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Research Article

Light and Ultrastructural Studies of the Sciatic Nerve after Inoculation with *Mycobacterium leprae* in Mice Foot Pad

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Abstract

The mouse foot-pad system represents the first truly useful and reproducible animal model of *Mycobacterium leprae* (*M. leprae*) infection. The simple and sensitive technique for demonstrating the viability of *M. leprae* in mice foot-pad system remains an essential tool for leprosy research. The pathogenesis of nerve destruction varies considerably along the spectrum. The light and ultrastructural study in sciatic nerve in infected mice was carried out of ascertain the changes in Schwann cells (SCs) containing myelinated and nonmyelinated axons. The SCs was observed the sign of degeneration by clumping neural filaments and neural tubes. However, axonal multiplication was clearly observed.

Keywords: Ultrastructure; Mice Footpad; Sciatic Nerve; *M. leprae*; Schwann Cell

Introduction

M. leprae the causative agent of leprosy, has a selective affinity for peripheral sensory nerves [1-3] specifically for SCs. *M. leprae* multiplies within SCs and can survive in them for long periods as 'persistor' or 'resistor' organisms [4,5]. Apart from macrophages and SCs are therefore considered the main natural host cells of *M. leprae*. Despite the historical importance of the mouse foot-pad technique in acquiring knowledge of *M. leprae* and leprosy, and it's continuing importance for the foreseeable future. Shepard's findings were subsequently confirmed by Rees and Pattyn among others, and the mouse foot-pad system became an important research tool in leprosy. Before Shepard's work, many workers had inoculated *M. leprae* into mice and other small mammals by a variety of routes. However, these workers sought evidence of a leprosy-like disease, whereas Shepard simply enumerated the AFB in the inocula and, after multiplication, in the inoculated tissues. When leprosy is not recognised and treated at early stage peripheral nerve damage ensues, leading to muscle weakness, paralysis and severe deformities [6]. In common practice, the clinical diagnosis of neuritis is made when there is pain or tenderness, or swelling of a nerve, a sensation of pinprick and tingling localized to that part of the skin supplied by the nerve. The clinical diagnosis of leprosy depends mostly on the degree of the nerve damage in patients. The histopathological demonstration of *M. leprae* in the nerve depends on the presence of an inflammatory granuloma in and around a nerve mandatory to confirm the diagnosis of leprosy. The inoculated mice are divided among groups of 10–20 mice each; Given the inherent imprecision of the technique, papers reporting the results of experiments in which the mouse foot-pad technique has been employed must present all of the data, so that they may be carefully evaluated in terms of the sources of error already described. However, very little is known about the mechanism of entry of *M. leprae* into SCs cells. In this study we have examined adherence of *M. leprae* to the SCs cell surface and we present data about the nature of the adherence.

The mechanism of interaction between *M. leprae* and neural cells has not been elucidated so far. No satisfactory interpretation exists as to the bacterium tropism to the nervous system in particular. The present study will be useful for understanding the structural characteristics of SCs cells, axons and other infiltrated cells including the interaction of *M. leprae* organism. This study has shed new light on some aspects of the infection of *M. leprae* and the phenomenon of nerve involvement has opened new avenue of research, possible mechanism of nerve damage after inoculation of *M. leprae* in mice food pad.

Materials and Methods

Nerve biopsies of sciatic nerves were obtained from 3 swiss albino mice infected with 10^7 *M. leprae* 1, 2, and 3 months after infection. One other part of the infected nerve biopsies was processed by fixing in 2.5% glutaraldehyde for electron microscopy. Thin layer covering or sputtering coating of a conducting material, typically a metal, such as a gold/palladium (Au/Pd) alloy was done. Conductive coating is needed to prevent charging of a specimen with an electron beam in conventional SEM mode (high vacuum, high voltage). The sciatic nerve was observed under SEM S3000N, Hitachi.

Subsequently, the another part of nerve tissue were dehydrated in grades of alcohol and embedded in Spurr's resin and blocks were made. Initially the semi-thin sections were cut and stained with toludine blue for the pathological studies and to make the orientation of the tissue for cutting ultrathin sections. The sections were put on to cupper grid and stained with uranyl actate and lead citrate. The stained ultrathin sections were examined in a Hitachi-7650 transmission electron microscope.

Results

The present experimental model was developed with a purpose of depicting the typical nerve lesions similar as seen in human leprosy and to study the early sequence of events surrounding the entry of *M. leprae* into the peripheral nerve. This study was undertaken to see if *M. leprae* inoculated into the mouse foot pad have the capacity to produce damage in its sciatic nerve, during the 1st month 2nd months and 3rd months. The mouse sciatic nerve used as a control was taken at the same time as those of the experimental group of inoculated mice and showed normal features. The semi thin sections consisted of one large, one small, and one very tiny funicle with a compact group of fibers. At the electron microscopic level, the normal nerve revealed an unmyelinated fiber group as compact multiple axonal units surrounded by a single Schwann cell (SCs) with a prominent nucleus. Myelinated fibers were well preserved with intact neuro tubules and neuro filaments (Figure 1).

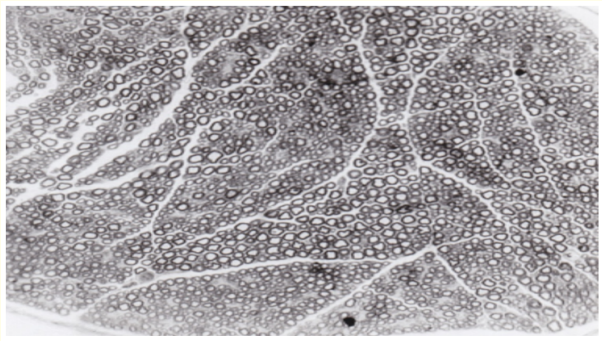

Figure 1: Semi-thin sections of Sciatic nerve of normal mice (Control) showing well preserved myelinated and unmyelinated nerve fibres with some endoneurial blood vessels X500.

Freshly harvested *M. leprae* injected into the mice foot pad and nerve biopsies were obtained 1st month 2nd months and 3rd months after inoculation in three mice (Figure 2) . Sciatic nerves were expose surgically and changes, such as adhesion, swelling and thickening if any at the site and along the length of the nerve, were recorded.

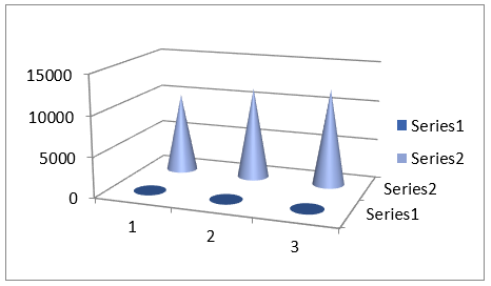

Figure 2: Showing the load of *M.leprae* after inoculation in mice foot pad during 1st , 2nd and 3rd months.

The mice were anesthetized using 4% isoflurane and two cm length of the nerve was biopsied subsequently, fixed in glutaraldehyde for processing light and electron microscopy.

Histopathological and Ultrastructural findings

At the light microscopic level semi-thin sections were cut and stained with toludine blue. There were no appreciable changes produced nor any bacilli were observed in any of the sciatic nerves. In the 1st months, light microscopic examination showed a normal complement of cells within the perineurial envelope. In the 2nd and 3rd month, at which time there was minimal SCs proliferation and minor degenerative changes in the nerves were evident. In *M. leprae* infected mice, the semi thin sections stained with toludine blue showed swollen of myelinated, undemyelinating fibers and proliferation of the endoneurial blood vessels (Figure 3,4). Observations at the 3rd post inoculation month indicated discrete areas and loss of few nerve fibers.

At the 3rd post inoculation month, both unmyelinated and myelinated fiber groups were involved. The changes observed were hypertrophy of the SCs of myelinated and unmyelinated fiber and their axons. Unmyelinated fibers showed axonal swelling, disintegration of neurotubules and neurofilaments, and extremely small axonal sprouts surrounded by folded basement membrane tubes, indicating minor degeneration of an unmyelinated fiber unit. The involvement of the unmyelinated fiber at the 3rd post inoculation month persists and progress with time (Figure 5).

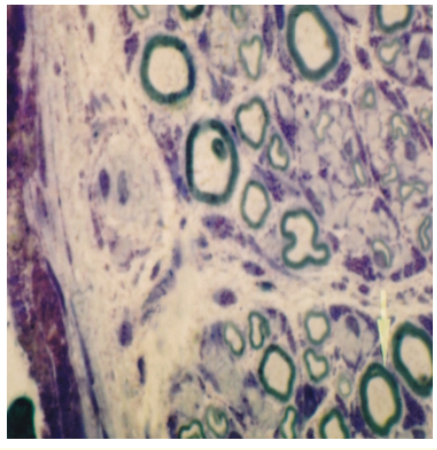

Figure 3: Semi-thin sections of Sciatic nerve of infected mice foot pad after 1st month inoculation with *M. leprae* showing swollen of myelinated and unmyelinated nerve fibres. Some endoneurial blood vessels is clearly visible. X800.

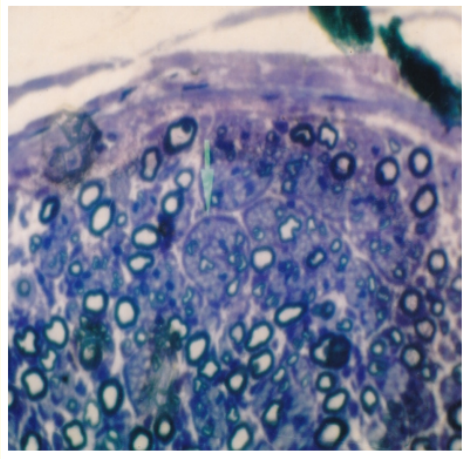

Figure 4: Semi-thin section of Sciatic nerve of mice foot pad after 3rd months inoculation with *M. leprae* showing disrupted of myelinated and unmyelinated fibres. Many small myelinate fibres and inflammatory cells are clearly visible. X 500.

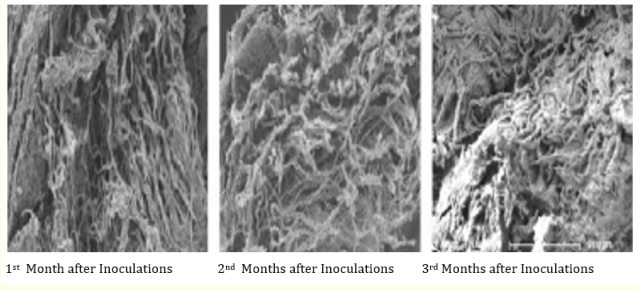

Figure 5: Scanning electron micrographs of the sciatic nerves after inoculation of *M. leprae* in mice foot pad after 1st Month, 2nd Months and 3rd Months showing disrupted elongated fascicles. X 5000.

The histopathological features of early nerve damage parallel the observation in patients and are similar in experimentally infected SW mice. At the 1st months to 3rd month intervals. Macrophages with extensive foamy cytoplasmic changes were seen as aggregates in the endoneurium and epineurium of macrophages (Figure 4). Cellular activity showed a peak between the 2nd and 3rd months and noticed vacuolated cytoplasm, thus indicating regression similar to the leprosy patients. In addition, SW mice SCs cells secreted increased levels of collagen *in vitro* which correlated with observations in sciatic nerves from experimentally infected SW mice and nerves of leprosy patients.

However, the presence of nerve abnormalities in the inflammatory cells reiterates the concept of a immunological mode of nerve damage in the initial stages, possibly due to aberrations in SCs functions. The clumping of the neurofilament and neural tubes was quite clear which characterized degeneration of SCs (Figure 6).

On the other hand, the absence of bacilli in SCs may merely reflect differences in the affinity or the nature of neural barriers in the human and mouse models.

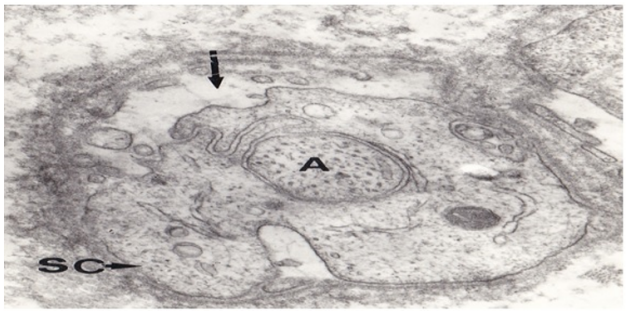

Figure 6: Transmission electron micrograph of SCs of Sciatic nerve of mice foot pad after inoculated with *M. leprae* showing degenerated of ↘ SCs by neural filaments and neural tubes. The connection of axon (A) to the basement membrane is clearly visible. X 20,000.

Discussion

The involvement of the peripheral nerve is the most constant feature of leprosy and seems to be very specific to infection by *M. leprae*. It is interesting to note that nerve damage occurs in all forms of leprosy, irrespective of the immunological status of the patient. Such peripheral nerve damage underlies sensory motor loss and accounts for most of the deformities and disabilities in leprosy. Therefore, it was decided to use the mouse sciatic nerve model to understand that *M. leprae* could produce the typical damage seen at the light and ultrastructural level in this model. A standard inoculum of 10^4 to 10^6 organisms was inoculated into the mouse foot pad as used for *M. leprae* and pathological changes were observed.

The Growth curves in the foot pad were fairly similar to those recorded for these mycobacteria by other observers no clinical or histological nerve damage has been reported by any of these authors, and no ultrastructural studies have been undertaken by them [7,8]. The only exception has been who described infiltration of MLM and macrophages containing acid-fast bacilli in the auricular and sciatic nerves of mice observed from the seventh month onward after inoculation [9]. Our study did not reveal minor infiltration in the sciatic nerve on light or electron microscopy.

In the present experimental model the sciatic nerve of freshly harvested mice was observed and found that at 1st inoculation the nerve is intact and well preserved. In semithin sections the few blood vessels, along with foamy cytoplasm was prominent. The myelinated and unmyelinated is clearly visible as observed in peripheral nerve in leprosy patients [10-12]. Further these workers suggested that SCs of unmyelinated fibers were frequently harboring the *M. leprae* organisms. These features in the initial phase of nerve damage have also been studied in the mouse model. The major limitation in the mouse model has been the absence of inflammatory cells and granulomatous reactions mimicking the spectral disease of the human, as well as the inability to demonstrate bacilli in SCs of the affected nerves. However, the presence of nerve abnormalities in the absence of inflammatory cells reiterates the concept of a non-immunological mode of nerve damage in the initial stages, possibly due to aberrations in SCs functions. On the other hand, the absence of bacilli in SCs may merely reflect differences in the affinity or the nature of neural barriers in the human and mouse models. The Swiss White (SW) mouse is a strain in which the response of host cells to *M. leprae* infection parallels those observed in lepromatous patients as opposed to the mice strain in which the response to *M. leprae* is similar to that observed in tuberculoid patients or normal individuals. The histopathological features of early nerve damage parallel the observation in patients and are similar in experimentally infected SW mice [13].

Possible mechanisms of entry of *M. leprae* in to nerve

M. leprae can enter the nerve by four different pathways. It was suggested that *M. leprae* enter the body through naked nerve filaments in the epidermis and spread centripetally along the axon [14]. The upward movement of the bacilli along the axonal flow was compared to fish swimming against the stream. Intra-axonal bacilli have been shown by several workers in electron-microscopic studies, but it is a rare occurrence.

The second suggestion was that *M. leprae*, on entering the skin, are phagocytosed by SCs in the upper dermis. Thus protected from the cells of the immune system, they multiply inside the SCs and travel along the nerve from one SCs to another by contiguity. Many workers consider that *M. leprae* has a special predilection for SCs and it remains as an important host cell of *M. leprae* [15].

The third possibility is that macrophages in the upper dermis initially take up the bacilli and these bacilli-laden cells aggregate around skin adnoxal structures, including nerve bundles. Bacilli released from these macrophages are ingested by perineurial cells, which pass them on to SCs or the macrophages containing the bacilli infiltrate the perineurium and invade the nerve.

Mechanism of nerve distraction

In paucibacillary patients leprosy has enough resistance and considerable delayed-type hypersensitivity to the antigen of *M. leprae*, and this hypersensitivity produces intense granulomatous reaction and sometimes necrosis at sites where antigen continue to appear. The peripheral nerves in human were present in the localized tuberculoid showed inflammation which destroyed large portion of the nerve. The inflammation is composed of epitheloid cells, giant cells and lymphocytes resulting the affected portion of the nerves shows localized enlargement. In BT diseases varying degrees of hypersensitivity to *M. leprae* and its antigen and varying degrees of ability to limit the diseases was evident. There is granulomatous inflammation composed of macrophages and lymphocytes in and around the nerves. The granulomatous infection characteristics of hypersensitivity are present in all the involved nerves. There is extensive distraction of the peripheral nerves by the granuloma, the nerves are finally replaced by fibres tissue. Large portion of the nerves shows marked of thickening and deformities due to nerve damage. The distribution of the lesions in the nerve trunks has been to be in areas where the temperature is lower and preferentially growth of the bacilli at these sites having been demonstrated [14,15]. A slight edema to the nerve due to minimal inflammation will produce enough thickening of its size to causes trauma during its passage in and out of the narrow tunnel. The distraction of the peripheral nerves might be possible because the perineurium and epineurium are tight structure composed of large amount of collagen and fibrous tissue.

The vasculopathy and nerve-affection were common in all types of leprosy. Thickening of vessel wall, was multi-layering and proliferation of basement membrane, irregularly enlarged EC, accumulation of infiltrating cells, were common findings [16-19]. Whatever may be source; this basement membrane material appears to be antigenic in nature and is capable of inviting an antibody-response. Such antibody response could have actually occurred was evidenced by the cells which had infiltrated in and around the vessels. These cells were lymphocytes, plasma cells, large mono-nuclear cells or macrophages, all of which are known to be immunologically competent in one form or other.

In addition, this is evident that endoneurial blood vessels (EBV) supply the nutrients to the nerve through blood for maintaining the metabolic activity of the SCs and proper nerve fibers functions. In paucibacillary cases our ultrastructural study clearly indicated that due to the hypertrophy of endothelial cell the lumen of the blood vessels become narrower and finally get closed resulting to hampering the blood supply causing the ischemia in the patients. In addition, to stop the supply of nutrients to the nerves and nerve become severely damaged. On the other hand, in multibacillary patients the lumen of the EBV is wide open, nucleus is small and blood circulation is normal, but ultrastructurally many bacilli have been observed in endothelial cell. These bacilli got multiply and pressure is increased inside the endothelial cell resulting to rupturing the endothelial wall and organisms comes into lumen and blood stream of the EBV.

Therefore, our study using light and electron microscope techniques has convincingly confirmed that beside the SCs, the EC of blood vessels also harbour *M. leprae* frequently. In paucibacillary leprosy the patients has enough resistance and considerable delayed type hypersensitivity to the antigen of *M. leprae*, and this hypersensitivity produces intense granulomatous reaction and some time necrosis at site where antigen continue to appear. The granulomatous inflammation characteristics of hypersensitivity is present in all of the involve nerve. There is extensive destruction of peripheral nerves by the granuloma, the nerve are finally replaced by fiberous tissue large portion of the nerves shows marked thickening and deformities due to the nerve damage. Thus, the typical sequential changes seen in the sciatic nerve of the mouse model may be used as one of the criteria for characterizing cultivable mycobacteria which are responsible for nerve damage.

Conflict of Interest

There is no any conflict of interest, between the authors and any institution or individuals in regard to the content of this paper.

Bibliography

1. Iyer CGS. "Predilection of M. leprae for nerves. Neurohistopathologic observations". *International Journal of Leprosy* 33 (1965): 634-645.

2. Khanolkar-Young., *et al*. "Nerve and skin damage in leprosy is associated with increased intralesional heat shock proteins". *Clinical and Experimental Immunology* 96 (1994): 208-213.

3. Dastur DK., *et al*. "Ultrastructure of nerves in tuberculoid leprosy". *Neurology* 20 (1972): 9-99.

4. Kahn P and Scott T. "The pathology of a radial nerve biopsy in leprosy: light and electron microscopy". *Journal of Pathology* 114.2 (1974): 97-100.

5. Jon CK. "Mycobacterium leprae in nerve lesions in lepromatous leprosy. An electron microscopic study". *Archives of Pathology* 89 (1970): 195-207.

6. Kumar V. "Emerging Concept on Peripheral Nerve damage in Leprosy". *International Journal of Research in Health Sciences* 2.1 (2017): 8-18.

7. Collins FM., *et al*. "Immune response to persistent myco-bacterial infection in mice". *Infection and Immunity* 20 (1965): 430-438.

8. H1lson, GRF. "Observations on the inoculation of M. leprae in the footpad of the white rat". *International Journal of Leprosy* 33 (1965): 662-666.

9. Wiersema JP., *et al*. "Nerve involvement. Comparison of experi-mental infections by Mycobacterium leprae and Mycobacterium lepraemurium". *International Journal of Leprosy* 33 (1965):617-633.

10. Kumar V., *et al*. "Ultrastructural Studies of peripheral nerves in lepromatous leprosy patients". *Indian Journal of Leprosy* 60 (1998): 360-363.

11. Kumar V., *et al*. "Ultra structural characteristics of Macrophages in dermal leprosy granulomas". *Japanese Journal of Leprosy* 58.4 (1989): 1985-190.

12. Kumar V., *et al*. "Isolation and characterization of infiltrates in the nerves of Neuritic leprosy". *Acta Leprologica* 7.92 (1990): 157-161.

13. Shetty VP., *et al*. "Immunohistological localization of mycobacterial antigens within the peripheral nervos of treated leprosy patients and thcir significance to nerve damage in leprosy". *Acta Neuropathology* 88 (1965): 300-306.

14. Job CK. "Pathology of peripheral nerve lesions in lepromatous leprosy - a light and electron microscopic study". *International Journal of Leprosy and Other Mycobacterial Diseases* 39 (1971): 251+268.

15. Job CK. "Nerve damage in leprosy". *International Journal of Leprosy and Other Mycobacterial Diseases* 57 (1989): 532-539.

16. Scollard DM. "Endothelial cells and the pathogenesis of lepromatous neuritis: insights from the armadillo model". *Microbes Infection* 2 (2000): 1835-1843.

17. Kumar V and Sen Gupta U. "Ultra structural study of Schwann Cells and Endothelial cells in pathogenesis of leprous neuropathy". *International Journal of Leprosy* 7 (2003): 328-340.

18. Kumar V. "Does clofazamine (B663) reach Mycobacterium leprae persisting in SCs and endothelial cells of endoneurial blood vessels in peripheral nerves?". *Microscope Research and techniques* 71 (2008): 614-618.

19. Kumar V., *et al*. "An ultra structural study of Schwann cells in peripheral nerves of leprosy patients". *Indian Journal of Leprosy* 64 (1992) : 81-87.

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