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## BIBLIOTECA

Neonataly Thymectomized Lewis Rats Infected with  
*Mycobacterium leprae*. 2. Histopathologic and  
Electron Microscopic Observations<sup>1</sup>Peter J. Dawson, Julius C. Ringus, and A. Howard Fieldsteel<sup>2</sup>

The use of the neonataly thymectomized Lewis rat (NLR) as a model for the study of leprosy has distinct advantages over both other murine hosts and the nine-banded armadillo (*Dasypus novemcinctus*). The intact mouse develops a self-limiting infection after footpad inoculation with a ceiling of about  $10^6$  organisms (17). A generalized infection does not occur after intravenous infection until 19 to 24 months after inoculation, and even then only comparatively small numbers of bacilli are found (21).

Rees, *et al.* (12, 15) have reported that footpad inoculation of *M. leprae* in thymectomized, lethally irradiated mice (reconstituted with syngeneic bone marrow) was followed by disseminated disease within 12 months. Other workers have not been entirely successful in reproducing these re-

sults, largely because of their inability to keep the mice alive long enough to finish the experiment (9, 18). Rees himself has now modified his procedure and gives 5 treatments of 200 rad at two week intervals (14). Although these mice survive well, they show only a tenfold increase in sensitivity compared to intact animals. While there is preliminary evidence that *M. leprae* replicate well in athymic (nu/nu) mice (1), these animals must be maintained under either germ-free or specific pathogen-free conditions (8). In contrast, *M. leprae* replicates readily in the armadillo (7, 20), but armadillos suffer from the disadvantage that they cannot be bred in captivity and have, in some areas, been reported to harbor other cultivable mycobacteria (10, 11). On the other hand, none of these objections appears to apply to the NLR. One of us (AHF) has previously reported that the animals are long-lived, relatively resistant to intercurrent infection, and support the replication of large numbers of *M. leprae* following either intravenous or footpad inoculation (4, 5). We are now reporting the histopathologic and electron microscopic findings in these rats following intravenous inoculation with *M. leprae*.

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TABLE 1. *Experimental details and bacterial counts on ears of NTLR inoculated with M. leprae.*

Animal #	Sex	Age (days)	Inoculation		Results of bacterial count <sup>a</sup>		Age at death (days)
			Route	No. of <i>M. leprae</i>	Day after inoc.	No. of <i>M. leprae</i>	
LL171L146.1	F	20	i.v.	$1.23 \times 10^7$	962	$4.10 \times 10^7$	968
LL171L145.2	M	30	i.v.	$1.23 \times 10^7$			
		33	IT <sup>b</sup>	$1.00 \times 10^4$	795 <sup>c</sup>	$2.85 \times 10^8$	797
LL34L315.3	F	48	i.v.	$3.17 \times 10^7$	545	$2.68 \times 10^7$	558
LL36L337.1	M	29	i.v.	$1.10 \times 10^7$	376	$2.36 \times 10^6$	770
LL36L339.3	M	27	i.v.	$1.10 \times 10^7$	376	$1.86 \times 10^6$	770
LL36L331.3	M	30	i.v.	$1.10 \times 10^7$	381	$3.02 \times 10^6$	770
LL36L332.2	M	30	i.v.	$1.10 \times 10^7$	384	$4.41 \times 10^6$	770
LL33L308.2	F	36	i.v.	$1.03 \times 10^7$	571	$2.03 \times 10^7$	573

<sup>a</sup> These were made on the left ear and are expressed per ear.

<sup>b</sup> IT = intratesticular.

<sup>c</sup> After i.v. inoculation.

## MATERIALS AND METHODS

Pregnant inbred rats of the Wistar/Lewis strain were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts, U.S.A. Although specific pathogen-free when obtained, neither they nor their offspring were maintained in this condition. Thymectomy was carried out in all instances between 5 and 16 hours after birth, using the method previously described (4). The strain of *M. leprae* used in these experiments was originally isolated from a leprosy patient by C. C. Shepard of the Center for Disease Control in Atlanta, Georgia. It has been maintained in mice and rats in the laboratory of one of us (AHF) for the past ten years and retains all of its original growth patterns and characteristics.

Thymectomized rats were inoculated intravenously with from  $1.10$  to  $3.17 \times 10^7$  *M. leprae* at 20 to 28 days of age. One rat received, in addition,  $10^4$  *M. leprae* intratesticularly 20 days after i.v. inoculation. The rats were killed 573 to 968 days after inoculation when bacillary counts, performed on one ear by the method of Shepard and McRae (19), indicated disseminated infection.

Complete autopsies were performed on eight rats. Tissues for histological examination were fixed in 10% formalin and stained with hematoxylin and eosin and Fite's stain (6). One mm cubes of tissue from footpads, snout, tail, sciatic nerve, and testes were fixed in 2% glutaraldehyde,

post-fixed in osmium tetroxide, and embedded in either Epon 812 or Araldite.

## RESULTS

The number of *M. leprae* in one ear of four animals was determined just before death and in the remaining animals approximately one year before they were killed, i.e., at about the midpoint of the experiment. The results (Table 1) indicated disseminated infection with massive replication of *M. leprae*. In general, there was a correlation between the numbers of organisms counted in the ear and those found in the various organs microscopically (Table 2).

Gross autopsies revealed no significant findings except in the animal inoculated intratesticularly where there was a small yellow area at the apparent site of inoculation. By light microscopy, significant histological changes were seen only in the nonhair bearing, distal parts of the body, namely, the footpads, snout, ears, tail, and testes. In all of these organs there was a rather wide variation in the degree of histologic change. In the footpads the principal change was edema (which was not seen grossly) and macrophage infiltration. This was most marked in the dermis and subcutaneous tissues where the number of macrophages varied greatly from animal to animal. In some footpads there were large granulomatous masses of cells with foamy cytoplasm, in others, the infiltration of macrophages was diffuse (Fig. 1). Occasional giant cells

TABLE 2. Average distribution of *M. leprae* in various tissues following inoculation.

Tissue <sup>a</sup>	Average (range) <sup>b</sup>
Snout	5 plus (4-5)
Footpads	5 plus (4-5)
Tail	4 plus (2-5)
Testes	3 plus (0-5)
Ears	3 plus (2-4)
Spleen	2 plus (0-3)
Lymph nodes	2 plus (0-2)
Bone marrow	2 plus (0-2)
Lung	1 plus (0-2)
Liver	1 plus (0-3)

<sup>a</sup> Organisms were not identified in other tissues.

<sup>b</sup> Because *M. leprae* were localized to only certain areas of the tissue it seemed preferable not to quantify them in terms of the number of fields examined. They were therefore quantitated on the following 1 to 5 plus scale:

- 1 plus = a single organism identified in the section;
- 2 plus = several organisms or clusters of organisms seen;
- 3 plus = organisms easy to find;
- 4 and 5 plus = large and very large numbers present.

were present. Generally, the immediate subepidermal zone of the dermis was spared. The muscles of the footpads were frequently edematous and infiltrated by foamy macrophages. Individual muscle fibers were often fragmented. Small nerves were also swollen and vacuolated. In one animal, macrophages were seen infiltrating the synovial membranes of the joints of the foot. Although mast cells were fairly numerous and occasional plasma cells were seen, lymphocytes and polymorphonuclear leukocytes were conspicuous by their absence. Fite stains revealed enormous numbers of *M. leprae* within macrophages, which in some instances could be seen in H and E sections as pale, amorphous, basophilic masses within the cytoplasm. These were equivalent to the numerous globi seen with the Fite stain. Large numbers of bacilli could also be seen in fibroblast-like cells between the muscle bundles while smaller numbers were present within the adventitia of blood vessels, the perineurium of nerves, and the sarcoplasm of muscle cells. Bacilli were not seen within the epidermis, which appeared normal microscopically. Many organisms appeared to be lying free in the tissues.



FIG. 1. Edema and histiocytic infiltration of dermis and underlying muscle of the footpad (H&E  $\times 40$ ).

The snout was always involved and often contained large numbers of macrophages reminiscent of lepromatous leprosy. These were found beneath the mucous membrane of the nasal passages, as well as beneath the skin (Fig. 2), and often were present around sebaceous glands. Moderate or large numbers of acid-fast bacilli were found within the macrophages (Fig. 3) with the formation of globi. Small numbers of bacilli were also seen in perichondrial fibroblasts as well as in striated muscle cells, sebaceous cells, and small nerves. In the ears the changes were mild by comparison. There was a scanty focal infiltrate of foamy macrophages again often in relation to sebaceous glands. Moderate numbers of *M. leprae* were present within macrophages as well as perichondrial fibroblasts. The few small nerves seen in the ears were not in-

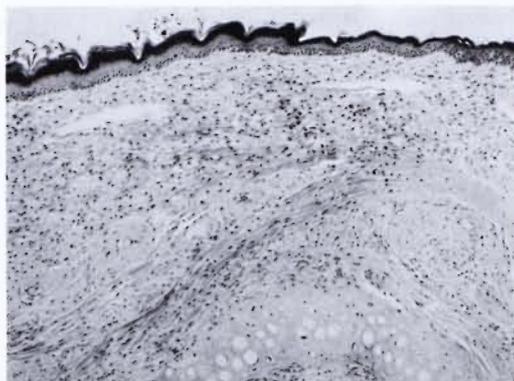


FIG. 2. Heavy infiltration by macrophages of the snout (H&E  $\times 40$ ).

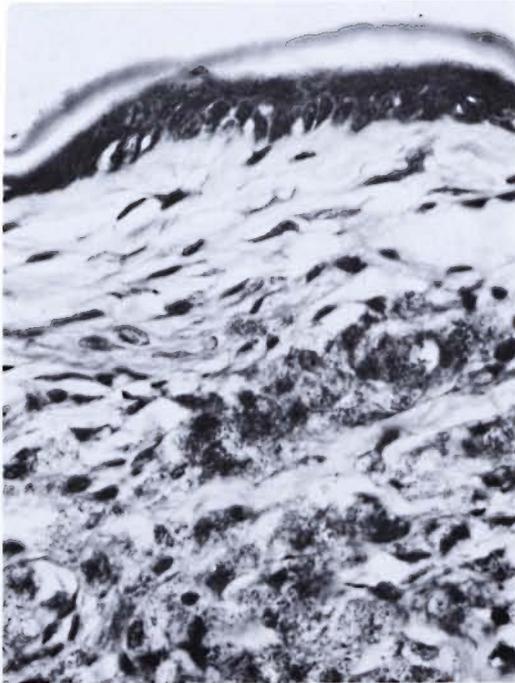


FIG. 3. *M. leprae* within macrophages in the snout (Fite  $\times 400$ ).

involved. The tail also showed a focal histiocytic infiltrate. The cells were lying individually beneath the epidermis and in the deep dermis and subcutaneous tissue. Organisms were generally numerous, particularly near the base of the tail around sebaceous glands. Organisms were also present in small nerves.

The testis was a major site of involve-

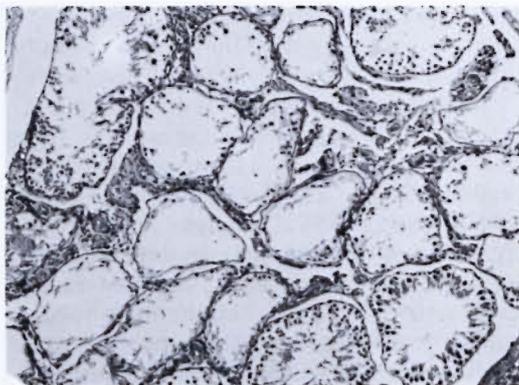


FIG. 4. Severely reduced spermatogenesis and focal macrophage infiltration of the interstitium of the testes (H&E  $\times 40$ ).

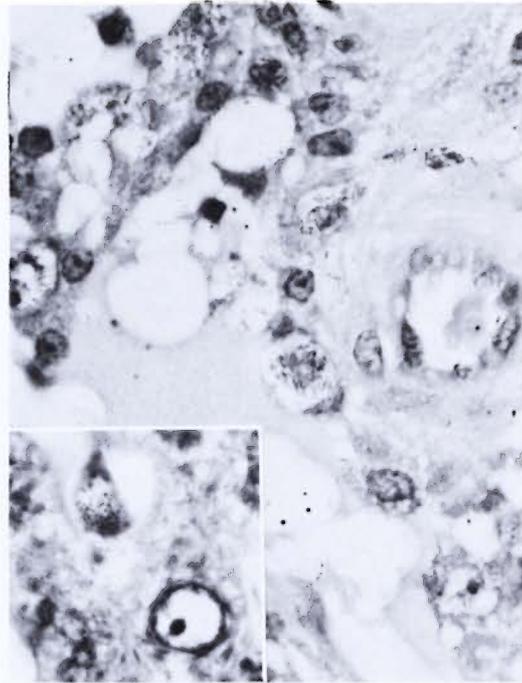


FIG. 5. *M. leprae* in the interstitium of testes. Inset. *M. leprae* within a Kupffer cell in the liver (Fite  $\times 1,000$ ).

ment in animals injected intravenously as well as in the rat inoculated intratesticularly. There was generally a complete absence of spermatogenesis, and the tubules were lined only by Sertoli cells. The interstitium was edematous and contained numerous small collections of foamy macrophages (Figs. 4 and 5). In some testes, these tended to be located beneath the tunica albuginea. In the rat inoculated intratesticularly there was focal fibrosis with dystrophic calcification and a scanty infiltrate of plasma cells with an occasional polymorphonuclear leukocyte, which were interpreted as being secondary to the inoculation. The testes contained large numbers of *M. leprae*, the majority of which were within macrophages, but some were present in the adventitia of blood vessels and an occasional organism was present intracellularly within the tubules, but it was not clear whether these were Sertoli cells or macrophages. Occasional *M. leprae* were found in reticuloendothelial cells in the liver (Fig. 5), spleen, bone marrow, lymph nodes, and lung, generally unaccompanied by other

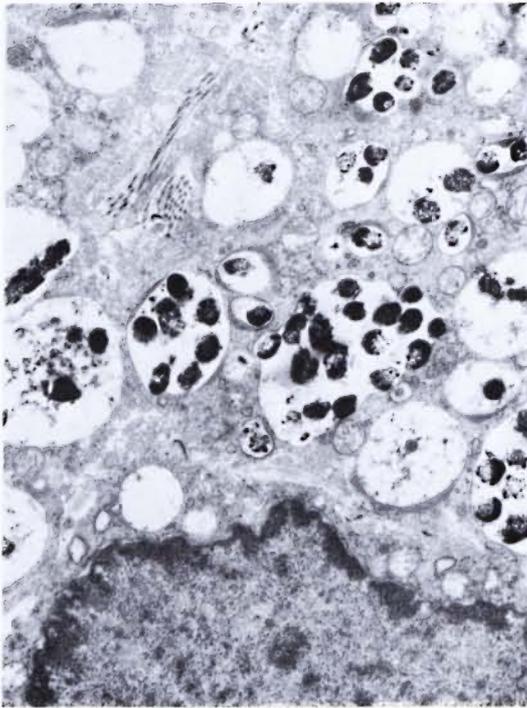


FIG. 6. Degenerating *M. leprae* lying within double membrane bound vacuoles in the cytoplasm of a macrophage in the snout ( $\times 10,000$ ).



FIG. 7. *M. leprae* lying free in the sarcoplasm of a muscle cell in the snout ( $\times 20,000$ ).

histopathological changes. A single macrophage containing organisms was seen in the medulla of one kidney. The remainder of the tissues was essentially unremarkable, and no organisms were seen in them. These included hair-bearing skin, sciatic nerve, leg muscles, diaphragm, intestinal tract, seminal vesicles, female genital tract, and brain. However, occasional foci of bronchopneumonia and chronic pyelonephritis were found in some animals.

Electron microscopy confirmed that the majority of organisms were within macrophages. Most of the fibroblast-like cells seen with the light microscope were found to have the ultrastructural characteristics of macrophages. The intracellular appearance of the bacilli was similar irrespective of the location of the cells. Both intact and fragmented *M. leprae* were contained within double membrane bound vacuoles (Fig. 6) while the cytoplasm was remarkable for the increased numbers of organelles, particularly heterogeneous lysosomes. An occasional bacillus was seen apparently lying free in the cytoplasm.

Electron microscopy of the footpads and snout showed intact bacilli in clusters of from 3 to 200 organisms lying free in the cytoplasm of striated muscle, i.e., not contained within an organelle (Fig. 7). The myofibrils adjacent to the organisms showed degeneration with the formation of numerous myelin figures. Bacilli were present in small numbers within the Schwann cells and perineural cells of nonmyelinated and myelinated nerve fibers (Fig. 8a, b). Several venules and lymphatic channels contained bacilli lying free within their lumens and within endothelial cells (Fig. 9). By light microscopy numerous organisms appeared to be extracellular, possibly because of the very heavy infiltration. However, by electron microscopy virtually all of these bacilli showed degenerative changes and were surrounded by fragments of degenerated cytoplasm, suggesting that they had been liberated by death of the macrophages which originally had phagocytosed them. Examination of the muscles proximal to the footpad showed involvement of the muscle with the presence of a small number of organisms. The sciatic nerve and thigh mus-

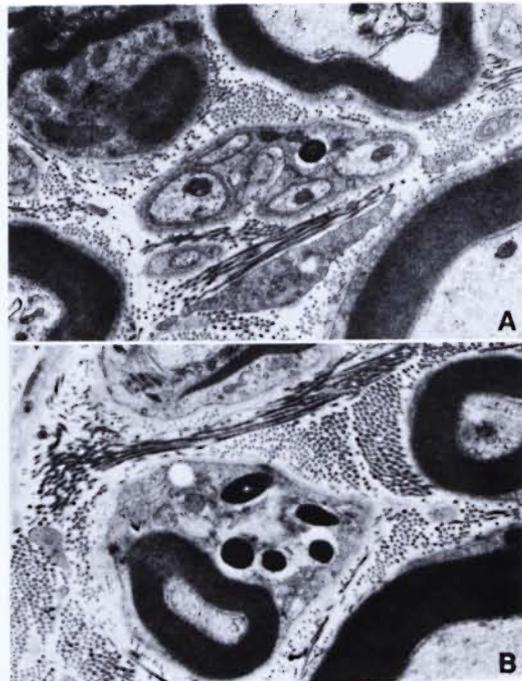


FIG. 8. *M. leprae* lying free in the cytoplasm of Schwann cells surrounding a) nonmyelinated and b) myelinated axons in the rat snout ( $\times 12,000$ ).

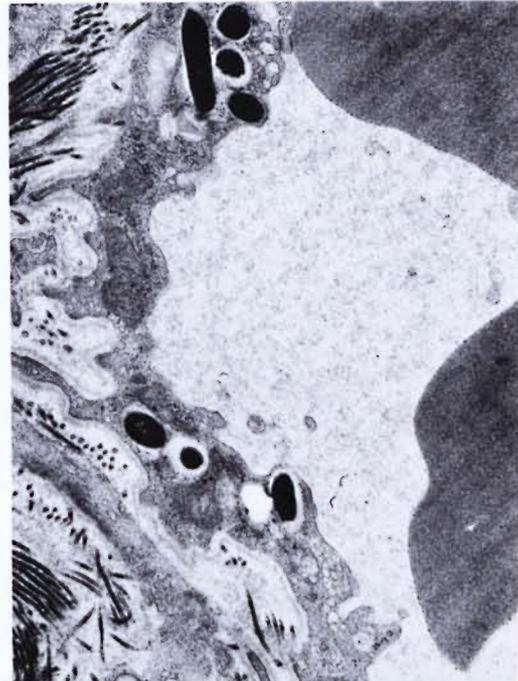


FIG. 9. *M. leprae* lying free within the cytoplasm of an endothelial cell in the snout ( $\times 16,000$ ).

cles appeared normal, and bacilli could not be demonstrated within them.

The very large numbers of *M. leprae* seen in the testes were for the most part contained within macrophages. Again intact and fragmented bacilli were found intracellularly within phagolysosomes while fragmented bacilli were found extracellularly. Organisms were also seen in the adventitial cells of some blood vessels. A very occasional bacillus was found within the seminiferous tubules, but we could not be certain if these were within macrophages or Sertoli cells.

#### DISCUSSION

Our results show that following intravenous inoculation of *M. leprae* in the NTLR, the infection localized primarily to the non-hair bearing areas of the body, namely the footpads, snout, ears, and tail. The most severe changes and the largest numbers of bacilli were found in the footpads although the actual number of bacilli varied widely. The most severely infected pads showed a heavy infiltrate with macrophages with ob-

vious degenerative changes in muscle and nerve, which did not extend to the thigh muscles and sciatic nerve. In contrast, moderate numbers of bacilli were found in sections of the ears with only a slight increase in inflammatory cells.

The snout was also remarkable for heavy bacillation accompanied by a marked macrophage infiltrate in areas covered by both skin and mucous membrane. While in some areas the lesions bore a superficial resemblance to lepromatous leprosy, both the character and distribution of the inflammatory infiltrate were different. One important difference from humans was the very large numbers of extracellular organisms seen in the rat, although the vast majority, if not all, appeared nonviable by the criteria of Rees and Valentine (<sup>13</sup>). Originally, it was felt that these were due simply to rupture of the cells either *in vitro* or during sectioning, but since this is not seen in human lepromatous leprosy, it is probably not the correct explanation. Rees and Weddell (<sup>16</sup>) and Esiri, *et al.* (<sup>2</sup>) have postulated that *M. leprae* replicate in muscle cells and that this is the source of the continued in-

fection since these cells cannot form phagosomes and hence are unable to kill the organisms, which eventually are liberated into the tissues. However, comparison of the numbers of bacilli found in muscle cells and macrophages makes it unlikely that the former are the source of the extracellular bacilli. Further, such organisms would be expected to appear nonbeaded.

Particularly striking was the ease with which organisms were found within reticuloendothelial cells in the liver, spleen, lungs, and lymph nodes. It is not clear whether these resulted from clearing of the organisms from the blood stream or from migration of macrophages from the primary sites of infection in the nonhair bearing extremities. Localization of disease was also noted in the interstitium of the testes even in animals inoculated intravenously. Here, as elsewhere, organisms were found extracellularly.

Our findings in the NTLR are similar to those previously reported by Evans and Levy in the footpads of intact BALB/c mice<sup>(3)</sup> and by Rees and colleagues in thymectomized and irradiated CBA mice<sup>(15,16)</sup>. The only major difference in the distribution of the organisms was our inability to find them within the sciatic nerve, a constant site of involvement in the mouse<sup>(21)</sup>.

The exact role of T-cell depletion on the cellular events leading to disseminated leprosy in the NTLR has not been explored. While the rats showed depletion of the thymic dependent regions in the spleen and lymph nodes, there is evidence to suggest that they retain some T-cell function since their lymphocytes are able to respond *in vitro* to concanavalin A, although to a reduced extent (Colston and Fieldsteel, unpublished). Evans and Levy<sup>(3)</sup> have described an alteration in the appearance of the macrophages which they correlate with the development of cellular immunity and termination of the logarithmic phase of bacterial growth in the intact mouse. Our model is, of course, quite different as the infection is generalized. But it is of interest that virtually all the macrophages examined by us with the electron microscope were activated, i.e., possessed numerous organelles and bacilli contained within double membrane bound phagolysosomes. It should be noted, however, that all of our animals

were examined one year or more after inoculation. Rees, *et al.*<sup>(15)</sup> have shown that replacement of the lymphoid tissue of thymectomized irradiated mice will restore the hosts' ability to limit the infection. This has not so far been attempted in the rat. The exact nature of the perturbation in lymphocyte/macrophage interaction that permits the progressive replication of *M. leprae* has yet to be worked out in either model.

### SUMMARY

We report the histologic and electron microscopic findings following intravenous inoculation of *M. leprae* into neonatally thymectomized Lewis rats, which were killed one to two years later. All organs appeared normal grossly. Histologic changes were confined to the footpads, snout, ears, tail, and testes, all of which were involved in every rat. The tissues were edematous and infiltrated by varying numbers of foamy macrophages. In the footpads muscle fibers were vacuolated, and small nerves showed degenerative changes. Large numbers of *M. leprae* were present in macrophages and striated muscle cells and smaller numbers in perineural cells and pericytes, as well as lying free in the tissues. Occasional intracellular bacilli were found throughout the reticuloendothelial system. Electron microscopy confirmed that the majority of organisms were within activated macrophages. Both intact and fragmented bacilli were contained within double-membrane bound vacuoles. Numerous *M. leprae* were lying free within the sarcoplasm of striated muscle cells. Virtually all of the extracellular organisms were degenerating.

### RESUMEN

En este trabajo estudiamos los cambios histológicos y los hallazgos al microscopio electrónico que resultaron de la inoculación intravenosa con el *M. leprae* en ratas Lewis timectomizadas al nacimiento, las cuales fueron sacrificadas 1 a 2 años después. Todos los órganos tuvieron una apariencia normal. Los cambios histológicos, en todas las ratas, estuvieron confinados a los cojinetes plantares, la trompa, las orejas, la cola y los testículos. Los tejidos estuvieron edematosos e infiltrados por macrófagos espumosos en números variables. En los cojinetes plantares, las fibras musculares estuvieron vacuoladas y los nervios pequeños mostraron cambios degenerativos. Se observó un gran número de *M. leprae* en los macrófagos

y en las células del músculo estriado y un pequeño número de bacilos en las células perineurales y en los pericitos, así como algunos bacilos residiendo libremente en los tejidos. Ocasionalmente se observaron bacilos intracelulares en el sistema retículo endotelial. La microscopía electrónica confirmó que la mayoría de los microorganismos estuvieron dentro de macrófagos activados. Los bacilos, intactos o fragmentados, se observaron dentro de vacuolas limitadas por una doble membrana. También se observaron numerosos *M. leprae* residiendo libremente en el sarcoplasma de las células del músculo estriado. Todos los microorganismos extracelulares estuvieron virtualmente degenerados.

### RÉSUMÉ

On relate dans cet article les observations histologiques et de microscopie électronique pratiquées à la suite de l'inoculation intraveineuse de *M. leprae* chez des rats nouveau-nés de Lewis thymectomisés, tués une ou deux années plus tard. Tous les organes sont apparus normaux à un examen macroscopique. Les modifications histologiques étaient limitées aux coussinets plantaires, au museau, aux oreilles, à la queue, ainsi qu'aux testicules, ces organes étant atteints chez tous les rats. Les tissus étaient oedématisés et infiltrés par un nombre variable de macrophages spumeux. Les fibres musculaires dans les coussinets plantaires étaient vacuolisées, et les filets nerveux présentaient une atteinte dégénérative. De très nombreux *M. leprae* ont pu être observés dans les macrophages, de même que dans les cellules musculaires striées; on en a également observés en plus petits nombres dans les cellules, périneural et dans les péricytes, et également dans les tissus, en dehors des cellules. Des bacilles intra-cellulaires ont également été relevés à l'occasion dans le système réticulo-endothélial. La microscopie électronique a confirmé que la majorité des organismes étaient situés à l'intérieur de macrophages activés. Des bacilles intacts, de même que des bacilles fragmentés, étaient hébergés dans des vacuoles tapissées d'une double membrane. On a observé de nombreux *M. leprae* libres à l'intérieur du sarcolemme des cellules musculaires striées. Presque tous ces organismes cellulaires étaient dégénérés.

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