

Nerve Damage Induced by Mycobacterial Granulomas in Guinea Pig Sciatic Nerves¹

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Nerve damage is the major cause of deformity in leprosy (²). Although the histology of nerves in leprosy has been well described (^{4, 15, 16}) the establishment of an animal model would enable an elucidation of the immunological and biochemical events which lead to nerve pathology.

Studies of the sciatic nerves of rats (²⁰) and mice (^{9, 22}) after injection of *Mycobacterium leprae* in the foot pad have not led to useful models due to the long development time and minimal damage found. Wisniewski and Bloom (²⁴) injected tuberculin intraneurally into sensitized guinea pigs; likewise, Mshana, *et al.* (¹²) injected rabbits intraneurally with sonicated *M. leprae*. However, both groups only describe histological observations after 3 days, with a predominantly perivascular, diffuse pleomorphic infiltration and minimal nerve damage. None of these attempts has successfully reproduced the observations on human nerves.

Experimental mycobacterial granulomas in the post-auricular lymph nodes of the guinea pig have been studied for several years (¹³). Intradermal injection of live BCG vaccine into the ear has been shown to give rise to an epithelioid cell granuloma in the draining lymph node, similar to that found in tuberculoid leprosy. In contrast, injection of cobalt-irradiated *M. leprae* induced a granuloma composed of phagocytic macrophages, as found in lepromatous leprosy. Similar granulomas have been reproduced within the sciatic nerves of guinea pigs by the intraneural injection of BCG vaccine and dead *M. leprae*, and the resulting dam-

age has been assessed by histological and electrophysiological techniques.

MATERIALS AND METHODS

Animals. Outbred Hartley-strain female guinea pigs, weighing 300–350 g, were obtained from David Hall (Newchurch, Staffs., U.K.). They were fed on RGP pelleted diet, supplemented with cabbage.

Mycobacteria. Pasteur-strain live *M. bovis* BCG, as a suspension in saline, was provided by the Pasteur Institut, Paris, France. Cobalt-irradiated (2.5 Mrad), armadillo-derived *M. leprae* was kindly given by Dr. R. J. W. Rees, National Institute for Medical Research, Mill Hill, London.

Sensitization. Live BCG (1×10^7), or 1×10^9 Co-irradiated *M. leprae*, suspended in 0.05 ml saline, were injected intradermally into the dorsum of each ear of the guinea pig. BCG-sensitized animals were left for 2 weeks before intraneural injection of antigens and *M. leprae*-sensitized animals for 5 weeks. This dose and time scale has been shown to give optimum sensitization in guinea pigs (¹⁴).

Induction of intraneural granulomas. The guinea pigs were anesthetized with 0.05 ml/kg Hypnorm (Janssen Pharmaceuticals, Wantage, U.K.) and 0.25 mg/kg midazolam ("Hypnovel"; Roche, Welwyn Garden City, U.K.) given intramuscularly into the foreleg. The right sciatic nerve was exposed aseptically, by parting the overlying muscles, lifted from the surrounding tissues, and 0.01 ml of mycobacterial suspension was injected intraneurally using a microsyringe and a 30 G needle. The skin was sutured with 3/0 catgut (Ethicon, Edinburgh, U.K.). The animals were monitored according to the criteria of Morton and Griffiths (¹¹). None of our animals showed any signs of pain, distress or discomfort. The procedure was approved by the Ethical Committee of the Royal College of Surgeons.

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Animals sensitized with BCG received an intraneural injection of 10^6 BCG organisms; those sensitized with Co-irradiated *M. leprae* were given 10^6 Co-irradiated *M. leprae* organisms intraneurally. Unsensitized animals were given 10^7 BCG organisms or 10^9 Co-irradiated *M. leprae* organisms intraneurally (i.e., the same number of bacilli as was found to give optimum sensitization by the intradermal route). Control animals were injected with 0.01 ml of sterile saline (0.85% w/v).

Animals were killed at various time intervals from 3 days to 12 weeks after intraneural injection, and both sciatic nerves were taken for histological examination to establish the course of granuloma formation within the nerve. For electron microscopic investigations and electrophysiological studies, animals were used at the time of optimum granuloma formation and nerve damage (1 week for sensitized animals; 2 weeks for the unsensitized BCG-injected animals; 5 weeks for the unsensitized Co-irradiated *M. leprae*-injected animals). Groups of five animals were used throughout and saline-injected control animals were always included in the studies. The contralateral (left) sciatic nerve was used as a control for each guinea pig; brachial nerves and spinal cord were examined; and normal guinea pig sciatic nerves served as additional controls.

Histology. Sciatic nerves taken for routine histology were fixed in formol saline, paraffin embedded, and cut longitudinally. Sections of $15\ \mu\text{m}$ were stained with Holmes' silver stain for axons⁽⁶⁾; $5\ \mu\text{m}$ sections were stained with hematoxylin and eosin, Ziehl Neelsen (for acid-fast bacilli) and picrosirius (for collagen). Sections stained by the picrosirius method were viewed with polarized light to visualize the collagen types present⁽⁷⁾. Sections of $5\ \mu\text{m}$ were also stained with a monoclonal antibody, ICRCS1, which labels myelin in Formalin-fixed, paraffin-embedded sections (kindly supplied by Professor B. Cohen and G. Elias, ICRF/RCS Histopathology Unit, London; manuscript in preparation) using the double-layer immunoperoxidase method as described elsewhere⁽¹⁾ following rehydration through grades of alcohol.

Immunohistochemistry. Sciatic nerves were snap-frozen in iso-pentane cooled

above liquid nitrogen, then $7\ \mu\text{m}$ sections were cut on a cryostat (model FS/FAS, Bright Instrument Co. Ltd, Huntingdon, U.K.) at -13°C . Sections were dried at room temperature, fixed in acetone for 10 min then stained using a double-layer immunoperoxidase method as described elsewhere⁽¹⁾.

Monoclonal antibodies. The following mouse monoclonal antibodies raised against guinea pig cell-surface antigens were used: CT5 and CT7, both putative pan T-cell markers; CT6, a putative T cytotoxic/suppressor-cell marker. These three antibodies were kindly supplied as ascites by B. T. G. Tan (Free University Hospital, Amsterdam, The Netherlands). They are all of antibody Class IgG₁ and were used at dilutions of 1:1000 (CT5), 1:100 (CT6), and 1:50 (CT7)⁽²¹⁾. The other mouse monoclonal antibodies were: MSgp4 (an IgG₁) which detects major histocompatibility complex (MHC) class I antigens and MSgp8 (an IgG₃) which detects MHC class II antigens (Healey and Turk, unpublished data); MSgp9 (an IgG₁), a B-cell marker (not immunoglobulin) (Healey and Turk, unpublished data); and anti-macrophage antibody (an IgG_{2a})⁽¹¹⁾. These last four monoclonal antibodies were raised within the Department of Pathology, Royal College of Surgeons, and were used undiluted as supernatants.

Electron microscopy. Anesthetized animals were perfused through the left ventricle with 100 ml RPMI medium, followed by 200 ml fixative (4% glutaraldehyde in Sorensen's buffer). The sciatic nerves were removed, fixed for a further hour, washed in buffer, post-fixed with 1% buffered osmium tetroxide, dehydrated through grades of alcohol, and embedded in araldite. Transverse sections of $1\ \mu\text{m}$ were cut on the ultramicrotome and stained with 1% toluidine blue in borax. Ultrathin sections were stained with lead citrate and uranyl acetate, and viewed on an AEI Corinth 275 transmission electron microscope.

Electrophysiological studies. To assess the propagation of extracellular compound action potentials across the granuloma, the sciatic nerves of anesthetized animals were exposed, mobilized from the sciatic notch to the knee, and insulated by a sheet of dielectric. The animal was earthed, a stimulating electrode applied proximal to the area

of granuloma (Model S8CR stimulator, Grass Medical Instruments, U.S.A.), and a monopolar recording electrode distal to the granuloma. Recordings were displayed on a Tektronix D13 5103N oscilloscope (Oregon, U.S.A.) with a 5A22N differential amplifier AC coupled with both filters (HF-3dB and LF-3dB) at 0.1 kHz.

RESULTS

Saline-injected animals showed normal nerve histology except for occasional diffuse pleomorphic perineurial infiltrate, or Wallerian type degeneration in a single fascicle, suggesting mild mechanical damage during the process of injection. The contralateral sciatic nerves, brachial nerves, and spinal cord were always normal.

Lesions in the unsensitized animals were extensive and showed granulomas with similar morphology and duration to those induced in lymph nodes by Narayanan, *et al.* (13). The cellular response to intraneural BCG injection was maximal at 2 weeks, resolving by 10–12 weeks. No acid-fast bacilli (AFB) were visible beyond 2 weeks. The granuloma was large and well organized, consisting of a necrotic center with polymorphonuclear leukocytes (PMNs), large pale-staining cells of the mononuclear phagocyte system (MPS) with an epithelioid appearance, surrounded by lymphocytes. Electron microscopy showed many of these large cells to have large round nuclei with prominent nucleoli, extensive cytoplasm with dilated rough endoplasmic reticulum (RER), an absence of phagocytosed material, paucity of other cytoplasmic organelles, and with the cell membrane often interdigitating with adjacent cells (Fig. 1). These are the distinguishing ultrastructural features of epithelioid cells (13). A spectrum of cell morphology between this cell type and highly phagocytic macrophages existed. Macrophages contained large amounts of myelin debris, making it impossible to identify phagocytosed organisms (13) (Fig. 2).

In contrast, the cellular response to intraneural *M. leprae* injection reached maximum size at 5 weeks, resolving by approximately 12 weeks. Intracellular AFB were visible until 4 weeks, usually within macrophages. The granuloma was comprised mainly of macrophages, often highly vacuolated, surrounding a necrotic center with



FIG. 1. Electron micrograph of a typical epithelioid cell found intraneurally in a BCG-injected, unsensitized guinea pig. Note the rough endoplasmic reticulum, lack of other cytoplasmic organelles, large round nucleus and distinct nucleolus (uranyl acetate and lead citrate stain $\times 6500$).

PMNs. Lymphocytes were arranged mainly peripheral to the macrophages, but not in such an ordered fashion as in the BCG granulomas.

Lesions in animals pre-sensitized with either BCG or *M. leprae* were not as extensive and did not give such great nerve damage as those in unsensitized animals. The time course was much shorter, with maximal cellular infiltration by 1 week and resolution by 3 weeks. No AFB were visible at 1 week. The granulomas produced were not as well defined as those in the unsensitized animals, and electron microscopy demonstrated a spectrum of MPS cell morphology, but no mature epithelioid cells were seen.

Nerve damage in all experimental animals correlated with the degree of endoneurial infiltration. It was quite common to have a perfectly normal nerve fascicle surrounded by an epineurial cellular infiltrate and which might be adjacent to a grossly infiltrated nerve fascicle.

Demyelination was demonstrated using the antimyelin antibody and appeared to precede axonal degeneration as seen by Holmes' stain. This was confirmed by electron microscopy, where disrupted myelin sheaths with widely spaced lamellae, or completely demyelinated but otherwise

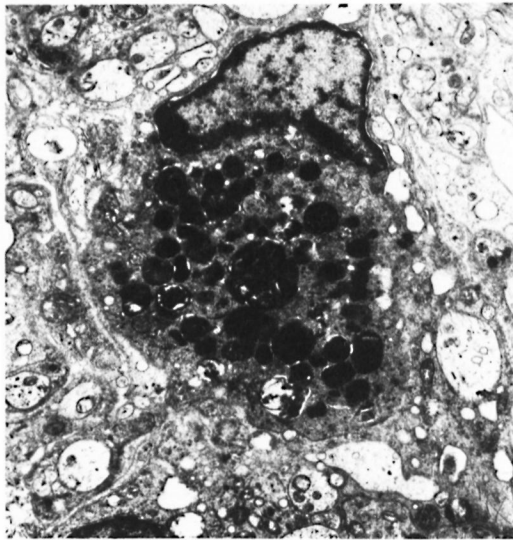


FIG. 2. Typical macrophage found epineurially in experimental animals. The abundant phagocytic vacuoles contain myelin debris. From a sensitized guinea pig 7 days after injection with BCG (stain as in Fig. 1 $\times 15,500$).

normal looking axons, were a constant feature (Fig. 5). Myelin debris was often observed in adjacent cells. Remyelination was apparent as one or two myelin lamellae being relaid around the axon.

Schwann cell proliferation was noted under light microscopy by increased numbers of elongated endoneurial nuclei and confirmed with electron microscopy by the presence of mitotic figures in cells associated with axons and basement membranes (characteristic of Schwann cells). Abnormal Schwann cell-axon associations were common, such as one Schwann cell enveloping both large and small axons or more than one Schwann cell associating with one axon.

Increased amounts of both endoneurial (Type III) and epineurial (Type I) collagen were observed by polarized light microscopy with the picosirius staining and by electron microscopy. These observations correlate well with those of Junqueira, *et al.* (8) on nerves in leprosy patients. Perineurial thickening, with infiltration of leukocytes and fibroblasts between the layers was also observed (Figs. 3 and 4).

Immunohistochemistry. In normal sciatic nerves only blood vessels and perineurial elements appeared to stain positively with MSgp4 (anti-Class I) and MSgp8 (anti-Class

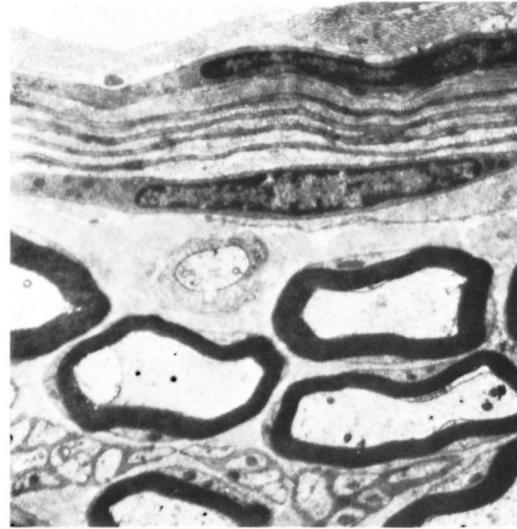


FIG. 3. Section of a contralateral sciatic nerve, showing many features of normal guinea pig nerves: layered perineurium; several large, myelinated axons; groups of small unmyelinated fibers surrounded by Schwann cell cytoplasm; a myelinated axon cut in transverse section at the node of Ranvier; a matrix of endo-, peri- and epineurial collagen fibers (stain as in Fig. 1 $\times 10,000$).

II). However, in the experimental nerves, infiltrated fascicles showed high levels of positivity with these monoclonal antibodies. Serial sections stained with other antibodies revealed that not all the Class I- and Class II-positive cells were lymphocytes or macrophages. These positive cells may be Schwann cells, since they have elongated nuclei and run proximal to and in parallel with the axons, or another endoneurial cell type, such as fibroblasts.

Staining with the antileukocytic monoclonal antibodies revealed that: a) Cells staining positive for the antimacrophage antibody were found in clumps in the center of all types of granulomas. b) CT5 (pan T marker) positive cells accounted for the majority of lymphocytes, were distributed diffusely throughout the granuloma and densely around the edge. c) CT7 (pan T marker) positive cells were fewer than CT5, particularly in the center of the granuloma. d) CT6 (putative T cytotoxic/suppressor-cell marker) positive cells were distributed throughout the granuloma but mainly on the periphery in all models. e) B cells were found as clumps on the periphery or slightly dis-

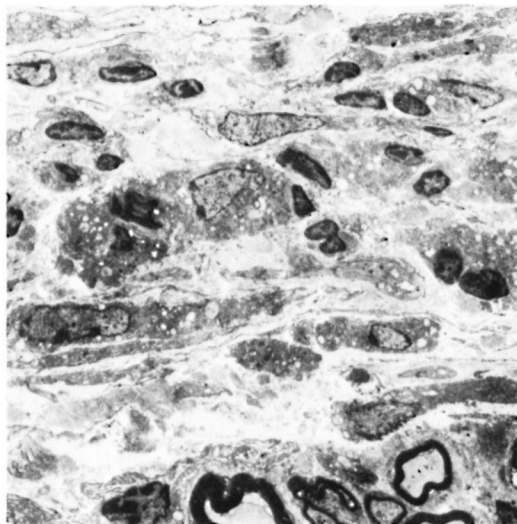


FIG. 4. Thickened perineurium from BCG-injected sciatic nerve of a sensitized guinea pig, showing disruption of the perineurial layers, infiltration by leukocytes, increased fibroblast activity, and presence of collagen (stain as in Fig. 1 $\times 2500$).

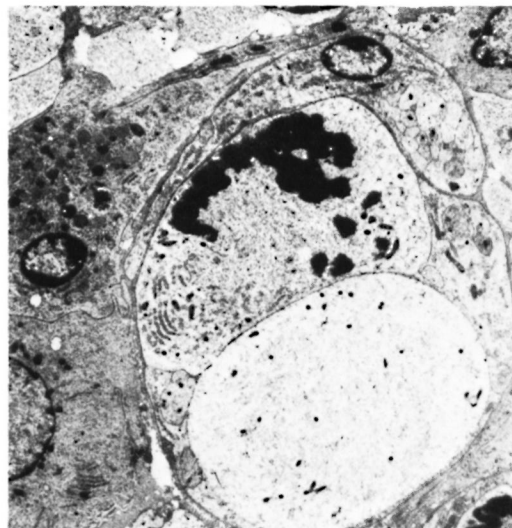


FIG. 5. Schwann cell undergoing cell division (as shown by the mitotic figures) adjacent to a large axon completely stripped of its myelin. From a BCG-injected, unsensitized guinea pig (stain as in Fig. 1 $\times 6500$).

tant to the area of granuloma and only rarely endoneurially, in contrast with T cells.

Electrophysiological studies. In the normal guinea pig sciatic nerve, monophasic recording technique detected up to four peaks within the compound action potential produced. The conduction velocities relating to these peaks could be clearly divided into four corresponding groups: a) >30 m/sec (mean = 64 m/sec S.D. = 16); b) 10–30 m/sec (mean = 16 m/sec S.D. = 2.6); c) 5–10 m/sec (mean = 7.6 m/sec S.D. = 0.81); d) <5 m/sec (mean = 3.7 m/sec S.D. = 1.0). Contralateral sciatic nerves of experimental animals and both sciatic nerves of the saline controls produced traces almost indistinguishable from those of the normal animal. Traces produced by the experimental nerves in all four groups showed a functional deficit, it generally being impossible to elicit a peak relating to the fastest conduction velocity (Fig. 6). However, it was not possible to distinguish any consistent differences between the experimental models. Figure 7 illustrates a typical example of traces from both left and right sciatic nerves of an experimental animal.

DISCUSSION

Nerve lesions in leprosy in man are associated with granulomatous infiltration of

nerves. In tuberculoid leprosy, the granuloma typically contains epithelioid cells and lymphocytic infiltration. In lepromatous leprosy, the infiltrating cell is characteristically the phagocytosing macrophage. In the nerve lesions of advanced lepromatous leprosy, there are highly vacuolated "lepra" cells, dense inflammation, and a thickened perineurium, with increased perineurial fibroblasts and collagen (^{4, 17}). All of these features have been found in the nerves of the unsensitized guinea pigs injected with *M. leprae*. In tuberculoid leprosy (^{4, 5}) there are epithelioid cells, necrosis, and myelin breakdown with axonal sparing. This has also been reproduced in the unsensitized guinea pigs injected with BCG. Previous experimental studies of nerve lesions produced by mycobacterial antigens (^{12, 24}) have been on delayed-type hypersensitivity reactions rather than granulomas resembling those seen typically across the spectrum of human leprosy.

Electrophysiological recordings across the experimental granulomas in the nerves were necessary to illustrate a functional deficit, since clinically (as ascertained by observing posture, running ability, and responses to pin pricks on the foot pad) the guinea pigs appeared normal.

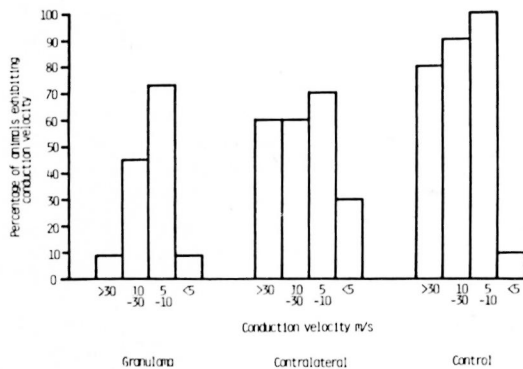


FIG. 6. Pattern of conduction velocities recorded for the different groups of nerves (granulomatous, contralaterals and controls from normal guinea pigs). Results are expressed as the percentage of animals in which a peak was elicited for the stated range of conduction velocities.

Other investigators^(3, 15, 18) have reported endoneurial MHC Class II-positive cells which are not leukocytes in leprosy and other peripheral neuropathies. These cells have been presumed to be Schwann cells. Our model produces a similar pattern of staining at the light-microscope level, however, the exact nature of the positive cells remains to be elucidated at the ultrastructural level. Evidence that Schwann cells can be induced to express Class II antigens *in vitro* with interferon gamma^(19, 23) and present endogenous myelin autoantigens to T lymphocytes *in vitro*⁽²³⁾, poses interesting questions about the possibility of Schwann cells being able to present mycobacterial antigens *in vivo*.

In our model we have not been able to demonstrate the presence of bacilli within Schwann cells and in this respect it differs from the situation in leprosy. However, the granulomas produced in the nerves of guinea pigs resemble those found in the nerves of leprosy patients. Therefore, it is hoped that our model may be useful in identifying the soluble mediators involved in nerve damage caused by mycobacterial granulomas.

SUMMARY

A possible model for nerve damage in leprosy has been developed in the sciatic nerve of the guinea pig. Intraneural injection of 10^7 BCG organisms into an unsensitized animal induces an epithelioid cell

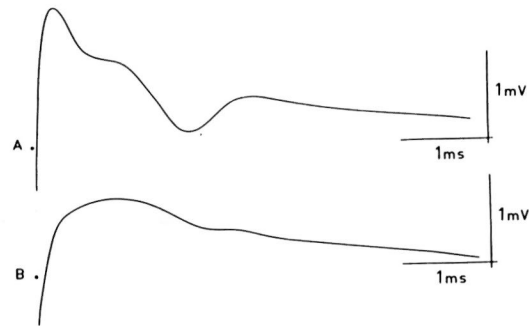


FIG. 7. Oscilloscope traces of guinea pig sciatic nerve extracellular compound action potentials. **A.** Contralateral (normal) nerve of an unsensitized guinea pig 2 weeks after intraneural injection of 10^7 BCG. **B.** Injected nerve.

Stimulus parameters were: single pulse; duration 0.01 msec; 8 volts. Distance recorded = 15 mm. Conduction velocities of peaks: **A.** 75 m/sec (1.7 mV), 15 m/sec (1.0 mV), 6.5 m/sec (0.6 mV); **B.** 15 m/sec (0.8 mV), 7.5 m/sec (0.5 mV).

granuloma in 2 weeks similar to that found in tuberculoid leprosy patients. In contrast, intraneural injection of 10^9 cobalt-irradiated *Mycobacterium leprae* organisms induces a macrophage granuloma in 5 weeks, similar to that found in lepromatous leprosy patients. Histological, immunohistochemical, electron microscopical and electrophysiological studies have demonstrated that the lesions induced in the experimental animals show many of the features documented in studies of nerve damage in leprosy patients.

RESUMEN

Se ha desarrollado un modelo de daño nervioso en lepra, usando el nervio sciático del cobayo. La inyección intraneural de 10^7 organismos BCG en un animal no sensibilizado induce un granuloma de células epiteloides en 2 semanas similar al encontrado en los pacientes con lepra tuberculoides. En contraste, la inyección de 10^9 *Mycobacterium leprae* irradiados con cobalto, induce un granuloma macrofágico en 5 semanas similar al encontrado en los pacientes con lepra lepromatosa. Los estudios histológicos, inmunohistoquímicos, al microscopio electrónico y los electrofisiológicos, han demostrado que las lesiones inducidas en los animales experimentales muestran muchas de las características descritas en los estudios sobre daño en nervios en los pacientes con lepra.

RÉSUMÉ

On a développé un modèle éventuel du dommage nerveux dans la lèpre dans le nerf sciatique du cobaye.

L'injection intraneurale de 10^7 organismes de BCG dans un animal non sensibilisé au préalable entraîne dans les deux semaines un granulome à cellules épithélioïdes semblable à celui qui l'on observe chez les malades atteints de lèpre tuberculoïde. Par contre, l'injection intraneurale de 10^9 organismes de *Mycobacterium leprae* irradiés au cobalt, produit dans les 5 semaines un granulome à macrophages, semblable à celui que l'on peut voir chez les malades atteints de lèpre lépromateuse. Des études histologiques et immunohistochimiques, de même que des investigations au microscope électronique et des explorations électrophysiologiques, ont démontré que les lésions produites chez les animaux d'expérience présentent la plupart des caractéristiques que l'on a pu relever lors d'études du dommage nerveux chez les malades de la lèpre.

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