INTERNATIONAL JOURNAL OF LEPROSY

Volume 33, Number 3
Printed in U.S.A.

Nerve Involvement. Comparison of Experimental Infections by *Mycobacterium leprae* and *Mycobacterium lepraemurium*[1]

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INTRODUCTION

During the mid-19th century when leprosy came under intensive study by Danielssen and Boeck, it was considered as a chronic disease entity affecting skin and nerves. The involvement of nerves has, therefore, long been recognized as a characteristic of leprosy, and it is still the most typical feature on which diagnosis can be made clinically and histopathologically. As a matter of fact the involvement of nerves can be observed histopathologically in any lesion of human leprosy, regardless of type, provided nerves are present in the affected part.

In 1956 one of us (C.H.B.) began animal transmission experiments with human leprosy, evaluating the results by histopathologic examination.[3] In several species of animals the cooler parts of the body, such as ears, foot pads, and testes, were inoculated with saline suspensions of specimens from leprosy patients with skin lesions rich in bacilli. Inoculated animals were followed until death occurred spontaneously. Microscopic nerve lesions were reported in the ears of 30 per cent of the hamsters in 20 human-to-hamster experiments(1). The nerve lesions developed very slowly and generally were not apparent until the second year after inoculation. However, if lesions were present in animals dying during the second year after inoculation, nerves were generally involved.

At the meeting of the Technical Committee on Pathology and Experimental Transmission, VIIIth International Congress of Leprology, Rio de Janeiro, September 1963 there was considerable discussion on the significance of nerve involvement in animals that had been inoculated with *Mycobacterium leprae*. The Committee included in its report the following: "Because in man leprosy is the only mycobacterial infection known to involve nerves, and although it is stated that murine leprosy does not affect nerves, the Committee recommends that this question be reinvestigated in murine leprosy."(2)

As a result of this recommendation, we decided to undertake an experiment with *Mycobacterium lepraemurium*, using the technics we had developed in following the outcome of the inoculation of human leprosy bacilli into hamsters and mice.

METHODS

The procedures we have used routinely for inoculating and histopathologically evaluating the results obtained in the ears and foot pads and for examining the auricular and sciatic nerves of small mammals are as follows:

Technic for ears. With a short (¼ in. or ½ in.) 26 or 27 gauge needle and a

[1]This work was supported in part by Grant AI-04005 and Grant AI-220 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

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[3]Transmission with *M. leprae* experiments. U. S. Public Health Service, Communicable Disease Center, Chamblee, Georgia, January 1956-June 1961; and Armed Forces Institute of Pathology, Washington, D. C., June 1961.

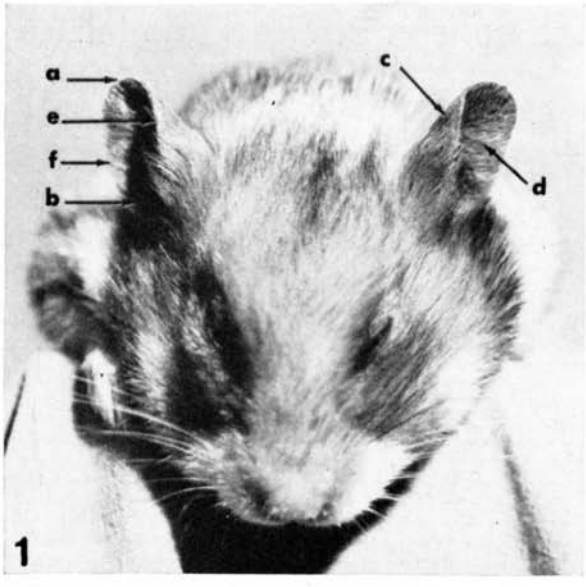

FIG. 1. Head of golden hamster showing anatomic orientation of external ears. **a** = apex; **b** = base; **c** = superior surface; **d** = inferior surface; **e** = anterior edge; **f** = posterior edge. AFIP Neg. 65-4220 (2).

tuberculin syringe approximately 0.05 ml. of inoculum is injected between the epidermis and the cartilage in the middle of the superior aspect of the external ear. This dose raises a bleb about 1 cm. in diameter. At the time of the autopsy the ears are cut off and placed between pieces of cardboard which are held together by paper clips. This insures that the ears are flattened during fixation. After fixation the hairs are cut off with scissors. The base of the ear (for anatomic orientation, see Figure 1) is trimmed to leave a straight edge, and three strips are cut from the inoculated basal half. With a small membrane punch the basal strip is marked with one notch, the middle with two notches, and the apical piece with one notch. The notches are made near either the anterior or the posterior edges of the strips (Fig. 2). The technician, in embedding the strips in the block, places the unnotched cut surfaces downward. The central piece, which has two notches, is placed first, and on either side the basal and apical pieces are placed, so that the notched edges appear at the same end of the block. The appearance of the sections in the finished slide is shown in Figure 3. The basal section is the thicker one, appearing as "C" in the photograph; the apical one appears as "A."

The pathologist will not experience difficulties in distinguishing from which area the sections of the ear are taken, since the apical part is shorter and more heavily pigmented than the basal part. By thus having these three levels of the ear arranged in this parallel fashion, the pathologist can follow lesions and nerves from one level of the ear to another.

Technic for feet. Using a 1 ml. tuberculin syringe and a short (¼ in. to ½ in.) 26 or 27 gauge needle, we inoculate the hind feet in the middle of the foot pad. In the hamster approximately 0.10 ml. of inoculum is injected into the soft tissue beneath the epidermis, resulting in the diffuse swelling of the foot pad. The foot pad of the mouse will receive about 0.05 ml. The legs are cut off above the ankle (Fig. 4). After fixation, the 2nd through 5th toes are cut off and the foot is divided into three pieces with scissors. The distal side of each piece is marked with India ink. After decalcification the technician embeds the pieces in paraffin with the unmarked sides facing downward. The appearance of the finished section is seen in Figure 5. The distal section is the flat pieces with five bones appearing as "A" in the photograph. The middle section is the oval piece with five bones appearing as "B" in the photograph. The proximal section is the round piece with two bones, indicated by "C" in the photograph. The plantar and dorsal sides of the foot can be distinguished without difficulty, because the plantar sides are nearly hairless and most of the muscle tis-

sue is located on the plantar side of the bones. The pathologist can follow lesions and nerves from one level of the foot to the other.

Technic for nerves. The great auricular and sciatic nerves are removed and flattened out on a piece of cardboard, (Fig. 6A). After drying for a few minutes the specimens stick to the cardboard and will remain attached during fixation. After fixation each nerve is cut separately into segments of about ½ cm. in length and the pieces are placed side by side on the surface of solid agar in a Petri dish (Fig. 6B). The pieces of nerve are then covered with melted agar (Fig. 6C). After the solidification of the agar in a refrigerator, a block containing the nerve pieces is cut from the plate (Fig. 6D). This block is embedded in paraffin. In sections, the great auricular nerves can be distinguished from the sciatic nerves by their smaller size (Fig. 7).

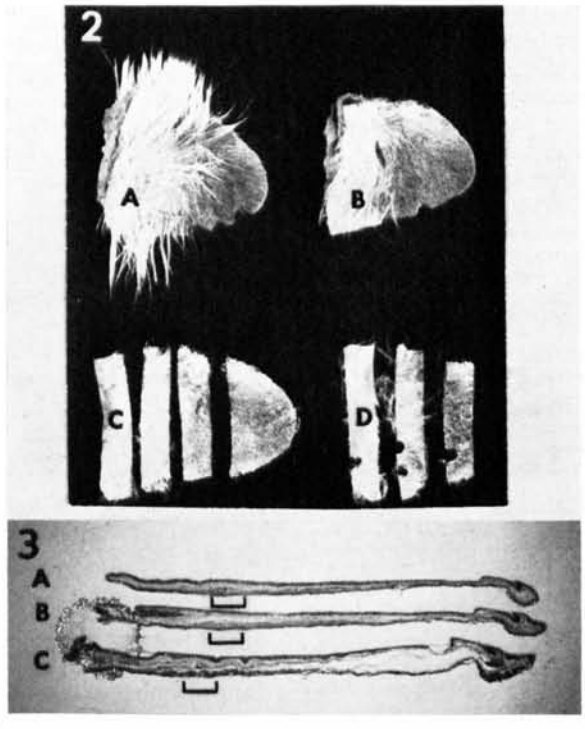

FIG. 2. Demonstration of standardized technic for cutting ears of experimental animals inoculated with leprosy bacilli. **A.** Flattened ear of hamster after fixation between two pieces of cardboard, which were kept together by paper clips. The notches in the edge of the ear are markings made at the time of inoculation as a part of a system for numbering the animals. **B.** The hairs have been cut off. **C.** The base of the ear was trimmed and three pieces were cut for microscopic examination from the inoculated basal half of the ear. **D.** The basal piece was marked with one punch, the middle piece with two punches, and the distal piece with one punch; the technician will embed the pieces in paraffin with the unmarked sides directed downward and the double marked piece placed in the middle, so that the marked edges are at the same end of the block. AFIP Neg. 64-2697 (2).

FIG. 3. Right ear of golden hamster, (L-6854) with hematoxylin and eosin stain, ×2. The pieces were cut and embedded in paraffin as described under Figure 2. The apical piece of the ear (**A**) is located at the top. In each piece the superior surface of the ear—recognizable by the presence of a muscular coat between the epidermis and the cartilage—is directed downward. Since this is the right ear, the anterior edge of each piece is on the right side of the figure. Now it is possible to locate the lesions in the ear (brackets) and to judge their extensions in the nerves and other tissues. AFIP Neg. 65-2094.

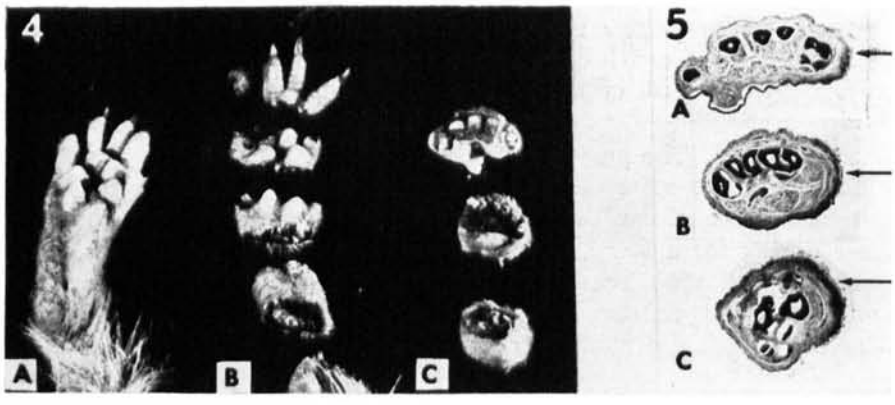

FIG. 4. Demonstration of standardized technic for cutting hind feet of experimental animals inoculated with leprosy bacilli. **A.** Fixed right hind foot of golden hamster. **B.** The 2nd through 5th toes were cut off; three pieces were cut from the foot for microscopic examination. On its distal surface each piece was marked with India ink. After decalcification, the technician will embed the pieces in paraffin with the unmarked sides facing downward. **C.** Facing upward are the unmarked sides from which microscopic sections will be cut. The three pieces can be distinguished from each other by their shape and the number of bones present in the cut surface. AFIP Neg. 64-2697 (3).

FIG. 5. Right hind foot of golden hamster (L-6915), hematoxylin and eosin stain, ×2. The pieces were cut and embedded in paraffin as described under Figure 4. The distal piece of the foot is **A**, the middle piece is **B**, and the proximal piece is **C**. The plantar and dorsal surfaces of each piece can be recognized by the distribution of muscle tissue, tendons and nerves. The plantar aspects are directed downward. Since it is the right hind foot, in each piece the lateral side is directed to the right. Now it is possible to locate lesions in the foot (arrows) and to judge their extension in the nerves and other soft parts. AFIP Neg. 65-2093.

NERVE LESIONS IN EARS OF GOLDEN HAMSTERS INOCULATED WITH *M. LEPRAE*

With our technic of cutting and embedding the ears we can usually locate the inoculum in the connective tissue and muscle tissue between the epidermis and cartilage in the middle of the apical and middle sections.

The mycobacterial lesions that may develop after several months are of microscopic size. They consist of mononuclear cell infiltrations, located outside the residual inoculum. Many, but not all, of the inflammatory cells contain acid-fast bacilli. Acid-fast bacilli are also frequently present in the epithelial cells of adjacent hair follicles. Generally, when growth results, the nerves present in the lesions are affected in animals that live longer than one year. However, we have noticed lesions in the nerves of several animals that died before the end of the first year after inoculation, sometimes as early as seven months after inoculation. We have the impression that the bacilli first appear in epineurial cells of the nerves that are located in, or close to, the initial inflammatory lesion. Later, intraneurally, acid-fast bacilli and mild cellular infiltration are observed. In the survey of the inoculated sites made possible by our standardized technic it appears that the lesions in the nerves become more extensive than those in the other soft parts. Affected nerves show many lesions with bacilli several millimeters from the inoculation site in the connective tissue.

This characteristic of the infection is demonstrated in Figure 8. The ear was inoculated 20 months before spontaneous death of the hamster with a suspension from a lepromatous skin lesion of a patient in the Philippines. Figure 8 presents the three sections of the right ear. The lesions are located close to the posterior edge of the ear in the areas that are indicated with braces (Fig. 3). Apparently, the same

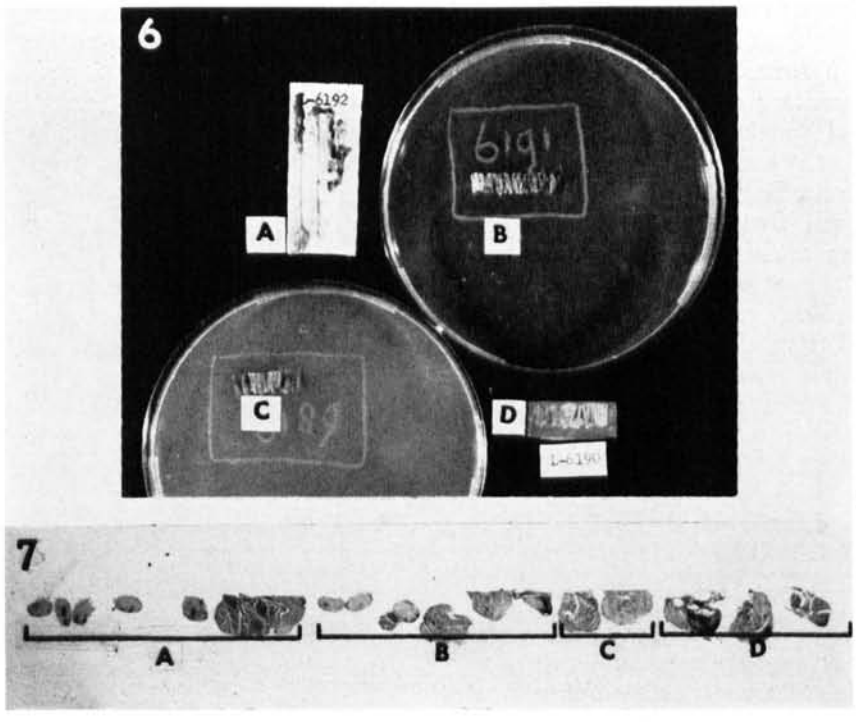

FIG. 6. Demonstration of standardized technic for cutting and embedding great auricular nerves and sciatic nerves of experimental animals. **A.** Piece of cardboard with great auricular and sciatic nerves of golden hamster. On the left, the left and right sciatic nerves. On the right, the left and right great auricular nerves. The proximal end of each nerve is directed upward. **B.** The nerves were cut in pieces each about ½ cm. in length. The pieces were put on an agar plate in the same order as they were cut, beginning with the proximal part of the left sciatic nerve. **C.** The pieces were covered with melted agar. **D.** After solidification of the agar, a block including the nerve pieces was cut from the plate for embedding in paraffin. AFIP Neg. 64-2697 (4).

FIG. 7. Photomicrograph of nerves from a block as shown in Figure 6D with hematoxylin and eosin stain, ×5. **A.** Left sciatic nerve. **B.** Right sciatic nerve. **C.** Left great auricular nerve. **D.** Right great auricular nerve. The distal parts of the sciatic nerves, and the great auricular nerves are surrounded by muscle tissue. AFIP Neg. 65-5438.

nerve is affected in all three sections (Fig. 8, A, B & C). The surrounding connective tissue reveals lesions in the apical and middle sections only. The spread of the infection through the nerve appears to be independent of the lesions in the surrounding tissue.

NERVE LESIONS IN HIND FEET OF GOLDEN HAMSTERS INOCULATED WITH *M. LEPRAE*

With our technic of cutting the feet, we locate the inoculum in the connective tissue and muscle between the epithelium and bones of the distal and middle sections.

The mycobacterial lesions that may develop after several months are so mild that observation can be made only microscopically. In most cases the lesions are located in the dermal connective tissue of the lateral side, quite a distance from the site of the inoculum. The lesions are similar to those occurring in the ears. Also, in the foot pads of hamsters the infection apparently spreads within the nerves, as is shown in Figure 9. The foot pad of this hamster had been inoculated with a suspension of a lepromatous skin lesion of a Surinam patient 24 months before spontaneous death. An inflammatory lesion rich in bacilli

was observed in the connective tissue of the distal section (Fig. 9B). A small nerve in this area revealed bacilli in epineurial and inflammatory cells (Fig. 9C). In the middle and proximal sections nerves are involved in the same areas (Fig. 9D), but in the surrounding tissues the lesions are slight. Small foci of inflammatory cells with a few acid-fast bacilli are observed in the distal part of the right sciatic nerve (Fig. 10). It is probable that the infection had passed from a small local lesion in the subepidermal connective tissue into an adjacent small nerve and then had spread through this nerve to the distal part of the sciatic nerve.

NERVE LESIONS IN HIND FEET OF LABORATORY MICE INOCULATED WITH *M. LEPRAE*

Usually the microscopic mycobacterial lesions are observed in the middle of the foot pad in the muscle and connective tissue, close to the site where the inoculum was injected. The infection of nerves occurs with a time lapse similar to that observed in golden hamsters. In mice, also, the infection spreads through the nerves. This is demonstrated in Figures 11-14, presenting lesions in the left hind foot of a hybrid black (Chatterjee) mouse, 23 months after inoculation. The inoculum for this animal had been obtained from foot pads of mice that were inoculated 13 months before with a suspension of a lepromatous skin lesion of a Surinam patient.

In Figure 11 arrows indicate the site of the lesions in the sections of the foot. Figure 12 presents lesions in the distal part. Acid-fast bacilli are present in small nerves and in surrounding tissues. Figure 13 reveals similar lesions in the middle of the plantar side of the proximal part. With respect to the large number of bacilli, it may be noted that the amount of inflammatory infiltration is very mild, as shown in Figure 13, which is a photomicrograph (hematoxylin and eosin stain) of the same area pictured in Figure 14. The corresponding sciatic nerve (Figs. 15 & 16) reveals numerous bacilli in the distal part and small foci of bacilli in the middle and proximal parts very close to the spinal cord.

STUDY OF NERVE LESIONS IN MURINE LEPROSY

Using an inoculum of *M. lepraemurium*, Hawaiian strain, we inoculated 125 NIH white Swiss general purpose female albino mice, in the right ear and right hind foot pad with approximately 5,000 bacilli in each site. After the second month postinoculation, inflammatory lesions with acid-fast bacilli were observed at the sites of inoculation. After the seventh month the inoculated feet of most mice were swollen; therefore, serial sacrifice of the animals was begun on a monthly basis. Microscopically the swollen feet revealed extensive inflammatory lesions composed of mononuclear cells laden with acid-fast bacilli. In the inoculated ears the development of lesions was irregular. Often the base of the ear and the corresponding lymph node were swollen, whereas the site of inoculation was grossly normal. Microscopically the swollen parts revealed lesions similar to those observed in the inoculated foot pads.

Dissemination of the infection occurred generally after the tenth month following

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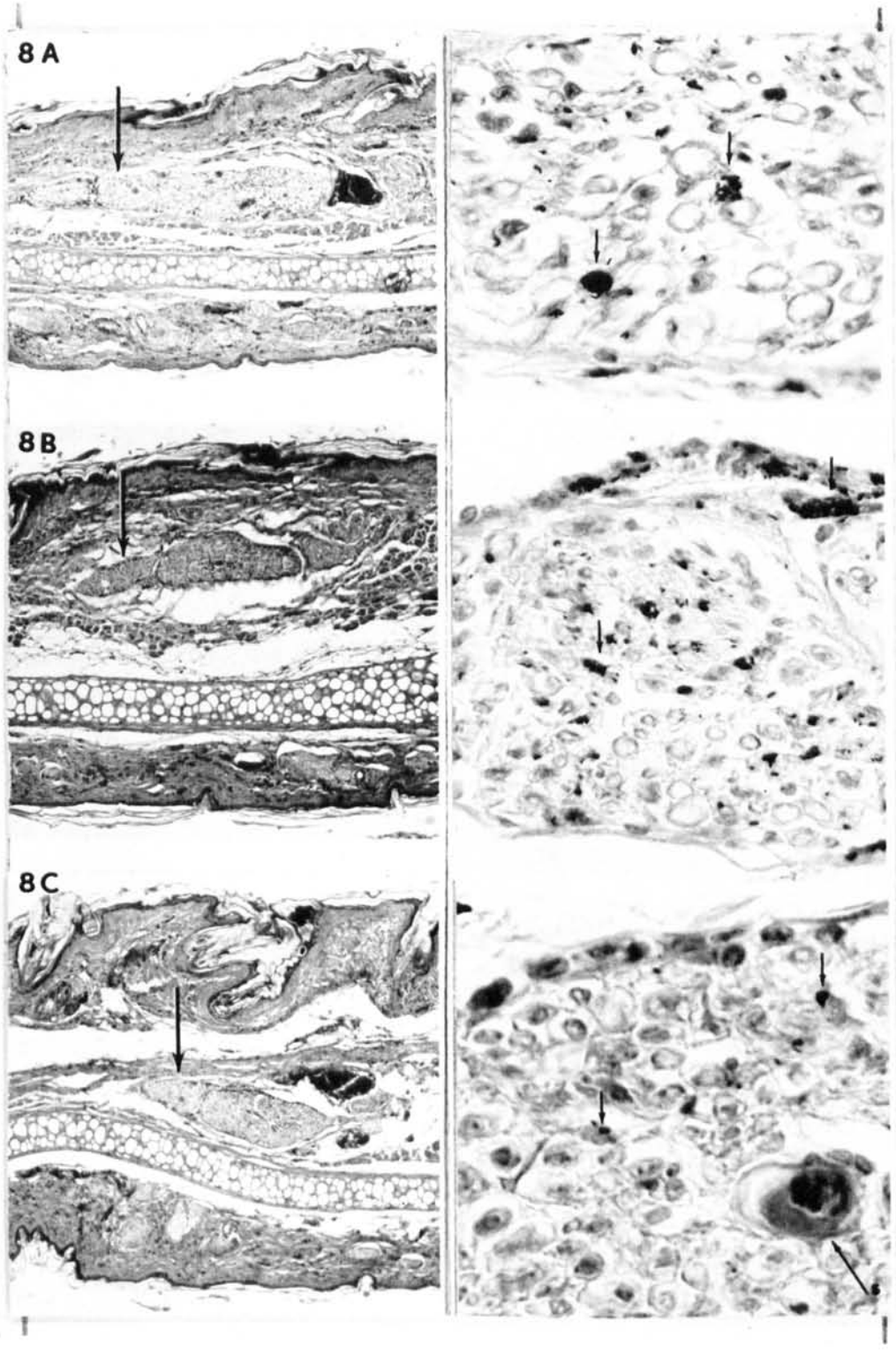
FIG. 8. Apical (**A**), middle (**B**), and basal (**C**) pieces of right ear of golden hamster (L-6854). Twenty months before spontaneous death of the hamster, the ears had been inoculated with a suspension from a skin lesion of a lepromatous leprosy patient of the Philippines. The microscopic fields are from the parts indicated by brackets in Figure 3, and in the middle of each photograph (arrows) show the same nerve. Inflammatory cells are present in the nerve and in the epineurium. On the left the photomicrographs were made from hematoxylin and eosin-stained sections, ×50. AFIP Negs. 63-5439, 65-5440, and 65-5441.

On the right the photomicrographs were made from a Fite-Faraco-stained section of the nerves indicated by the arrows on the left. The acid-fast bacilli (arrows) are shown in packets and globular masses. Solitary bacilli are also seen. Under the microscope the bacilli were usually observed to be intracellularly located. A Schaumann body is shown in **C**, ×650. AFIP Negs. 65-5442, 65-5443, and 65-5444.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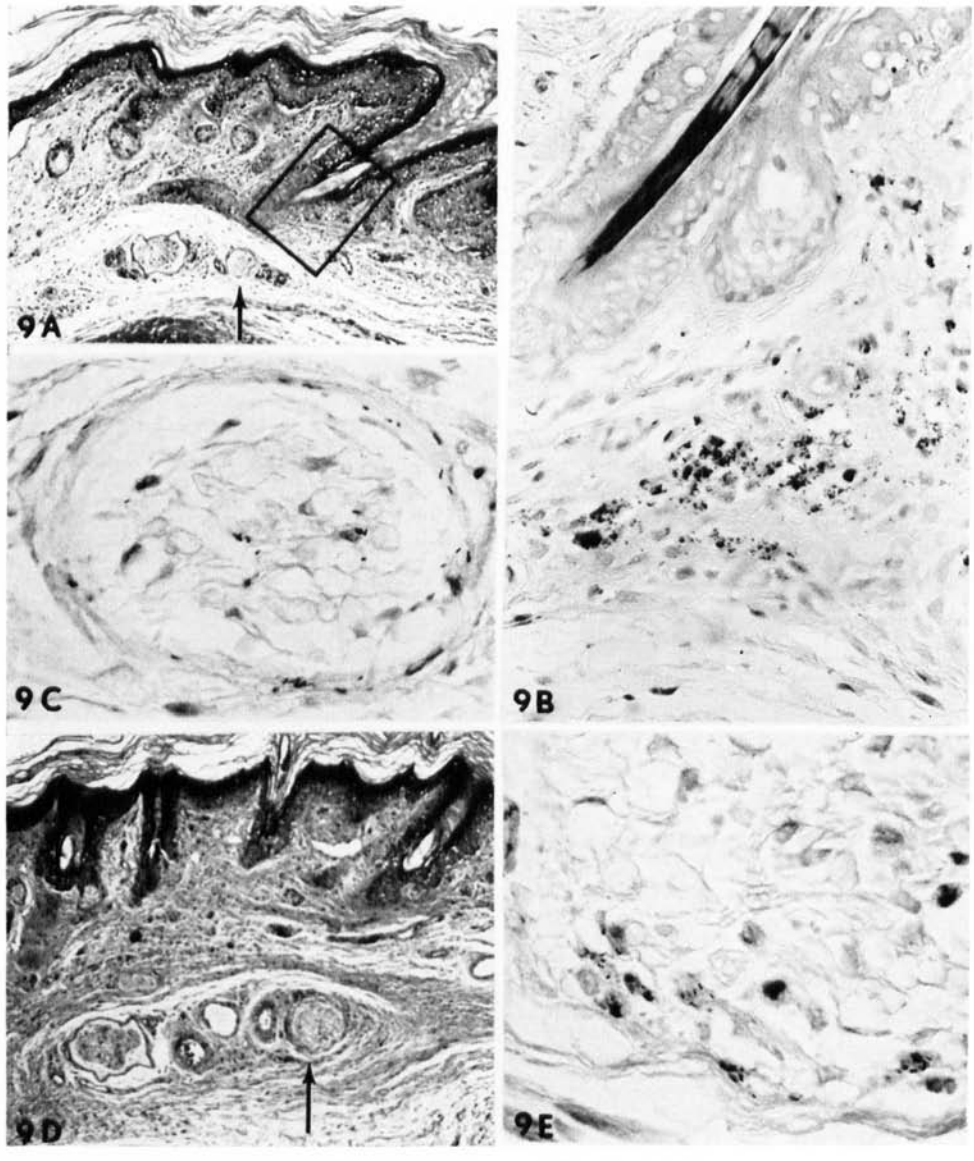
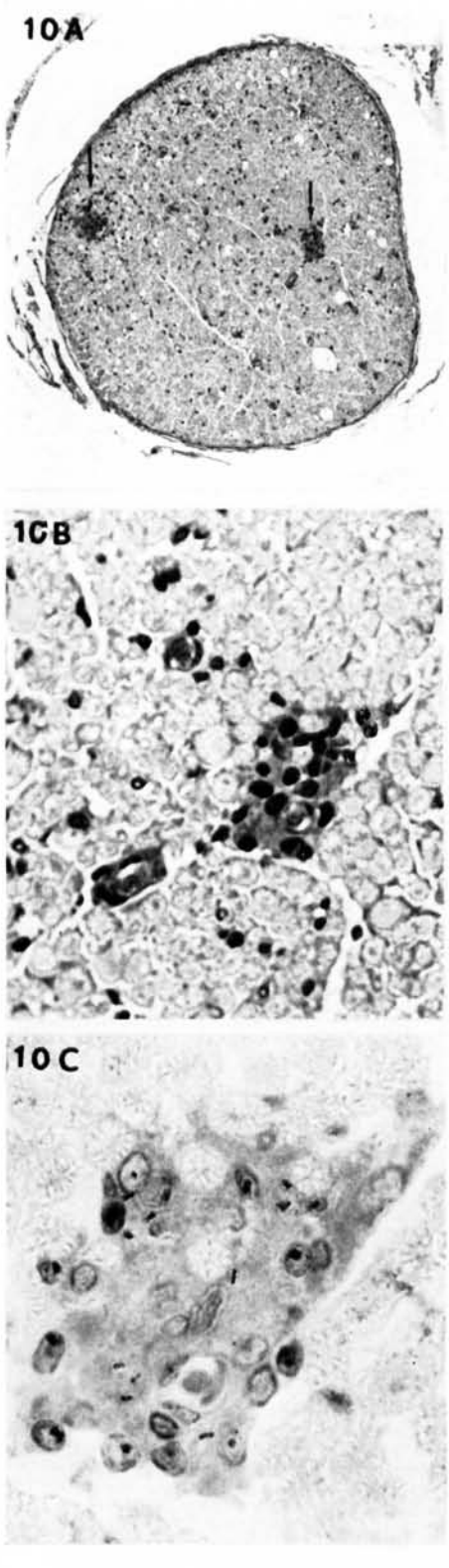


FIG. 9. Photomicrographs from right hind foot of hamster (L-6915). **A.** Distal piece, hematoxylin and eosin stain, ×50. The microscopic field is indicated by an arrow in Figure 5A. The hind feet and ears were inoculated with a suspension from a skin lesion of a lepromatous leprosy patient in Surinam 24 months before spontaneous death of the hamster. AFIP Neg. 65-5445. **B.** Outlined area of **A,** Fite-Faraco stain, ×265. Around a hair follicle numerous acid-fast bacilli are observed in inflammatory cells. AFIP Neg. 65-2890. **C.** Nerve indicated by arrow in **A,** Fite-Faraco stain, ×650. Acid-fast bacilli are observed within the nerve and in the epineurial cells. AFIP Neg. 65-5446. **D.** Proximal piece of hind foot, hematoxylin and eosin stain, ×50. The microscopic field is indicated with an arrow in Figure 5C. In connective tissue there are mononuclear inflammatory cells; some contain acid-fast bacilli. AFIP Neg. 65-5447. **E.** Part of nerve indicated with arrow in A, Fite-Faraco stain, ×650. Acid-fast bacilli are observed within the nerve and in the epineurial cells. AFIP Neg. 65-5448.



inoculation. Most severely involved were organs and tissues of the reticuloendothial system, such as lymph nodes, liver, spleen, and bone marrow. Frequently, lesions were observed also in the lung and skin. In several mice microscopic lesions were noticed in the kidneys and heart muscle. In the majority of the animals by the time metastatic lesions occurred there was local necrosis with polymorphonuclear cell infiltration at the site of inoculation. Animals with grossly visible metastatic lesions were emaciated, and several died spontaneously from the widespread infection.

Animals found dead, if not decomposed, and all animals killed, were autopsied and the tissues were studied histopathologically. The experiment was terminated at the end of 15 months. Seventy-seven mice were autopsied. The inoculated parts, right ear and right foot and corresponding great auricular nerves and sciatic nerves, were investigated routinely. The infiltration of nerves by *M. lepraemurium* was observed from the seventh month after inoculation. As the experiment progressed the incidence of nerve infiltration did not increase. The infiltration of nerves by acid-fast bacilli was observed 28 times in 21 mice, but only in the inoculated parts in which well-developed, often tumor-like granulomas were noted. The incidence of nerve lesions in ears and feet is shown in Table 1.

The affected nerves were infiltrated with macrophages containing many acid-fast bacilli. It appeared that the affected nerves were involved by an extension of the severe granulomatous inflammatory process from the adjacent tissue. This is demonstrated in Figures 17 and 18, presenting the lesions of the right hind foot of a mouse inoculated 11 months before. The foot was swollen

FIG. 10. Photomicrographs from right sciatic nerve of same hamster as in Figure 9. **A.** This part of the sciatic nerve is taken approximately from the middle of the leg. There are two small inflammatory lesions (arrows). Hematoxylin and eosin ×95. AFIP Neg. 65-5449. **B.** Section shown in A, hematoxylin and eosin stain, ×400. AFIP Neg. 65-2888. **C.** Fite-Faraco-stained section of area shown in B, ×975. AFIP Neg. 65-2882.

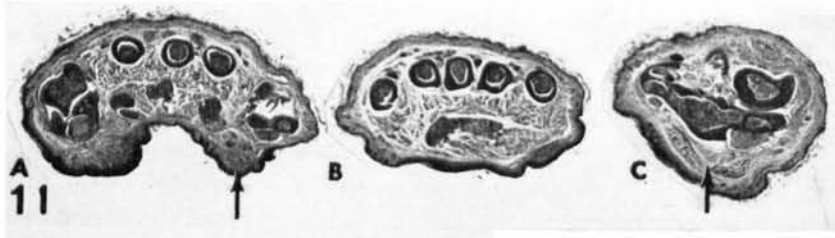


FIG. 11. Left hind foot of hybrid black mouse (Chatterjee), hematoxylin and eosin stain, ×6. **A** is the distal piece, **B** the middle piece, and **C** the proximal piece. The plantar surface of each piece is directed downward. The mouse lived 23 months after inoculation of the foot pads with bacilli obtained from foot pads of hybrid black mice, which were inoculated 13 months before with a suspension prepared from a skin lesion of a lepromatous leprosy patient in Surinam. AFIP Neg. 5450.

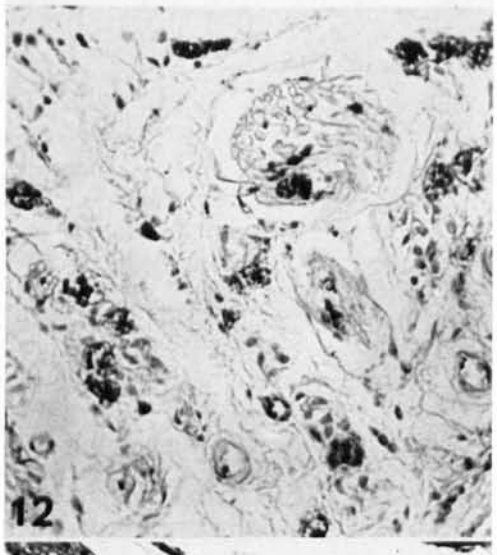


FIG. 12. Area indicated by arrow in Figure 11A, Fite-Faraco stain, ×265. Many bacilli and groups of bacilli are observed in nerves and surrounding tissues. AFIP Neg. 65-2869.

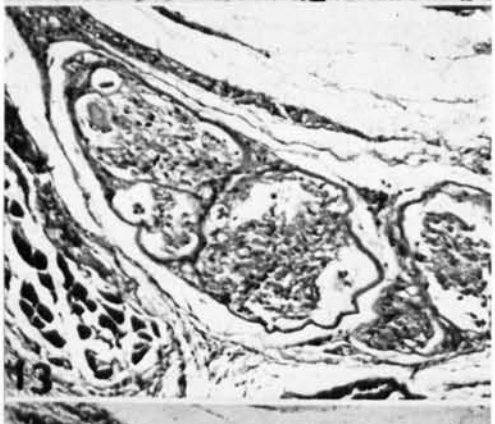


FIG. 13. Area indicated by arrow in Figure 11C, hematoxylin and eosin, ×100. Nerve with mild inflammatory lesions. AFIP Neg. 65-2872.

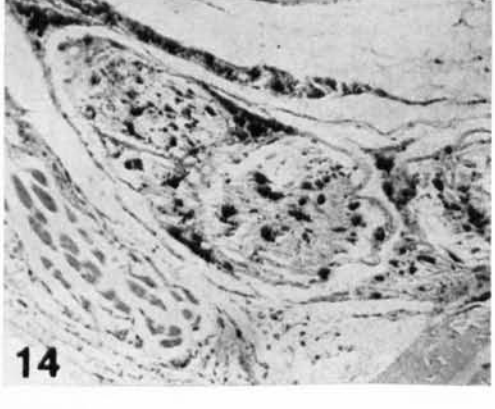
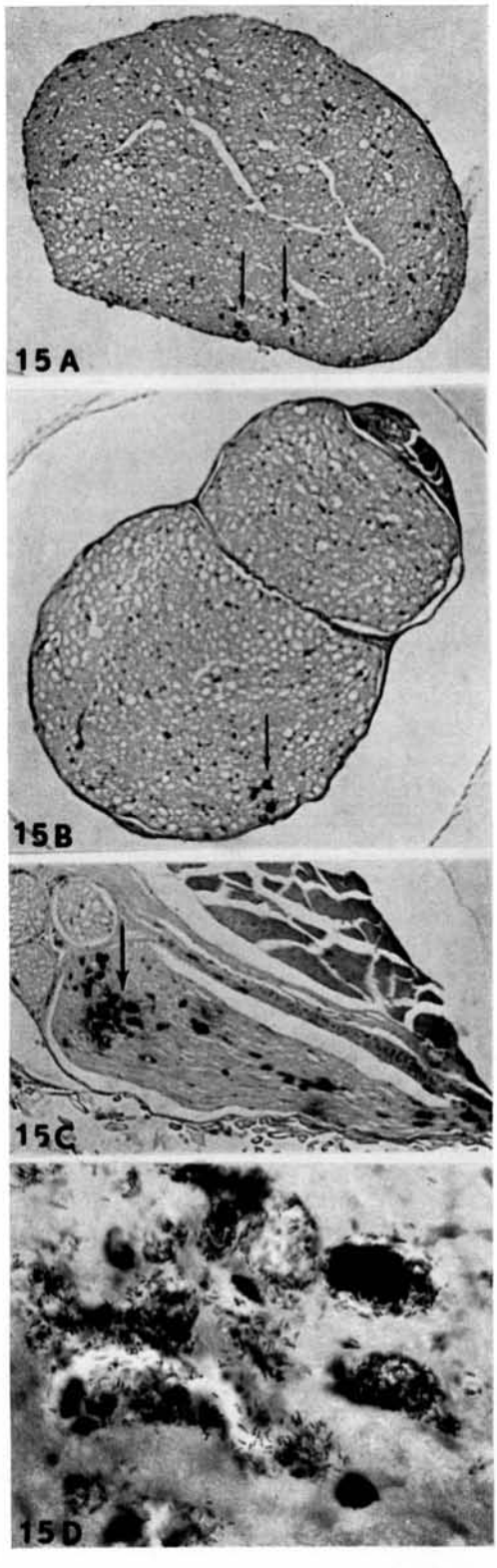


FIG. 14. Same as Figure 13, Fite-Faraco stain, ×100. The acid-fast staining reveals numerous bacilli and groups of bacilli. AFIP Neg. 65-2871.



and ulcerations had developed at several places. The middle of the foot is shown in Figure 18A, at low magnification. Most of the original tissue had been replaced by the granulomatous process. In the entire section two nerves were seen that were partly replaced by inflammatory cells. One nerve lesion is shown in Figures 18A and B, and Figure 19. In other parts of the foot such nerve lesions were not observed, but nerves along with the other structures may already have been destroyed completely.

In four mice the right great auricular or right sciatic nerves were affected. In all cases the nerves were surrounded by a large mass of granulomatous tissue with many acid-fast bacilli. The nerves were partly replaced by similar inflammatory cells in a small segment only.

CONCLUSIONS

1. Mycobacterial lesions occurring in ears and hind feet of golden hamsters and laboratory mice after inoculation with human leprosy bacilli remain of microscopic size during the life span of the animals. Involvement of nerves is generally observed in animals with lesions who live longer than one year after inoculation. With regular use of a special technic for cutting and blocking the inoculated parts,

FIG. 15. Illustrations of left sciatic nerve of mouse mentioned in Figure 11. **A.** The proximal piece of the removed sciatic nerve, location corresponding with (a) in Figure 16. Groups of bacilli (arrows) are observed between nerve fibers. Fite-Faraco stain, ×130. AFIP Neg. 65-2867. **B.** This is the middle piece of the removed sciatic nerve; location corresponds with (b) in Figure 16. Fite-Faraco stain, ×130. Groups of acid-fast bacilli are indicated by arrow. AFIP Neg. 65-2866. **C.** The distal piece of the removed sciatic nerve. Location corresponds with (c) in Figure 16; Fite-Faraco stain, ×80. Numerous acid-fast bacilli are present within one of the branches of the sciatic nerve. AFIP Neg. 65-2865. **D.** The microscopic field is indicated by an arrow in **C.** Fite-Faraco stain ×650. AFIP Neg. 65-2873.

TABLE 1.—*Incidence of infiltration of nerves in ears and feet of mice infected with* M. lepraemurium.

	Number investigated histopathologically	Number with mycobacterial granulomas	Number with nerve infiltration
Right ear	77	71	8
Right hind foot	77	77	16

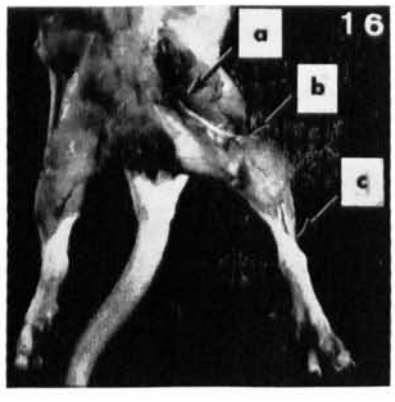


FIG. 16. Mouse with exposed proximal part of right sciatic nerve. Arrows indicate levels that correspond with the sections of the sciatic nerve in Figure 15. AFIP Neg. 64-894 (4).

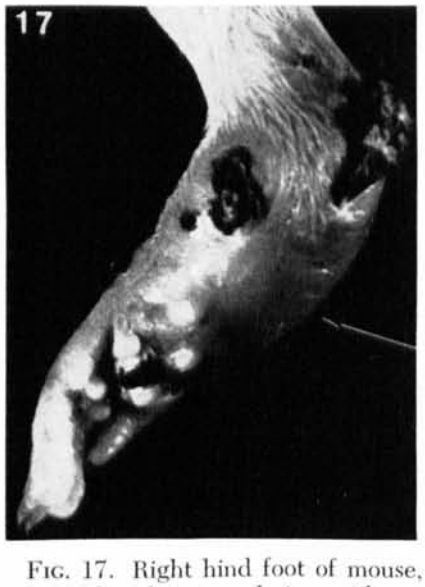


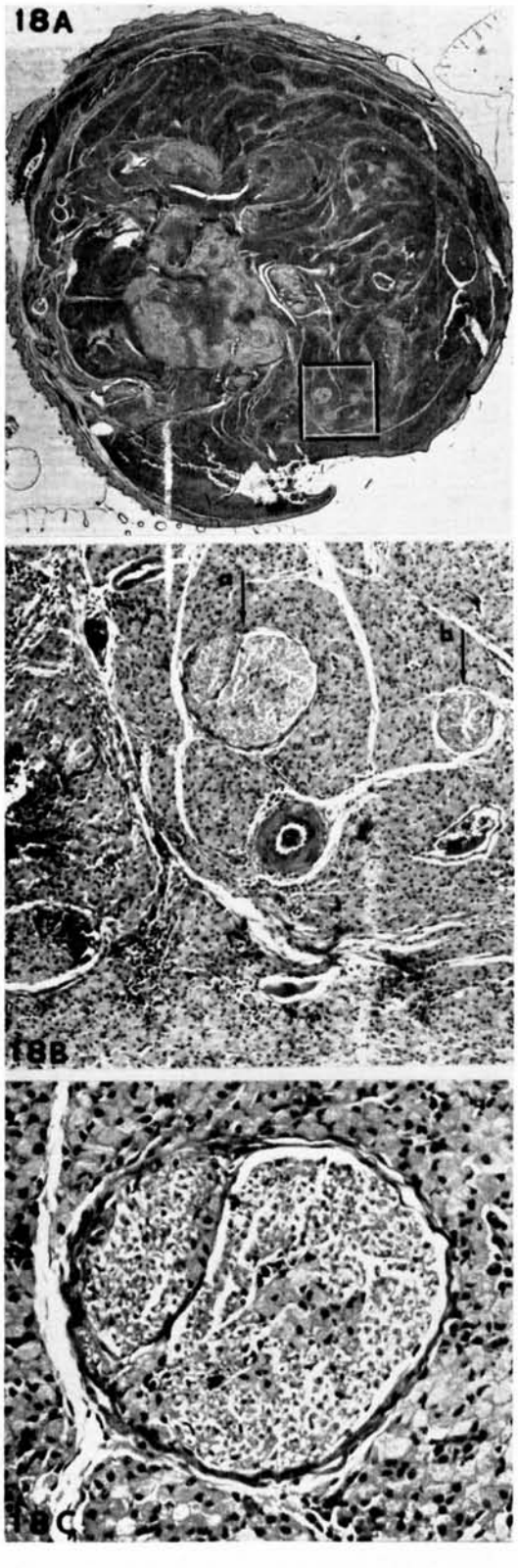
FIG. 17. Right hind foot of mouse, 11 months after inoculation with rat leprosy bacilli. AFIP Neg. 64-6637.

it appears on microscopic examination that the spread of the infection through the nerves is independent of infection in the other soft parts. We propose to call this type of nerve involvement seen in animals inoculated with *M. leprae* "primary nerve invasion."

2. The nerve lesions observed in the ears and feet of hamsters and mice inoculated with human leprosy bacilli, resemble those occurring in indeterminate and early lepromatous leprosy in man. In the experimental lesions caused by *M. leprae* it appeared that the bacilli had gained entrance into the nerves by first invading the epineurial cells, where many months after inoculation small inflammatory lesions had developed in the perineurial tissues. Later bacilli could be observed in the intraneural tissue.

3. Mycobacterial lesions occurring in the ears and hind feet of albino mice many months after inoculation with *M. lepraemurium* became tumor-like. Some involvement of nerves was observed in those animals that lived longer than 7 months, but on use of the same technic for cutting the inoculated parts as was used for the animals inoculated with the human leprosy bacilli, nerves were affected only locally in areas with extensive granulomatous lesions. There is no evidence that the infection spread distally or proximally through the nerves. Such extension into nerves should be possible in any severe necrotizing, chronic inflammatory lesion that replaces tissue. We propose to call this type of nerve involvement "secondary nerve infiltration."

In susceptible animals, rat leprosy spreads by the hematogenous route; therefore, small lesions were frequently observed in the noninoculated left ear and left hind foot of the animals. However, in such



metastatic lesions nerve involvement was not observed. This observation is in agreement with that of Fite([3]), who, in experimental *M. lepraemurium* infection of rats by intravenous inoculation, very seldom noticed affection of nerves. As a result of this study we do not think that *M. lepraemurium* demonstrates the predilection for nerves that is a characteristic feature of *M. leprae*.

FIG. 18. Section of right hind foot in Figure 17. **A.** This section is from the level of the foot indicated by arrow in Figure 17. Fite-Faraco stain, ×91. Most of the former structure of the foot has been replaced by the granulomatous inflammatory lesion. AFIP Neg. 65-2892. **B.** Outlined area of **A**, hematoxylin and eosin, ×70. One nerve **(a)** is infiltrated by the granulomatous inflammatory process. Another nerve **(b)** is not damaged. AFIP Neg. 65-2862. **C.** Nerve of **B** indicated with arrow **a**; hematoxylin and eosin, ×195. The nerve is partly replaced by inflammatory cells. AFIP Neg. 65-2860.

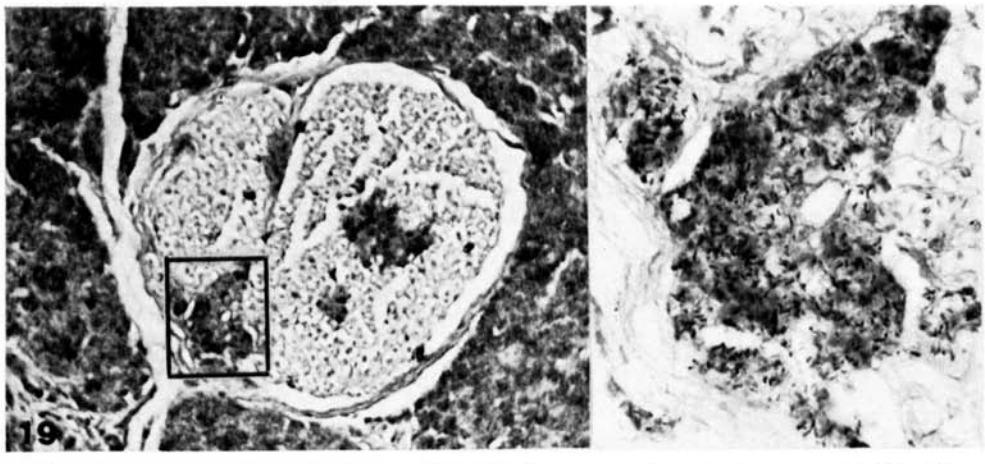


FIG. 19. Same area as shown in Figure 18C, Fite-Faraco stain, ×195 (inset ×755). The right part of the picture is a high power micrograph of the outlined area. AFIP Negs. 65-2861 and 65-5451.

SUMMARY

We have described the methods we have developed for the preparation of the ears, feet, and large nerves of hamsters and mice that have enabled us to follow the course of the invasion of nerves by *M. leprae*. Applying similar technics in mice inoculated with *M. lepraemurium*, we have observed the effects of that bacillus on nerves. When the granulomatous infection it produces becomes advanced, *M. lepraemurium* may infiltrate and destroy nerves along with the other tissues in the inoculated ears or feet, but it does not show the tendency selectively to invade nerves that characterizes the human leprosy bacillus.

Acknowledgment. Thanks are due to Dr. Gert L. Laqueur, Chief, Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland, for helpful suggestions on the manuscript.

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DISCUSSION

Dr. Mason. Thank you very much, Dr. Wiersema. I am sure there will be much discussion of this paper.

Dr. Hanks. In looking at the situation of *M. leprae* in the nerves of the mouse, I gained the impression that perhaps some of these bacillary clusters might have been proliferating outside of cells. In the cases where *M. lepraemurium* had invaded nerves, it seemed to me quite obvious that the bacilli were beautifully encased within Schwann type or histiocytic cells. If these impressions are wrong, please correct me.

Dr. Wiersema. In some areas it seems as if bacilli were found in nerve tissue that looked normal. Whether they are located in Schwann cells or within the nerve fibers is not evident in microscopic sections. Electron microscopic examination may be helpful in answering this question.

Dr. Middlebrook. Dr. Wiersema, as we all know, the level of tuberculin hypersensitivity elicited by *M. tuberculosis* in hamsters is much greater than that in the mouse. I wonder if you have, or if anyone to your knowledge has, prepared a heated extract of *M. lepraemurium* and tested it for delayed type hypersensitivity in the skin of hamsters and mice infected with these organisms.

Dr. Wiersema. I do not have any experience with this type of investigation.

Dr. Rees. First let me congratulate the speaker on investigating a problem that our Committee on Animal Transmission particularly requested at Rio, and also note the wisdom of the Committee in asking that this be done. I do this because I would like to remind the audience that all classical textbooks on murine leprosy say that nerves are not involved, although one of our Japanese colleagues at our Committee meeting did refer us to Japanese literature indicating that, grossly, nerves are involved in murine leprosy. But he has very carefully tried to say, at the same time, that, although the literature was wrong, and nerves are involved in murine leprosy, it can be clearly distinguished from the type of nerve involvement seen in human leprosy in the hamster and mouse. The most distinctive feature in murine leprosy is that the most extensive infection of surrounding tissues results in only a relatively minor infection of nerves, whereas in experimental human leprosy even minor tissue infections show nerve involvement with bacilli in Schwann or perineurial cells. In collaboration with Drs. Weddell and Palmer at Oxford I am particularly interested in this problem and, while I do not want to go into this very special subject, I would like to say we agree with Dr. Wiersema that the cells in nerves in murine leprosy look like the cells in any other tissue in murine leprosy. In experimental human leprosy, on the other hand, they look different. I believe there is no way of defining these in the human leprosy infection without either special staining, or more particularly, electron microscopy. Already we have fairly good evidence suggesting that the cell types in which the human leprosy bacilli are found are either perineurial or Schwann cells, which are easily distinguished from inflammatory cells in electron microscopy by the possession of a basement membrane. At this level it should be possible to clinch this once and for all.

Dr. Pappagianis. I would like to suggest an experiment which is perhaps not practical. Could one infect both hind foot pads, section the sciatic nerve on one side, and by so doing perhaps demonstrate that the nerve does or does not provide a trophic role for the development of the human leprosy bacillus? If sectioning of the sciatic nerve prevents the development of the bacilli on the side of the section, this would suggest that some nutrients are provided by the nerve itself, and not just by the Schwann cells.

Dr. Shepard. I would like to ask what line of mice these were?

Dr. Wiersema. We have observed this result in both white and black mice. The case I showed you was of a black mouse 24 months after inoculation. The black mouse was a Chatterjee mouse. The white mice were from the National Institutes of Health.

Dr. Pattyn. I would like to ask what is the earliest time at which the nerve involvement is apparent?

Dr. Wiersema. In lepraemurium infection of mice we observed nerve involvement, first, seven months after inoculation, when tumorous lesions had developed at the site of inoculation. In experimental human leprosy of hamsters and mice we have observed nerve involvement in inoculated ears and feet six months after inoculation. However, it generally takes about one year before nerve involvement becomes evident in our routine sections in experimental human leprosy.

Dr. Winslow. I wonder if you have any idea, Dr. Wiersema, how the leprosy bacilli are transmitted along the nerve in murine and human leprosy.

Dr. Wiersema. In experimental lepraemurium infection of mice some nerves are locally infiltrated and apparently destroyed. We have no indication that lepraemurium bacilli are transmitted by the nerves. After the bacilli have penetrated nerves in experimental human leprosy of hamsters and mice, the infection spreads proximally or distally within the nerves. The mechanism of this transmission is not clear.

Dr. Mason. There is one matter that I would like to raise which has bothered me ever since the beginning of Dr. Shepard's work with the foot pad. How much of the inoculum remains at the site an hour after it is injected?

I ask this because as an anatomist I find one of our best ways of demonstrating the movement of fluids in lymphatics to students is to inject the foot pad of any laboratory animal with trypan blue or India ink. In a matter of minutes these same substances can be detected in the popliteal lymph nodes or further proximally. I have not heard much comment on the possible involvement of lymphatic drainage from the foot pad area.

Dr. Shepard. We have been concerned from the beginning with the number of organisms lost from the foot, especially in the light of the long course of the experiments. We have tried to find out how many organisms it takes to produce an infection, and this study gives us some knowledge of it. It turns out that you need on the order of only 10 viable organisms to produce an infection; so you can not be having a very significant loss in the total number of organisms. Fluid is one thing that we are not actually talking about. Here we are talking about loss of the organisms. And if you put in some particulate material that traces the inoculum you can generally find it locally until it deteriorates there some time later.

Dr. Rees. Regarding murine leprosy and involvement of nerves, I should have said that our own group and two other groups in England have confirmed the kind of findings that Dr. Wiersema has given. Coming back now to a question—several of the questions—that have been asked. For other reasons we have been sectioning sciatic nerves of mouse foot pads inoculated with *M. leprae,* and have found that this makes no difference for multiplication of acid-fast bacilli within the foot pad. In fact, we were not doing these experiments for this reason. We were much more concerned as to whether this influenced the infection of the sciatic nerve itself or nerves within the foot pad. To our disappointment sectioning or regular crushing of the nerve, which would stimulate Schwann cells, has not led to a greater infection of the nerves. One point keeps coming up among the speakers, which I think from our own work is clear: in murine leprosy, although nerves appear to be involved, this is really not true endoneurial infection. I think the great difference between human leprosy and murine leprosy, as seen in the mouse, or the hamster, is that the bacilli really are not within the endoneurium in murine leprosy but are quite often and more frequently within the endoneurium in human leprosy.

Dr. Kirchheimer. This is in answer to the question about disappearance of *M. lepraemurium* from foot pads. We have tested our method by trying to recover *M. lepraemurium* from foot pads following injection of known numbers. What percentage recovery do we really get? If about two million organisms are inoculated in the foot pad, and we try immediately to recover the organisms, we are able to recover about 95 per cent of the organisms inoculated. After 30 minutes we still recover about 85 per cent. When I get back home, I may find that we have results at 1 hour, 2 hours and so on, following injection of known numbers.

Dr. Hilson. I too have made some estimates of the rate of loss of *M. lepraemurium* from mouse foot pads, and, broadly speaking, they agree with Dr. Kirchheimer's. But I can say that after two days there is only 25 per cent retention of bacilli in the foot pad. In other words, 75 per cent of injected bacilli have left the foot pad. In the case of the testis, something like 75 per cent of the bacilli are retained. Another

point I wanted to query was in connection with transmission of bacilli up nerves. I think Professor Payling Wright was the first to show the drift of tetanus toxin up nerves and the flow of poliomyelitis viruses. I wonder if *M. leprae* gets up nerves more easily because of its smaller size. This is just an impression and I do not know if it is valid.

Dr. Binford. Yesterday Dr. Reich mentioned the cultivable mycobacterium that we had obtained in hamsters after inoculation with human leprosy. We have had several strains—we call two of them "NQ" according to the designations of the experiment in which these were isolated. This organism, four to six months after inoculation, causes a histiocytic granuloma in the ears and foot pads of hamsters. One of the reasons, apart from the fact that the bacillus was cultivable, that convinced us that it was not the human leprosy bacillus was that it did not demonstrate any predilection for the small dermal nerves of the hamster. Occasionally—I would say in not more than a dozen instances in perhaps a thousand animals—we have found nerves involved. This involvement, we think, was accidental or environmental. So far we have seen no mycobacterium that affects nerves, in man or animal, in a manner similar to that which occurs in human leprosy or in animals experimentally infected with *M. leprae.*

I would like to emphasize, with reference to Dr. Dharmendra's remarks yesterday on rat leprosy, that we have never had any rat leprosy in our own colony at the Armed Forces Institute of Pathology or in the colony we previously maintained at the Communicable Disease Center in Atlanta. Dr. Y. T. Chang very willingly agreed to cooperate in this study on nerve lesions in rat leprosy and arranged for the mice to be kept at the National Institutes of Health, so that we would run no risk of having murine leprosy introduced into the animal colony used in our human leprosy experiments.

Dr. Mason. We shall now have to bring this session to a close. I wish to thank Dr. Pattyn and Dr. Wiersema for the important contributions that they have made to the program.