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Intracellular Location of *Mycobacterium leprae* in Macrophages of Normal and Immune-Deficient Mice and Effect of Rifampin

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Soon after more than 10^6 *Mycobacterium leprae*, freshly harvested from armadillo liver or harvested and ^{60}Co irradiated, were inoculated into the hind footpads of either normal or thymectomized and irradiated (T900R) mice, the organisms were found to reside within phagosomes of polymorphonuclear and mononuclear cells. On the other hand, 7 and 8 months after 10^4 freshly harvested *M. leprae* were inoculated into the footpads of normal or T900R mice and the organisms had multiplied to their maximum in the normal mice, many organisms, largely intact by electron-microscopic criteria, were found to reside free in the cytoplasm of the footpad macrophages, whereas damaged organisms were contained within phagosomes. After 11 months, many intact organisms were found to lie free in the cytoplasm of the macrophages of T900R mice, whereas only damaged intraphagosomal *M. leprae* cells were observed in the macrophages of normal mice. Finally, a remarkably large proportion of damaged extraphagosomal *M. leprae* was found in T900R mice administered rifampin for 2 days in a bactericidal dosage. It appears that *M. leprae* multiplies free in the cytoplasm of the footpad macrophages of infected mice, whereas the *M. leprae* cells resident within the phagosomes of the macrophages are dead. As the result of treatment with rifampin, the organisms appeared to have been killed in their extraphagosomal location, only afterwards being incorporated into phagosomes. However, the intracellular site in which *M. leprae* is killed in the course of an effective immune response remains unclear.

Most intracellular parasites are found within the phagosomes (phagocytic vacuoles) of the macrophages of their hosts, where they multiply. *Mycobacterium leprae*, an obligatory intracellular parasite, appears to represent an exception to this generalization. In the tissues of leprosy patients, *M. leprae* has been found within and outside phagosomes (3). This is also true of the footpad macrophages of experimentally infected mice (8), in which the bacilli have also been seen to lie free in the sarcoplasmic matrix of muscle fibers, with no surrounding phagosomal membrane (7). In the course of the experimental infection of mice, the bacilli have been found to lie extraphagosomally during logarithmic multiplication; during the stationary or "plateau" phase, most of the bacilli appear to have degenerated and to reside within phagosomes (8, 16).

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It is not clear whether the natural site of multiplication of *M. leprae* in mouse footpads is extra- or intraphagosomal, nor is it known in which site the bacilli are killed. In an effort to obtain additional information that might be relevant to the questions of where *M. leprae* multiplies and where it is killed, experiments were carried out in which the intracellular situation of the bacilli was examined during multiplication and death of the bacilli in the tissues of both immunologically normal and immune-deficient mice.

MATERIALS AND METHODS

Female CBA/Ca mice 8 to 10 weeks of age were obtained from the Animal Division of the National Institute for Medical Research, London, England. Mice were anesthetized by the intraperitoneal administration of tribromoethanol (Avertin; Winthrop Laboratories, Surbiton-Upon-Thames, Surrey, England) at 364 mg/kg of body weight. A parasternal incision was made, the thymic lobes were aspirated, and the incision was closed with 3-0 silk. More than 98% of the mice survived the procedure. Completeness of thymectomy was confirmed at autopsy.

TABLE 1. Intra- and extraphagosomal distribution of *M. leprae* after a large inoculum

Mice	Fresh *M. leprae*			Irradiated *M. leprae*		
	Total no.	% Extraphagosomal[a]	% Intraphagosomal	Total no.	% Extraphagosomal	% Intraphagosomal
Normal	112	<1 (0–<5.60)	100 (>94.4–100)	120	<1 (0–<5.24)	100 (>94.8–100)
T900R	109	<1 (0–<5.74)	100 (>94.3–100)	148	<1 (0–<4.27)	100 (>95.7–100)
Total	221	<1 (0–<2.89)	100 (>97.1–100)	268	<1 (0–<2.29)	100 (>97.7–100)

[a] The bacilli (<10% of the total) whose location was doubtful are not shown in this table. Numbers within parentheses are 95% confidence limits.

Two weeks after thymectomy, the mice were exposed to 900 rads of gamma radiation from a ^{60}Co source and infused on the same day with 4.9×10^7 nucleated bone marrow cells obtained from normal syngeneic donors. Hereafter, these mice are termed thymectomized-irradiated (T900R) mice. For 2 weeks thereafter, the animals were administered oxytetracycline in the drinking water in a concentration of 125 mg/ml; the antibiotic solution was replaced daily. During this period, mortality was 20%.

Suspensions of *M. leprae* in Hanks balanced salt solution were prepared from livers of heavily infected armadillos, and both hind footpads of normal or T900R mice were inoculated. Bacilli were harvested and counted by an established method (13, 20).

The proportion of viable (i.e., infective for mice) *M. leprae* in a suspension was assessed by inoculation of mice with 10-fold serial dilutions of the suspension (29). At least 1 year later, individual, foot-by-foot harvests of *M. leprae* were performed from 10 footpads of the mice inoculated with each dilution. The most probable number of viable bacilli in the original suspension was calculated from the results of the harvests by means of the equation of Halvorson and Ziegler (11).

For electron microscopy (EM), mouse footpad tissues were cut into small pieces and fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 20 mM calcium chloride and 0.2 M sucrose. The samples were rinsed several times with the same buffer, postfixed in 1% osmium tetroxide for 2 h at 4°C, rinsed again with buffer, and stained en bloc with 0.5% uranyl acetate in 0.1 M sodium acetate for 1 to 3 h at room temperature. The specimens were subsequently dehydrated in graded alcohol or acetone and embedded in Spurr low-viscosity resin (27). Semithin sections cut with a glass knife were stained with toluidine blue and used to locate the areas of granulomatous response. Thin sections were cut with a diamond knife, stained with a saturated solution of uranyl acetate in 50% ethanol and 3% lead citrate in 0.15 N sodium hydroxide, and screened with a Phillips 300 electron microscope operated at 60 kV.

Interrupted series of thin sections were screened systematically for macrophages. The bacilli contained in the macrophages were counted and scored as "intact" or "damaged" and, in obscure cases, as "doubtful." Bacilli were considered to be damaged if there was any evidence of abnormality, such as disorganization of cytoplasm or of the nuclear region (7). The intracellular situation of the bacilli was also assessed; *M. leprae* cells were scored as "intraphagosomal" or "extraphagosomal" and, in obscure cases, as "doubtful."

Differences between proportions were tested for statistical significance by means of the quadratic normal approximation (5) or the X^2 one-sample test (24).

RESULTS

Fate of a large inoculum. A portion of a suspension of *M. leprae* prepared from armadillo liver was killed by exposure to 2.5 megarads of gamma radiation from a ^{60}Co source, and normal and T900R mice were inoculated into both hind footpads with 1.8×10^6 freshly harvested or irradiated bacilli per footpad. Animals of each group (normal mice inoculated with fresh *M. leprae*, T900R mice inoculated with fresh *M. leprae*, normal mice inoculated with irradiated *M. leprae*, and T900R mice inoculated with irradiated *M. leprae*) were sacrificed 24 or 48 h or 1 or 2 weeks after inoculation, and samples of footpad tissue were fixed for light microscopy and EM. For each group of animals,

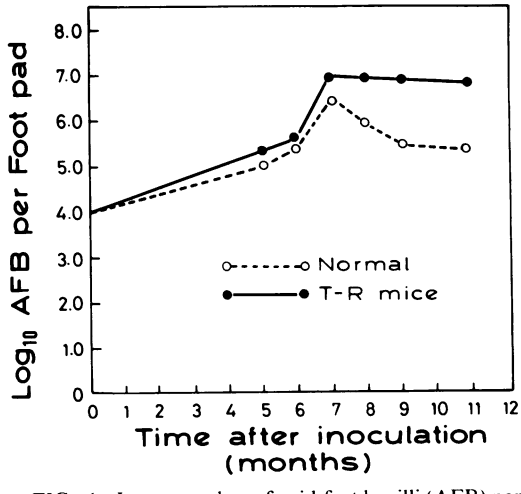

FIG. 1. Log_{10} number of acid-fast bacilli (AFB) per footpad of normal and T900R mice as a function of time after inoculation in both hind footpads with 10^4 *M. leprae* per footpad. Values represent the mean of four footpad harvests of *M. leprae*.

TABLE 2. Intra- and extraphagosomal distribution of *M. leprae* after a small inoculum

Time after inoculation (mo)	Mice	Results of harvest		No. of bacilli scored	Results of EM examination					
					% Extraphagosomal[b]			% Intraphagosomal		
		% Viable	MI[a] (%)		Total	Intact	Damaged	Total	Intact	Damaged
7	Normal	2.4	29	90	12.2 (6.6–21.2)	12.2 (6.6–21.2)	<1.1 (0–<6.9)	87.8 (78.8–93.4)	16.7 (9.9–26.3)	71.1 (60.5–79.9)
	T900R	12	47	120	63.7 (54.5–72.0)	62.1 (52.9–70.5)	1.6 (0.3–6.3)	36.3 (28.0–45.5)	19.4 (13.0–27.6)	16.9 (11.0–25.0)
8	Normal	0.33	5.0	139	<0.7 (0–<4.5)	<0.7 (0–<4.5)	<0.7 (0–<4.5)	>99.3 (>95.5–100)	<0.7 (0–<4.5)	>99.3 (>95.5–100)
	T900R	12	34	141	58.1 (49.5–66.3)	56.0 (47.4–64.3)	2.1 (0.6–6.6)	41.8 (33.7–50.5)	22.7 (16.3–30.7)	19.1 (13.2–26.8)
11	Normal	0.02	2.0	126	<0.8 (0–<5.0)	<0.8 (0–<5.0)	<0.8 (0–<5.0)	>99.2 (>95.0–100)	<0.8 (0–<5.0)	>99.2 (>95.0–100)
	T900R	1.9	46	167	37.7 (30.4–45.6)	35.9 (28.8–43.8)	1.8 (0.5–5.6)	62.3 (54.4–69.6)	22.2 (16.3–29.4)	40.1 (32.7–48.0)

[a] MI, Morphological index.
[b] The bacilli (<10% of the total) whose morphological appearance or location was doubtful are not shown. Numbers within parentheses are 95% confidence limits.

more than 100 *M. leprae* within polymorphonuclear (PMN) and mononuclear (MN) cells were scored.

By 24 and 48 h after inoculation, an acute inflammatory response had developed in the subcutaneous tissues of the footpads of animals from all four groups. *M. leprae* was found mostly within PMN and MN cells. During this time, all of the intracellular bacilli in all four groups of animals were located within phagosomes, and none was found free in the cytoplasm (Table 1). Because no differences were observed among the four groups of animals, it appears justified to pool the results.

A total of 12% of the *M. leprae* inoculated appeared to be solidly stained by light microscopy (i.e., the morphological index [28] was 12%), and the most probable number yielded an estimate of 2.7% viable *M. leprae* in the fresh inoculum. Among a total of 221 bacilli, proportions of 12/100 solid and 2.7/100 viable bacilli are significantly different from a proportion of 1/100 (actually 0/221) ($P < 0.01$). Therefore, the total of 221 bacilli found to reside within phagosomes probably included viable bacilli.

In the tissues of the animals sacrificed 1 or 2 weeks after inoculation, PMN cells had largely been replaced by MN cells. Only a few bacilli were found in macrophages, all of which were within phagosomes. However, the numbers of *M. leprae* were too small to permit analysis.

Fate of a small, fresh inoculum. Normal and T900R mice were inoculated into both hind footpads with 10^4 freshly harvested *M. leprae* per footpad. At intervals thereafter, harvests of *M. leprae* were performed from the footpads of normal and T900R mice. The results of harvests of *M. leprae* from footpads of these mice at intervals after inoculation are shown in Fig. 1. At 7, 8, and 11 months after inoculation, one or two mice from each group were also sacrificed, and samples of footpads were taken for EM studies. The most probable number of viable bacilli in each harvested suspension was measured, and the morphological index was also determined.

The proportions of solidly staining and mouse-infective *M. leprae* were always larger among the bacilli harvested from T900R mice than among those from normal mice (Table 2), and the proportions of bacilli that appeared intact by EM were always larger than the proportions of solid and infective *M. leprae*. On the other hand, the results of these three measurements appear to have been roughly proportional.

Two locations of the *M. leprae* within macrophages could be distinguished: (i) an intraphagosomal location, in which the bacilli were surrounded by a double membrane (Fig. 2); and (ii) an extraphagosomal location, in which the bacil-

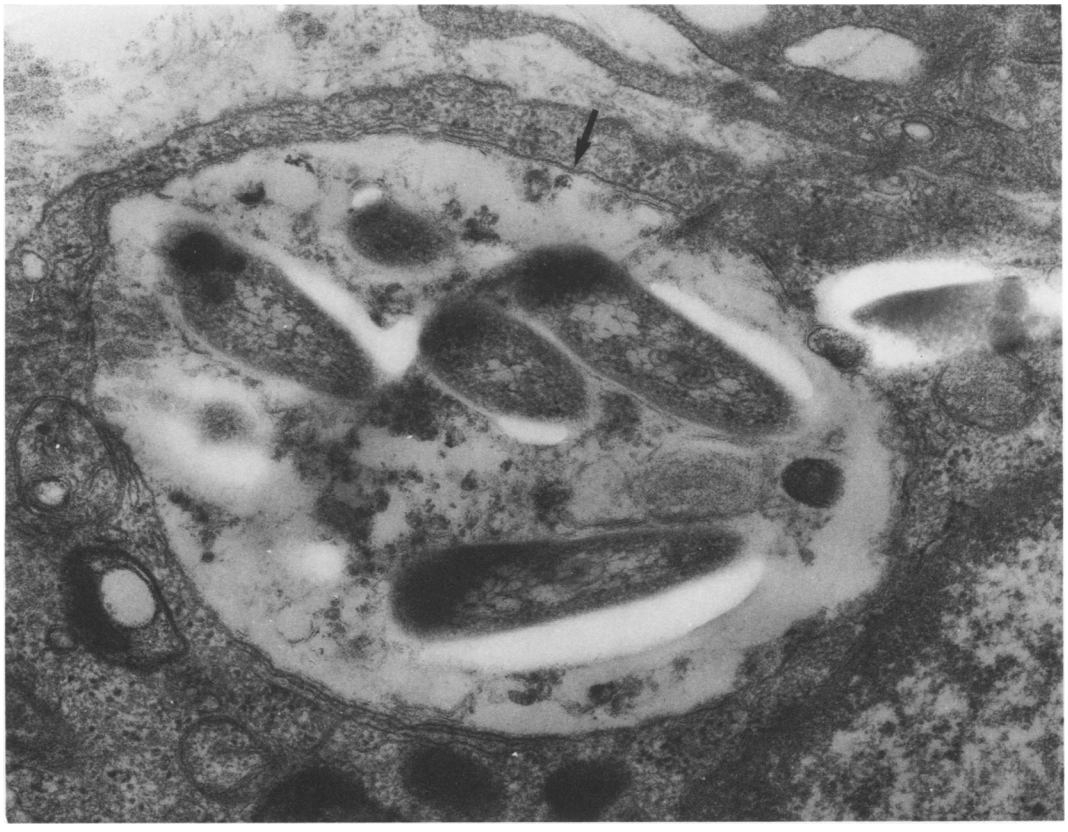

FIG. 2. Group of *M. leprae* surrounded by a phagosomal membrane (arrow) in a macrophage of a normal mouse 7 months after inoculation. Magnification, ×36,000.

li were free in the cytoplasm (Fig. 3). Seven months after inoculation, at which time the number of *M. leprae* had become maximal in normal mice, many extraphagosomal bacilli were observed in both normal and T900R mice. In the normal mice, a minority of the bacilli were extraphagosomal; all of these appeared intact. Most of the *M. leprae* cells appeared damaged and were located within phagosomes. In addition, a small proportion of the intraphagosomal bacilli appeared intact. At 8 and 11 months after inoculation, all of the *M. leprae* cells scored in the tissues of normal mice appeared damaged and were located within phagosomes of macrophages.

In the T900R mice, on the other hand, bacilli were found in the extraphagosomal location for a longer period than in normal mice. Seven months after inoculation, the majority of the *M. leprae* cells were found to reside free in the cytoplasm of macrophages, and virtually all of these bacilli appeared intact. At the same time, approximately half of the bacilli located within phagosomes appeared damaged. The situation 8 months after inoculation was not significantly different from that after 7 months. By 11 months after inoculation, however, the proportion of intraphagosomal bacilli appeared to have increased at the expense of extraphagosomal bacilli ($P < 0.05$). Still, virtually all of the extraphagosomal *M. leprae* cells appeared intact, whereas a majority of the intraphagosomal bacilli, accounting at this time for about two-thirds of the bacilli scored, appeared damaged.

In the normal and T900R mice 7 months after inoculation, the macrophages had a histocytoid appearance. There was no evidence of macrophage activation, as shown by the lack of interdigital connections among macrophages and the small numbers of lysosomes and mitochondria. The extraphagosomal bacilli were frequently surrounded by abundant amorphous, electron-transparent material (Fig. 3). Disintegration of cytoplasmic components of the macrophages adjacent to bacilli sometimes was seen, accompanied by accumulation of amorphous electron-transparent material (Fig. 4), suggesting that the presence of the bacilli may be damaging to the macrophage. On the other hand, the macrophages of normal mice sacrificed 8 or 11 months

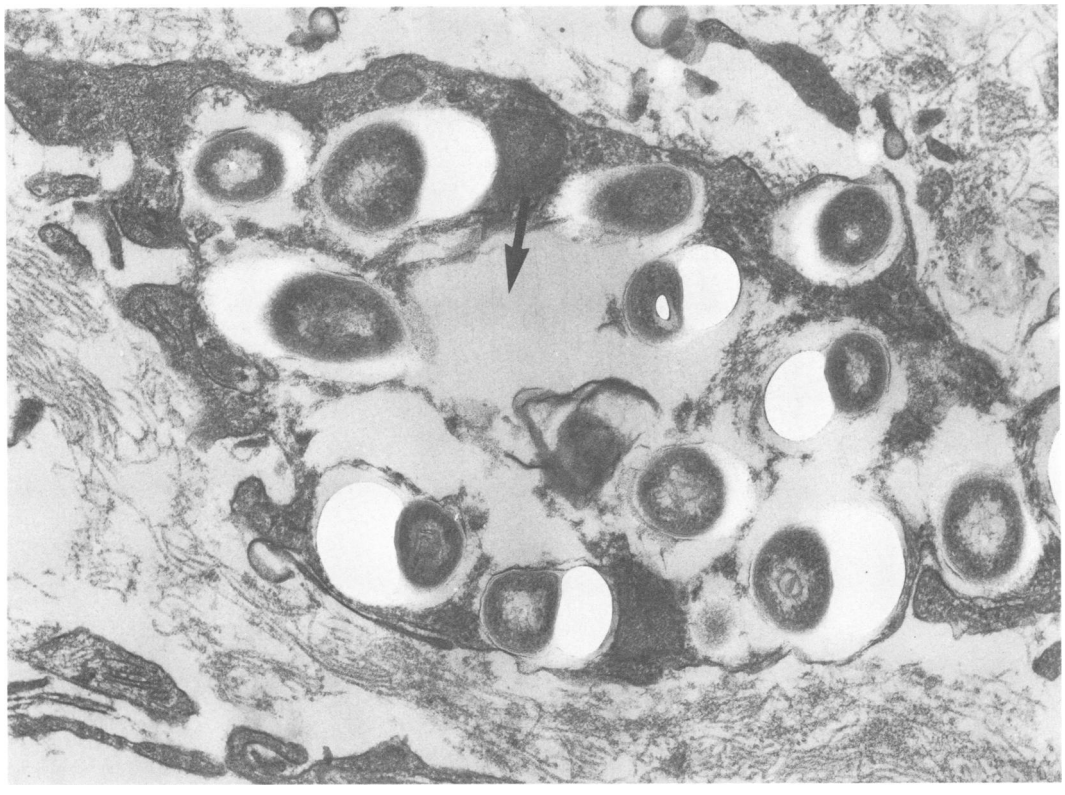

FIG. 3. Group of *M. leprae* in a macrophage of a T900R mouse before treatment with rifampin. Note abundant amorphous electron-transparent material around the bacilli (arrow). Magnification, ×36,000.

after inoculation appeared to be activated, as shown by interdigital connections and numerous lysosomes, mitochondria, and vacuoles. At no time did the macrophages of T900R mice show these changes.

In the T900R mice, especially those sacrificed 11 months after inoculation, an occasional finding was focal degeneration of the cytoplasm of the macrophages, accompanied by release of bacilli to the extracellular area. Macrophages with cytoplasmic processes entrapping bacili were also observed (Fig. 5). Intact, damaged, extraphagosomal, and intraphagosomal bacilli were sometimes found within the same macrophage. The phagosomal membrane was not always complete; various degrees of disruption of the membrane were seen (Fig. 6).

The results obtained from normal mice inoculated with a small number of freshly harvested *M. leprae* confirm those reported earlier by Evans and Levy (8) and provide direct proof of their claim that the intraphagosomal location is characteristic of dead *M. leprae*. The results obtained from T900R mice are also consistent with those of Levy et al. (16). Multiplication of *M. leprae* in T900R mice is also limited by a ceiling, and the bacilli are killed once the ceiling has been achieved (19). As was the case in normal mice, killing of *M. leprae* in T900R mice is accompanied by disappearance of extraphagosomal bacilli and their replacement by intraphagosomal damaged bacilli.

Influence of rifampin treatment on the intracellular location of *M. leprae*. T900R mice were inoculated in both hind footpads with 10^4 *M. leprae*. Harvests of *M. leprae* from the footpad tissues were carried out at intervals until the number of bacilli reached 10^6 per footpad; the remaining mice were then divided into two groups. One served as the control group, whereas the mice of the second group were administered rifampin, incorporated into the mouse diet in a concentration of 0.1 g/100 g of diet, for 48 h to kill the *M. leprae* rapidly. At intervals thereafter, animals were sacrificed, the samples of footpad tissues were taken for EM.

The proportion of extraphagosomal bacilli remained essentially unchanged during the first week after completion of rifampin treatment (Table 3). Two weeks after treatment, however, only a very few extraphagosomal bacilli were encountered. The proportion of intact-appearing

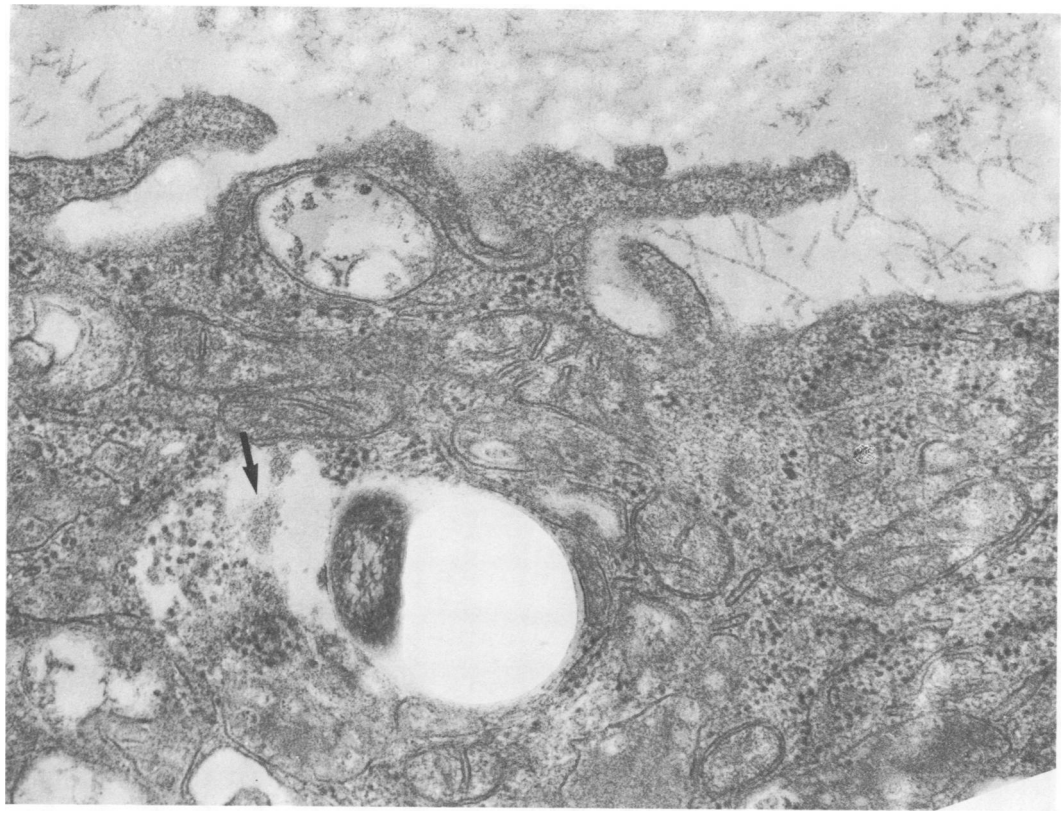

FIG. 4. Intact extraphagosomal *M. leprae* in a macrophage of a T900R mouse 11 months after inoculation. Note the amorphous material adjacent to bacilli disrupting surrounding cytoplasm (arrow). Magnification, ×38,000.

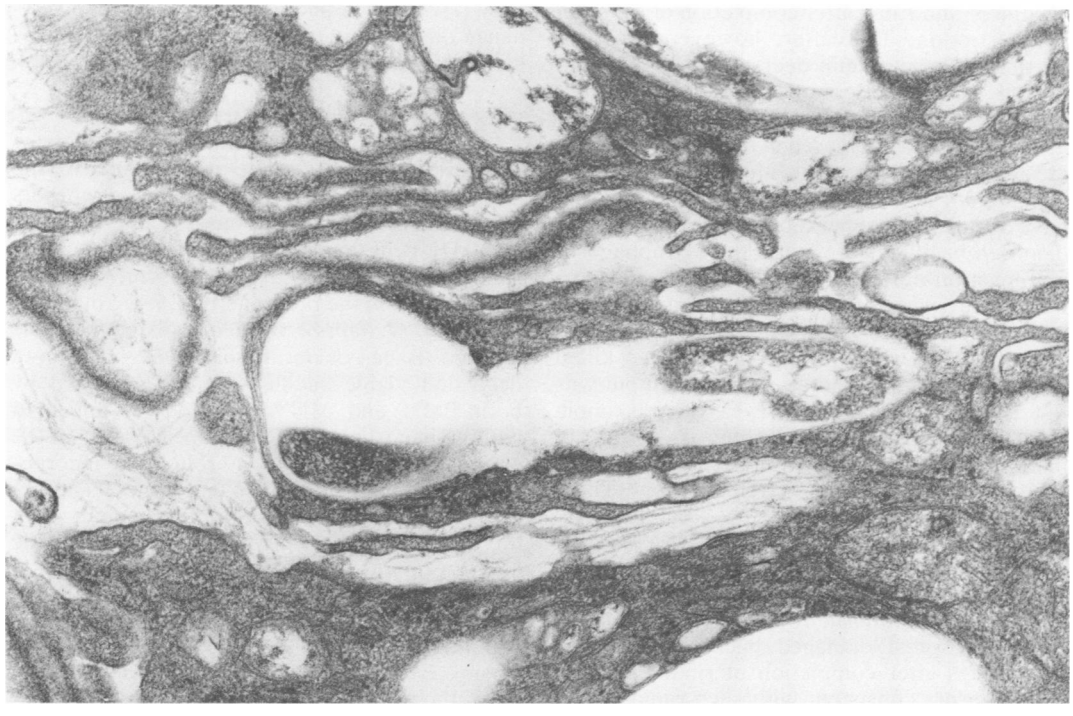

FIG. 5. Macrophages of T900R mouse engulfing bacilli 48 h after rifampin treatment. Magnification, ×48,000.

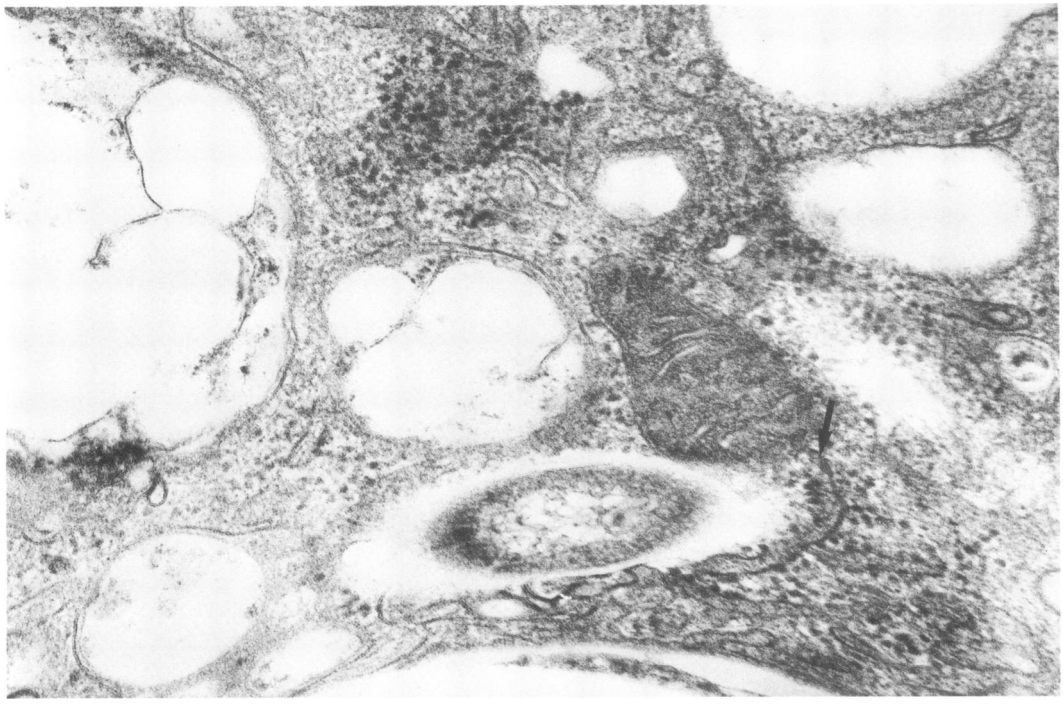

FIG. 6. Intact *M. leprae* in a macrophage of a T900R mouse 11 months after inoculation. Note interrupted phagosomal membrane (arrow). Magnification, ×55,000.

M. leprae varied between 58 and 73%, but did not change significantly between the start of treatment and 1 day after completion of rifampin administration. Thereafter, however, the proportion of intact bacilli decreased significantly, to 43% 3 days after completion of the treatment and to 28% after 1 week. The proportions of intact *M. leprae* decreased in both the extra- and intraphagosomal locations, and no shift of intact bacilli from one location to another appears to have occurred. A remarkably large proportion of damaged bacilli was found in the extraphagosomal location during the first half of the experiment; later, virtually all of the damaged bacilli were found within phagosomes.

The rate at which the *M. leprae* was killed during treatment of the mice with rifampin was not measured directly. However, there is ample evidence from studies both in humans (17) and in mice (22, 23) that the bactericidal action of rifampin on *M. leprae* is extremely rapid. Thus, it appears very likely that the great majority (99.9%) of the viable *M. leprae* cells were killed during the 48-h period of rifampin administration and the first 2 days after completion of the treatment. The unusually large proportions of extraphagosomal damaged bacilli observed 3 and 7 days after completion of rifampin treatment appears consistent with such rapid killing of *M. leprae*.

Damaged bacilli in a stage of division were occasionally found in rifampin-treated mice (Fig. 7). Such a finding provides graphic evidence of the rapidity with which *M. leprae* was killed during treatment.

DISCUSSION

In a report of their histopathological study of the early response in the mouse foot to the inoculation of *M. leprae*, Evans and co-workers (9) characterized the response as one of PMN and MN cells and observed that intracellular bacilli were located within phagosomes in these cells. When, in the present study, large numbers ($>10^6$) of *M. leprae* were inoculated into the footpad tissues of normal or T900R mice, both dead and viable bacilli were phagocytized by both PMN and MN cells, and all appeared within phagosomes. The immune capacity of the animals appeared to play no role in the incorporation of the bacilli into phagosomes. The appearance of the bacilli within phagosomes is, at this stage, probably nonspecific, as might be the case for any phagocytized foreign material.

The viable *M. leprae* cells appeared to survive their initial interaction with PMN and MN cells, coming subsequently to reside free in the cytoplasm. Because, in both normal and T900R mice, the great majority of intraphagosomal bacilli were damaged, whereas the majority of

extraphagosomal bacilli were intact, it appears likely that the natural habitat of viable, multiplying *M. leprae* is free in the cytoplasm of the macrophages. That multiplication of *M. leprae* continued to a higher level in T900R than in normal mice, accompanied by a more prolonged extraphagosomal residence of the bacilli and a higher proportion of intact bacilli, is consistent with this formulation. The study described here suggests that the natural site of multiplication of *M. leprae* is in the cytoplasmic matrix of the host cells. This is in contrast with *Mycobacterium tuberculosis*, *Mycobacterium microti*, and *Mycobacterium lepraemurium*, which multiply within phagocytic vacuoles (2, 4, 12).

The site at which *M. leprae* is killed is obscure. It appears certain that, when *M. leprae* was killed in the course of treatment by rifampin, killing occurred in the extraphagosomal location, but the abrupt killing was not followed immediately by incorporation of the dead bacilli into phagosomes. This suggests that, although the bacilli were dead, they were not immediately recognized by the host macrophages, as recognition would mean that the bacilli would be engulfed by phagosomes.

In fact, the dead, extraphagosomal bacilli came gradually to be contained within phagosomes of nonactivated macrophages. Similarly, damaged bacilli had been found within phagosomes of nonactivated macrophages of both normal and T900R mice. Thus, there was no requirement for activation of macrophages for *M. leprae* to be incorporated into phagosomes; on the other hand, incorporation into phagosomes appeared clearly to precede activation of macrophages, which was a late event in normal mice. This stands in contrast to the observation of Evans and Levy (8) that appearance of the bacilli within phagosomes was correlated with evidence of activation of the macrophages.

Evidence has been presented for steps of a process involving disruption of macrophages, release of bacilli into the extracellular area, and phagocytosis of the bacilli by "new" macrophages. Moreover, arguing simply from the greater-than-100-fold increase of the number of *M. leprae*, the failure to find large numbers of bacilli within individual macrophages also suggests that the bacilli are redistributed to other macrophages. This is in accord with the kinetics of macrophages in granulomas (1). Therefore, incorporation of the bacilli into phagosomes probably does not result from the activity of autophagosomes (10) in the same macrophage, but rather from rephagocytosis by other macrophages. It appears likely that continuous turnover of macrophages accompanied by redistribution of the *M. leprae* resulted in the appearance of the bacilli within phagosomes.

TABLE 3. Intra- and extraphagosomal distribution of *M. leprae* in T900R mice treated with rifampin

Time after beginning treatment (days)	No. of bacilli scored	% Extraphagosomal[a]			% Intraphagosomal		
		Total	Intact	Damaged	Total	Intact	Damaged
0	139	42 (33.5–50.4)	40 (32.2–49.0)	2 (0.25–5.6)	58 (49.6–66.5)	31 (23.5–39.4)	27 (20.3–35.7)
2	123	38 (29.7–47.4)	33 (25.2–42.5)	5 (2.0–10.8)	62 (52.6–70.3)	40 (31.2–49.1)	22 (15.2–30.5)
3	182	31 (24.3–38.1)	28 (21.8–35.2)	3 (1.0–6.6)	69 (61.9–75.7)	30 (23.8–37.5)	39 (32.0–46.8)
5	182	31 (24.3–38.1)	24 (18.3–31.2)	7 (3.6–11.5)	69 (61.9–75.7)	19 (13.9–25.9)	50 (42.5–57.5)
9	199	28 (22.1–35.0)	9 (5.6–14.1)	19 (14.0–25.4)	72 (65.0–77.9)	19 (14.0–25.4)	53 (45.6–59.8)
16	146	3 (0.88–7.3)	2 (0.53–6.4)	1 (0.03–4.3)	97 (92.7–99.1)	17 (11.6–24.4)	80 (72.6–86.1)
30	212	0 (0–<3.7)	0 (0–<3.7)	0 (0–<3.7)	100 (>97.0–100)	3 (1.2–6.3)	97 (93.7–98.8)
(30)[b]	171	35 (28.1–42.8)	33 (25.9–40.4)	2 (0.75–6.3)	65 (57.2–71.9)	28 (21.6–35.5)	37 (29.7–44.6)

[a] The bacilli (<10% of the total) whose morphological appearance or location was doubtful are not shown. Numbers within parentheses are 95% confidence limits.
[b] Untreated control mice.

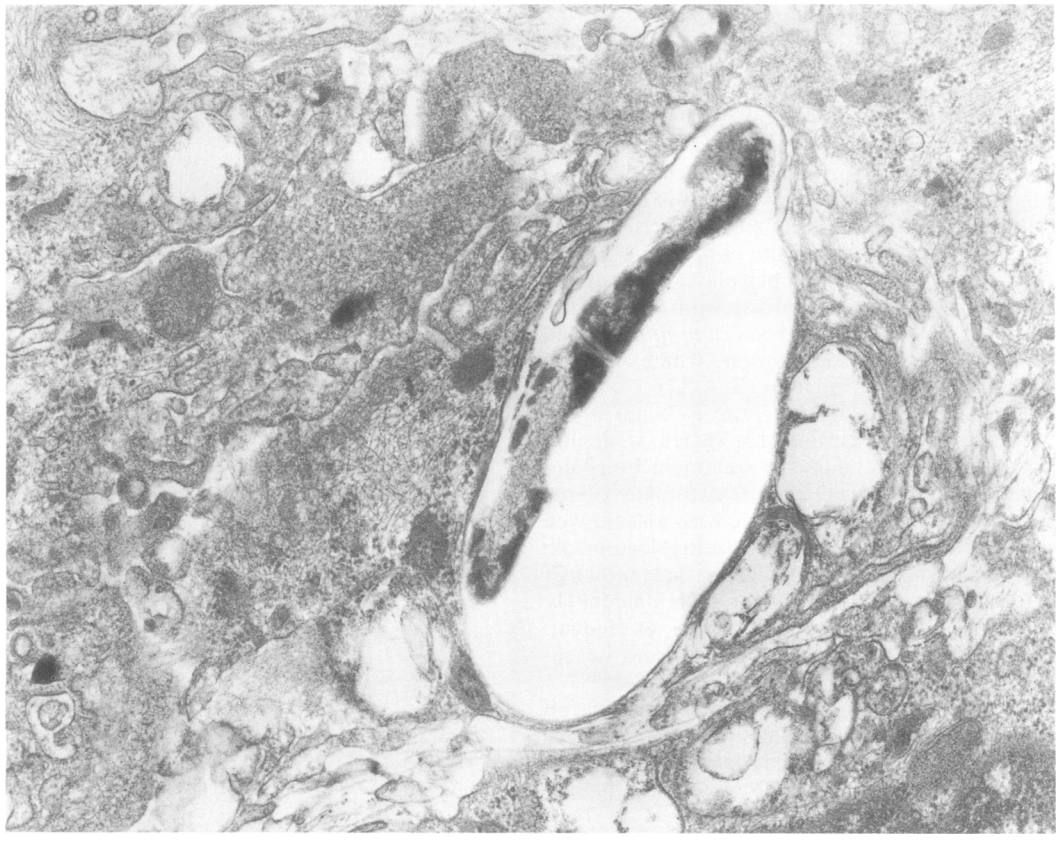

FIG. 7. Dividing, damaged *M. leprae* in a macrophage of a T900R mouse 1 month after rifampin treatment. Magnification, ×32,000.

It is likely that the rephagocytized bacilli reside initially within phagosomes, from which they subsequently escape. A similar process of escape from phagocytic vacuoles has been described for some viruses (6, 26), rickettsiae (21, 25), and *Trypanosoma cruzi* (14, 18). The mechanisms by which *M. leprae* interacts with the phagosomal membrane of the host cell, promoting its disappearance, are not yet clear. A possible mechanism is lysis of the phagosomal membrane by a product of the bacilli. It is apparent that the process does not produce a complete dissolution of the membrane, but rather produces a limited reaction in which the membrane is partially disrupted. This possibility is favored by the occasional finding of membrane remnants around the bacilli.

The differences observed between proportions of mouse-infective and intact bacilli may have resulted from limitations of the various techniques employed. In the course of EM study, only a thin section of the bacillus is examined; the appearance of the section may not always be representative of the entire bacillus. If the bacillus appears damaged, there can be no question of its status. If, however, the bacillus appears intact, it may possibly reveal damage in another plane. This is particularly likely to be the case during the early stages of degeneration after the death of the bacillus. Thus, the results of EM study may overestimate the proportion of viable bacilli. In addition, the method employed for determination of the most probable number may underestimate the number of mouse-infective *M. leprae*. Because as much as 80% of an inoculum may be lost from the footpad soon after inoculation (15), the smallest inoculum (10 bacilli) may fail to infect, even when the proportion of viable *M. leprae* approaches 80%.

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