood vessels after successful injection of bacilli, it seems reasonable to postulate that most of the bacilli that leave the inoculation site do so via the lymphatics. To study the magnitude of this traffic, bacterial counts were performed on the popliteal and inguinal lymph nodes which were supposed to drain the foot. The evidence presented in this article indicates that this supposition is correct only for the popliteal node; the inguinal lymph node should be regarded as a distant node receiving its bacilli via the blood stream. This is in harmony with Cuq (8), who states that the route of drainage from the hind limb is from the popliteal via the external sacral to the retroperitoneal lymph nodes and from them to the thoracic duct. Since the present work does not include any study of the retroperitoneal lymph nodes at an early stage of the infection, we shall not know whether these nodes received more of the primary inoculum than did for instance the inguinal node. At a later stage they contained more, i.e., 5 to 10 times as many bacilli, as the inguinal lymph node. Apart from the view presented by Rees (19) that the inguinal node drains the footpad, surprisingly little attention has been paid to this question by those using the footpad model to study *M. leprae* infection in mice (10, 17, 21, 24).

Recovery of bacilli in the footpad during the first 2 days after inoculation was 30 to 50% and 45 to 65% in the C3H and C57/BL strain, respectively. These figures are somewhat higher than those reported by Levy et al. (17) for *M. leprae*. They injected the bacilli in a fairly large volume (0.05 ml) and a large part of the bacilli may have been drained away together with the excess fluid, preferably to the popliteal lymph node. The use of a smaller inoculum (10 µl) might then explain the higher recovery in the present experiments. This hypothesis also gains support by the finding of Levy et al. (17) in that when they inoculated dead mice, the swelling of the foot persisted until harvest and more of the inoculum was recovered after 1 h as compared with living mice in which the swelling quickly disappeared. However, other factors such as the

prevention of leakage through the needle path or the efficiency of the harvesting technique may also contribute to the relatively high recovery in the present experiment.

As pointed out by Gray (12), *Mycobacterium tuberculosis* has been shown to loose its acid fastness temporarily during the first 2 h after being injected into animals or added to cell cultures in vitro. Assuming that loss of acid fastness is related to a metabolic process in the bacterium, this phenomenon would be expected to occur at a later time in such extremely slow-growing species as *M. leprae* or MLM than in *M. tuberculosis*, e.g., after 24 h. From the data presented by Levy et al. (17), it appears that, in most instances, the recovery of *M. leprae* from the footpad was less after 24 h than after 72, 120 or 216 h, whereas there was no increase in recovery from 72 to 216 h. The supposition that some of the MLM temporarily loose their acid fastness after injection would explain the apparent lack of lag period and the surprisingly high viability of the bacilli, which both are inferable from Fig. 3; the marked increase in the number of bacilli occurring between 1 and 8 days after inoculation being due to increased stainability of the MLM and not to bacterial multiplication. Recovery 1 week after inoculation might then be a better estimate of the number of bacilli remaining in the footpad; the number of MLM found in the C3H and C57/BL strains were at that time almost the same and recovery would have been 78 and 85%, respectively. If these considerations are correct it is possible to account for 85 to 90% of the bacilli injected in the present experiment.

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