Hypophosphorylation of NF-H and NF-M subunits of neurofilaments and the associated decrease in KSPXK kinase activity in the sciatic nerves of Swiss white mice inoculated in the foot pad with Mycobacterium leprae

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Summary
Objective To study the phosphorylation state of neurofilament (NF) proteins and activity of KSPXK kinase in the sciatic nerves of Swiss white (S/W) mice inoculated in the hind foot pads with M. leprae.

Design Test group includes S/W mice inoculated in the foot pads with freshly harvested human derived (viable) M. leprae. Control groups were constituted by (1) Age matched un-inoculated mice, (2) Mice similarly inoculated with M. smegmatis and (3) heat killed M. leprae. Phosphorylation state of NF was studied using Western blot analysis and phosphor-specific NF antibody (SMI 31; Sternberger Monoclonals, Inc.). The KSPXK kinase activity was assayed by using KSPXK fusion protein in a radiometric method using γ32P ATP.

Results Several fold increase in M. leprae numbers was seen in viable M. leprae group while M. smegmatis failed to show any fold increase in the foot pads of S/W mice. Western immunoblot analysis of cytoskeletal preparation from sciatic nerves of un inoculated mice and mice inoculated with M. smegmatis showed immunoreactivity to SMI 31 antibody and protein bands corresponding to both NF-H and NF-M at all the time points from 4–20 months post inoculation. In case of viable M. leprae; SMI 31 reactive protein bands were seen at 4 months but not at any of the later intervals i.e. between 6–20 months. With heat killed M. leprae transient loss of immunoreactivity to SMI 31 was seen. Decrease in KSPXK kinase activity was recorded in sets inoculated with viable and heat killed M. leprae, and corroborated with loss of immunoreactivity seen in WBs reacted with SMI 31 antibody.

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Conclusions
Alterations in the sciatic nerve NF cytoskeleton was seen following inoculation in the hind foot pad with both viable and heat killed *M. leprae*. The hypophosphorylation of NF observed in this study corroborates with the earlier observations in human leprous nerves.

Introduction

Mouse is a well established experimental model for leprosy and has been used as an adjunct to understand the pathomechanism(s) of nerve damage in leprous nerves. Earlier studies have shown that inoculation of viable *M. leprae* in the hind foot pad (FP) in immuno-competent mice result in limited/localised multiplication of the bacilli and a slow progressive neuropathy in the sciatic nerve. Morphological changes recorded in the nerves of *M. leprae* inoculated mice were specific and find several parallels with the insidiously occurring nerve damage seen in human leprous nerves. However the underlying mechanisms remain unclear.

It is now well established that the axonal calibre, hence the nerve conduction velocity, is governed by the phosphorylation status of lysine-serine-proline (KSP) repeats of COOH termini of high [NF-H; 200 kilo dalton (kDa)] and medium (NF-M; 150 kDa) molecular weight subunits of neurofilament (NF) protein. Whilst phosphorylation of KSP repeats, in particular both KSPXXK epitopes of NF-H and NF-M subunits, has an important role in the maintenance of axonal calibre and hence the nerve conduction velocity, low molecular weight NF subunit (NF-L; 68 kDa) forms the core of NF. Protein kinases, in particular the cyclin dependent kinase 2 (cdc2) related kinases and mitogen activated protein kinase (MAP kinase) have an important role in phosphorylation of KSP repeats in the carboxyl termini of NF-H and NF-M. The finding that the characteristic sensory/motor neuropathy seen in leprosy is associated with decrease in axon diameter makes it an interesting proposition to study the status of neurofilament phosphorylation in an experimental model as well. Earlier immunohistotochemical studies on human leprous nerves at our centre has demonstrated loss or decrease of immuno reactivity against phosphorylated epitopes of NF. Hence, it is of interest to look into the status of NF phosphorylation and possibility of the alteration in KSPXXK kinase activity in nerves from experimental mouse model of leprosy as well. Hence the state of NF phosphorylation and the phosphokinase activity was assessed in the sciatic nerves of mice following inoculation with *M. leprae*. PK activity assessed using specific peptide substrate (KSPXXK) was further correlated to NF phosphorylation in these nerves. The results thus obtained are presented and discussed in this paper in the context of its relevance to nerve damage in leprosy.

Materials and Methods

**ANIMALS**

Adult S/W mice aged 2–3 months were used for the study. They were fed with standard diet and were kept in a 12-hour light/dark cycle in an air-conditioned room and all care was taken to minimise their suffering. A total of 192 mice were divided into (a) test group of 48 mice that received injections of freshly harvested *M. leprae* suspension derived from a human leproma in both the hind foot pads; duplicate sets were studied, (b) three control groups (48 mice each) included, i.e. (I) uninoculated, (II) inoculation with viable
M. smegmatis and (III) inoculation with heat killed M. leprae. Two dilutions were studied viz. 1 × 10^4 M. leprae and 1 × 10^5 M. leprae. Volume of the injection per FP in all the groups was maintained at 30 μl.

**Antibodies**

SMI 31 antibody; directed against phosphorylated NF-H and NF-M (Sternberger and Sternberger, USA) and antibodies against NF triplet proteins (Boehringer Mannheim) were employed for western blot (WB) analysis.

**Preparation of bacterial suspension for foot pad inoculation**

M. leprae suspension from a freshly obtained nodule biopsy (from an untreated lepromatous leprosy patient) was prepared using the standard protocol. M. smegmatis grown in Lowenstein Jensen/Dubos broth was harvested and washed in saline. An aliquot of M. leprae was heat killed by autoclaving (121°C for 20 min. at 15 lb pressure). The M. leprae and M. smegmatis bacillary suspensions in saline were adjusted to a density of 10,000 per 30 μl/FP.

**Inoculation of M. leprae and M. smegmatis**

M. leprae and M. smegmatis bacillary suspensions thus prepared were injected subcutaneously in both hind foot pads (30 μl/FP) of mice as described by Shepard.

**Sciatic nerve biopsy**

Mice were anesthetised using pentabarbitone (0.3–0.4 mg/kg body wt.) and the sciatic nerve biopsies were obtained at 4, 6, 12, 16 and 20 months post inoculation, from a minimum of 5 mice (10 nerves) per interval. Whole length of the nerve from both the hind limbs were exposed, dissected out, pooled and stored at −70°C till further use.

**Foot pad harvests**

Following sciatic nerve biopsy, the M. leprae and M. smegmatis inoculated mice were killed by cervical dislocation. The FP were cleaned thoroughly and harvested using a mechanical homogeniser. The acid fast bacilli (AFB) were stained with Ziehl Neelson’s Carbol Fuschsin (ZNCF) and were quantified to assess the fold-increase in bacillary number at different time points using a standard protocol.

**Neurofilament rich cytoskeletal preparation**

Neurofilament (NF) rich fraction of the cytoskeleton was prepared by modified method of Tokutake and co-workers as described by Guru and co-workers. In brief, sciatic nerves (stored at −70°C) were cleaned off any extraneous tissue. Ten nerves were pooled and were homogenised (1:20 w/v) in an extraction buffer [25 mM phosphate buffer, pH 6.8, containing NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), sodium orthovanadate (1 mM), 0.5% (v/v) Triton X-100 and protease inhibitors [phenyl methyl sulphonyl fluoride (1 mM) and benzamidine (35 ng/ml)]. The homogenate was centrifuged at 20,000 g for 30 min at 10°C. The supernatant obtained was stored for assay of KSPXK kinase activity. The pellet was...
re-homogenised in the extraction buffer containing 1 M sucrose, followed by centrifugation at 20,000 g for 30 min at 10 °C and the supernatant with floating myelin rich fraction was discarded. The latter step was repeated till myelin floating was totally absent. The NF rich pellet was re-suspended in the extraction buffer without Triton X-100 and centrifuged as above and the detergent free pellet was collected.

SOLUBILISATION OF CYTOSKELETAL PREPARATION AND PROTEIN ESTIMATION

The Triton X-100 insoluble cytoskeletal preparation was homogenised in 6 M urea in 50 mM Tris-HCl buffer, pH 7.4 and centrifuged at 15,000 g for 20 min at 25 °C. Supernatant was used for protein estimation and sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS PAGE) analysis. Protein content of both urea solubilised cytoskeletal preparation and the Triton X-100 soluble supernatant was determined by Lowry’s method.32

SDS-PAGE AND WESTERN IMMUNOBLOT

The NF triplet proteins from urea solubilised fraction of cytoskeletal preparation were adjusted to 1 μg protein/μl. These were further subjected to SDS–PAGE and resolved using 7.5% acrylamide gel. A 10 μl sample was loaded per well from both the test and the control groups. Gels were stained with Coomassie Brilliant Blue (CBB). Separate gels were electro blotted on to PVDF membranes. The membranes were blocked with 0.25% (volume/volume) Tween 20 in Tris buffered saline (TBS, 0.25 mM) for 15 minutes. This was followed by incubation with primary antibody (SMI 31 (1:5000 dilution) or antibodies against individual NF triplet proteins [NF-H, NF-M and NF-L (1:1000 dilution) in TBS containing 0.25% bovine serum albumin (BSA)] at ambient temperature for 60 minutes. Alkaline phosphatase labelled rabbit anti mouse antibody (Krikegard and Perry, 1:5000 dilution in TBS containing 0.25% BSA) was used as secondary antibody. Immuno blots were developed using 5-Bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium (BCIP/NBT) as chromogen substrates.

Western blots were scanned using Bio Rad gel documentation system and molecular weights were determined using Quantity 1 software from Bio Rad.

KSPXK KINASE ACTIVITY

Kinase activity from Triton X-100 soluble extracts of sciatic nerves from control and M. leprae infected mice were assayed by using KSPXK fusion proteins by radiometric method described earlier except that KSPXK fusion protein repeats expressed in E. coli was used as acceptor substrate instead of synthetic peptide.32P labelled adenosine triphosphate (ATP) (Board of Radiation and Isotope Technology, Department of Atomic Energy, Govt. of India) was used as a donor substrate.

Assay procedure

KSPXK kinase activity was measured in a final assay volume of 50 μl containing Tris/HCl buffer (0.1 M), pH 7.4 [containing MgCl2 (5 mM), dithiothreitol (1 mM), vanadate (1 mM)], KSPXK fusion protein repeats (1 mM), 32p ATP (0.1 mM) and sciatic nerve extract protein (range 0.1–1.16 mg/ml) as enzyme source. Enzyme reaction was initiated by adding radio-labelled ATP (donor substrate) to a pre-incubated enzyme/buffer, substrate (acceptor)
cocktail. After incubation for 30 minutes at 30°C, an aliquot (40 μL) of the assay volume was placed on p18 phosphocellulose paper and allowed to dry, followed by passing through wash cycles (three of 15 minutes each) in 75 mM phosphoric acid and final washing in ethanol. Dried filter papers containing radio-labelled peptides were measured for radioactivity by liquid scintillation counting.

Each assay was carried out in quadruplicate, two each for the control (in the absence of acceptor substrate) and the test (in presence of KSPXK fusion protein).

KSPXK kinase activity was expressed as picomoles of $^{32}$P transferred from γ-labelled ATP by milligram protein per minute (pm/mg/min). This was further equated to nM ATP transferred per gram of nerve tissue (nM/gm nerve).

**STATISTICAL ANALYSIS**

Graph Pad Prism 4 software was used for statistical analysis. As a first part mean and standard deviation was calculated for the samples at different time points. Difference within and between the groups was further assessed by two way analysis of variance (ANOVA).

**ETHICAL CONSIDERATION**

The Institutional Ethics Committee approved the animal experiments within the framework laid down by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India (Registration number 424/01/a/CPCSEA).

**Results**

**MULTIPLICATION OF M. LEPRAE IN THE FOOT PADS**

Viable *M. leprae* showed a steady increase (100-fold) between 6–16 months post inoculation. The highest average acid fast bacillary (AFB) yield was $172 \times 10^4 \pm 99-49/FP$ observed
at 16 months followed by decline at 20 months. On the other hand *M. smegmatis* showed presence of AFBs up to two months (7.01 × 10^3 ± 3.96/FP). Absence of AFBs beyond 6 months post inoculation indicated that the mouse FP did not sustain the growth of *M. smegmatis*. Heat killed *M. leprae* inoculated mice also showed the presence of granular AFBs up to 6 months but there was no fold increase (Figure 1).

Statistical significance was determined using two way ANOVA. Significant difference was seen between viable and heat killed *M. leprae* and *M. smegmatis* inoculated groups (*P* < 0.0001).

**SDS-PAGE AND WESTERN IMMUNOBLOT ANALYSIS**

*Age matched uninoculated mice*

SDS poly acrylamide gel stained with CBB showing Triton X-100 insoluble cytoskeletal preparation from uninoculated mice revealed the presence of protein bands corresponding to all three subunits of NF proteins namely NF-H, NF-M and NF-L viz. 200 kDa, 150 kDa and 68 kDa respectively (Figure 2a).

Further Western immunoblot analysis, employing specific antibodies, viz. SMI 31 antibody detected phosphorylated NF-H and NF-M (Figure 2b).

![Figure 2](image-url)

**Figure 2.** (a) Typical SDS PAGE profile of cytoskeletal preparation from sciatic nerves of normal mice, (b) WB with SMI 31 and (c) Using antibodies to individual NF protein. Key: Molecular weight marker (M; lane 1), NF preparation from normal rat spinal cord (C; lane 2) and NF preparations from normal S/W mouse sciatic nerves collected at different time points from 2–20 months (lanes 3–8) are depicted.
A cocktail of antibodies from Boehringer Mannheim directed towards phospho-independent NF-H, NF-M and NF-L also identified the protein bands corresponding to NF-H, NF-M and NF-L (Figure 2c).

**Inoculation with M. smegmatis**

The Triton X-100 insoluble preparations were studied between 4–20 months. Protein bands corresponding to NF-H and NF-M were detected with SMI 31 antibody at all the study intervals and are depicted in Figure 3.

**Inoculation with heat killed M. leprae**

Inoculum of $1 \times 10^4$ HKML

The Triton X-100 insoluble preparations were studied between 2–20 months. When reacted with SMI 31 antibody; protein bands corresponding to NF-H and NF-M were not seen at 2 months interval. At 4 months positive reaction was seen at protein band corresponding to NF-H, but very faint NF-M band was seen. Total absence of SMI 31 immunoreactivity was seen at 6 months post inoculation. Whereas faint protein bands were seen at molecular weight corresponding to NF-H and NF-M at the later intervals i.e. at 12–20 months post inoculation (See Figure 4).

Inoculum of $1 \times 10^5$ HKML

The Triton X-100 insoluble preparations were studied between 2–20 months intervals. The SMI 31 antibody reactive NF-H and NF-M bands were observed at 2 and 4 months, but were absent at 6 and 12 months. Protein bands corresponding to NF-H and NF-M were observed at 16 months (of lower intensity) and 20 months post inoculation.

**Inoculation with viable M. leprae**

The Triton X-100 insoluble pellet preparation of sciatic nerves from mice inoculated with viable *M. leprae* ($1 \times 10^6$; two sets were studied) were reacted with SMI 31 antibody. The presence of NF-H and NF-M proteins was revealed at 4 months interval (Figures 5a and 5b); but at the subsequent intervals of 6, 12, 16 and 20 months SMI 31 reactive NF-H and NF-M bands were not seen (Figure 5a). At 12 months post inoculation very faint NF-M band was observed in one set (Figure 5b).

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![Figure 3](image-url) Western blot with SMI 31 antibody. Sciatic nerve NF preparations from mice inoculated with *M. smegmatis* in the foot pad is seen along with other controls. Key: Mol. Wt. marker (M; lane 1); NF preparation from normal rat spinal cord (C; lane 2), normal S/W mice age 4 months (N4; lane 3) and mice inoculated with *M. smegmatis* (4–20 months; lanes 4–8).
NF preparation from one set (ML-1) was also reacted with antibodies to individual NF proteins from Boehringer Manheim as described earlier. It is interesting to note that NF-H, NF-M and NF-L bands were seen at 4 months. But from 6–20 months post inoculation only faint NF-L band was detected. NF-H and NF-M bands were not seen (results not shown).

WBs reacted with specific antibodies were further scanned using Bio Rad gel documentation system. Molecular weight determination was done using ‘Quantity 1 software’ from Bio Rad.

SALIENT OBSERVATIONS AND INFERENCES

Uninoculated age matched control mice and mice inoculated with *M. smegmatis* demonstrated protein bands corresponding to NF-H and NF-M from 4–20 months post inoculation. While inoculation with heat killed *M. leprae*, resulted in transient absence of NF-H and NF-M bands. Both NF-H and NF-M reappeared at 12 months (in case of HKML $\times 10^4$) and at 16 months (in case of HKML $\times 10^5$) showing some dose related effect. On the other hand, with viable *M. leprae* inoculation NF-H and NF-M subunits immunoreactive to SMI 31 antibody were observed at 4 months but were absent at all the later intervals i.e. between 6–20 months.

**Figure 4.** Western blot with SMI 31 antibody is depicted. Sciatic nerve NF preparations from mice inoculated with heat killed *M. leprae* in the foot pad is seen along with other controls. Key: Mol. Wt. marker (M; lane 1); NF preparation from normal rat spinal cord (C; lane 2), and mice inoculated with heat killed *M. leprae* $1 \times 10^4$ and $1 \times 10^5$ (both studied between 2–20 months).

**Figure 5.** Sciatic nerve NF preparations from mice inoculated with viable *M. leprae* in the foot pad. Western blots with SMI 31 antibody from two sets of mice ML1 (5a) and ML2 (5b) along with controls is depicted. Key: Mol. Wt. marker (M; lane 1); NF preparation from normal rat spinal cord (C; lane 2), and mice inoculated with *M. leprae* (4–20 months; lanes 3–7) are depicted.
Thus NF protein alterations seen in case of heat killed \textit{M. leprae} were transient where as in case of viable \textit{M. leprae} the NF alterations persisted till the end.

**KSPXK Kinase Activity**

The Triton X-100 soluble fractions of sciatic nerves from four groups of mice were subjected to radiometric assay. The counts of $^{32}$P labelled ATP detected were in the range of 200–400 CPM. Kinase activity (expressed as nM ATP/gm nerve) against KSPXK peptide substrate in Triton X-100 soluble fraction of uninoculated mice was in the range of 0.076 to 0.740 nM. The KSPXK kinase activity in sciatic nerves of \textit{M. smegmatis} inoculated mice was in the range of 0.149 to 0.919 nM/gm nerve. While the viable \textit{M. leprae} inoculated mice showed KSPXK kinase activity in the range of 0 (below detectable level) to 0.219 nM/gm nerve. In case of heat killed \textit{M. leprae} a transient decrease in kinase activity was seen (at 4 and 6 months) that was followed by an increase, with final levels more than that of \textit{M. smegmatis} inoculated group (0.038–1.39 nM) (Figure 6). The observed pattern of KSPXK kinase activity also corroborates with the results from WB analysis depicting SMI 31 immunoreactivity of cytoskeletal preparation.

**Discussion**

The pattern of \textit{M. leprae} growth seen in the present study corroborates with typical growth curve of \textit{M. leprae} as described by Shepard\textsuperscript{1} and good fold increase was observed.

The control uninoculated mice showed the presence of protein bands corresponding to all three NFs in WBs reacted with specific antibodies. Decreased immunoreactivity to SMI 31 antibody and faint or absent NF-H and NF-M protein bands in both viable and heat killed \textit{M. leprae} inoculated mice in the Triton X-100 insoluble pellet fraction that represents the cytoskeletal proteins was demonstrated in the WB analysis (Figures 4 and 5). In \textit{M. leprae} inoculated group these NF alterations were seen at 6 months post inoculation and coincide with increase in \textit{M. leprae} numbers (Figure 1). No such NF alterations were seen with

![Figure 6. KSPXK kinase activity in Triton X-100 soluble fraction from sciatic nerves of uninoculated mice (UN), mice inoculated with \textit{M. smegmatis} (MS), heat killed (HKML) and viable (VML) \textit{M. leprae} studied between 4–20 months post inoculation is depicted.](image)
M. smegmatis, a non-pathogenic acid fast bacilli; implying the specificity of changes. Decrease in KSPXK kinase activity was observed in case of viable M. leprae (between 4–20 months) and heat killed M. leprae (at 4 and 6 months) and corroborates well with the changes in NF proteins seen in WB (Figures 4–6). These findings imply dephosphorylation of NF proteins and are in accordance with NF dephosphorylation reported earlier by our group in human leprosy nerves.26,27

Phosphokinase activity in the sciatic nerve compartment following inoculation of M. leprae and its components is being studied at our centre and results presented in this manuscript are part of our initial experiments. Decrease in KSPXK kinase activity in sciatic nerve preparations of M. leprae inoculated mice as compared to mice inoculated with M. smegmatis was observed. Similar decrease in kinase activity was seen in the mice inoculated with heat killed M. leprae but was transient in nature. WB results also showed decrease in SMI 31 immunoreactivity in these groups.

PK activity recorded in the present study using KSPXK fusion protein as substrate corresponds to specific proline directed kinases i.e. cyclin dependent kinases (CDKs) and mitogen activated protein kinase (MAP kinase) that phosphorylate serine residues on the KSP repeats present on the carboxy terminal region of NF-H and NF-M. Among these cyclin dependent kinase 5 (CDK5) and extra-cellular signal regulated kinase 1/2 (ERK1/2) are the principal kinases. Phosphorylation at this site is known to regulate inter-filament distance and hence axon calibre. Whilst, the Ser/Thr residues on Glu-rich region on NF tail domain; are phosphorylated by casein kinases,18,36 c – Jun N – terminal kinase (JNK)/stress activated protein kinase (SAPK) has also been associated with aberrant phosphorylation of NFs seen in diabetes.37

Phosphorylation of KSP repeats, in particular KSPXK and KSPXXK epitopes, is brought about by kinase(s) belonging to CDK and MAPK super families.14,19,20,22 While, CDK5 is known to phosphorylate KSPXK epitopes only, ERK 1/2 can phosphorylate both KSPXK and KSPXXK epitopes.19,22 Hence, the assay of protein kinase activity using specific peptide substrate i.e., KSPXK fusion peptide in this study, in essence represents the combined activity due to CDK5 and ERK1/2. Thus, it is possible that the depletion in KSPXK kinase activity could be due to the decrease in either CDK5 or ERK 1/2 per se or both.

A further set of experiments confirmed the results of present study and a decrease in CDK5 and ERK 1/2 activity was recorded in the sciatic nerve compartment of mice following inoculation in the hind foot pad with M. leprae. Similar but transient decrease was seen in PGL-1 inoculated mice (submitted elsewhere). Kinase activity recorded with KSPXK fusion protein also carry the same message and serve as an indicator of decreased CDK5 and ERK1/2.

The CDK5 and MAP kinases together form important signaling systems. Alteration in the levels of CDK5 and ERK 1/2 could initiate a cascade resulting in gross abnormality in the axonal NF cytoskeleton. However these changes are more likely to be quiescent and could result in slow progressive nerve damage.

Decreased phosphorylation of NF proteins at the C-terminal region could result in decrease in inter-filament distance. This could further result in collapse of NF cytoskeleton and arraying. A rearrangement in NF cytoskeleton seen as compaction and arraying at the axon periphery was reported by us in atrophied axons of human leprosy nerves.27 Similar NF rearrangement was also seen in mouse leprosy nerves. It is interesting to note that NF-M has been identified as target protein for the myelin directed outside-in signaling cascade that involves myelin associated protein (MAG) and mediates radial axonal growth.38
Though present results have not yet been supplemented by morphometric analysis or nerve conduction velocity (NCV) testing, the rearrangement of NF cytoskeleton seen in the axonal compartment is quite suggestive. It is important to note at this point that morphological as well as immunohistochemical studies demonstrate presence of NF protein in the axonal compartment of both the test and the control group of nerves (results not shown). Control groups also showed NF bands at their expected molecular weights in WB. But the sciatic nerve preparations from *M. leprae* inoculated groups failed to show normal NF profile and faint or absent immunoreactivity to protein bands corresponding to NFs was observed.

Altered C-terminal phosphorylation could result in change in the three dimensional structure of NF or bring about change in NF protein interaction with other components of cytoskeleton. This could make it more vulnerable to degradation by proteases. Results of an ongoing study suggest activation of calpain proteases in *M. leprae* and PGL-1 inoculated mice but not in the control mice inoculated with normal saline (submitted elsewhere). Activated calpain could contribute to NF cytoskeleton abnormality seen in the present study. Earlier reports also suggest that dephosphorylation of NF proteins renders them susceptible to degradation by proteases such as calpain. A study by Eustis-Turf and co-workers showed the presence of antibodies against neural proteins, including NF, in the sera of leprosy patients thus supporting this view.

The changes seen in the sciatic nerve of S/W mice infected in the FP with *M. leprae* seem to depict the changes seen in the early/quiescent phase of *M. leprae* infection in human cases of leprosy. In the mouse model it has been shown that involvement of ‘C’ fibres occur as early as 4 months, morphological changes suggestive of axonal atrophy and demyelination of myelinated fibres at 12 months and later. The present study demonstrates NF alterations indicating dephosphorylation as early as 6 months post inoculation, thus implying that the biochemical changes in axonal compartment probably precede gross structural changes in the myelinated fibres. These changes occur in the absence of integral bacilli and inflammatory cells in the sciatic nerves of *M. leprae* infected mice suggest role for diffusible molecules of bacterial and/or host origin.

Notably inoculation with heat killed *M. leprae* results in loss of immunoreactivity to SMI 31 much ahead of viable *M. leprae*, and the effect was transient in nature, unlike that induced by viable *M. leprae*, which was delayed in onset and was persistent (Figures 4 and 5). Restoration of NF-H and N-M phosphorylation by 12 months post inoculation in heat killed *M. leprae* (1 $\times$ 10$^9$) group as evidenced by the appearance of immunoreactivity to SMI 31 antibody corroborate with the increase in KSPXK kinase activity and suggest possible nature of the biochemical events underlying the *M. leprae* induced alterations in NF.

The increase in KSPXK kinase activity among heat killed *M. leprae* group could be an indicator of repair mechanism. Presence of aberrantly myelinated fibres (poly-axonal myelination) and large regenerating units were seen in sciatic nerves of mice inoculated with heat killed *M. leprae* (results not shown); indicating an aberrant regenerating response. Thus subsequent to transient down regulation; a surge in KSPXK kinase activity aiding regeneration and repair mechanism could assist in functional recovery of these nerves. In this regard it is of importance to note the reported role of CDK5 and ERK1/2 during development, cell differentiation and regeneration following degeneration.

While there has been tremendous interest in aberrant phosphorylation of cytoskeletal proteins in central nervous system disorders such as Alzheimer’s disease and amyotrophic lateral sclerosis and its relation to degenerative changes, the alterations, if any, in NF
phosphorylation of peripheral nerves from these disorders is not completely understood. Hypophosphorylation of NF-H and NF-M components in sciatic nerves of mice infected in the FP with \( M. \text{leprae} \) is probably the first experimental evidence to demonstrate that infectious agents on their own or via an aberrant host response could as well lead to disturbance in the dynamics of cytoskeletal components in nervous system. Hypophosphorylation of NF proteins seen in the human leprous nerves\(^{26,27} \) corroborates with the reported absence of senile plaques in brains of leprosy patients as compared to normal ageing and Alzheimer’s disease.\(^{51,52} \) Hence it is important to identify and characterise the molecular constituents and the biochemical events that bring about alterations in the NF cytoskeleton that could further result in peripheral neuropathy. This could assist in further understanding aberrant phosphorylation and the consequent accumulation of cytoskeletal proteins in the neuronal cell bodies seen in neurodegenerative disorders.

To conclude, the results obtained in mouse model for leprous neuropathy indicate the biochemical alterations in the NF, viz., hypophosphorylation of NF-H and NF-M. Similar NF changes were also observed in human leprous nerves.\(^{19,20} \) Dephosphorylation of NF corroborated with the decrease in KSPXK kinase activity and was specific to \( M. \text{leprae} \) infection and not seen in \( M. \text{smegmatis} \) inoculated group. With heat killed \( M. \text{leprae} \) the NF alterations occur much earlier but were transient in nature as compared to viable \( M. \text{leprae} \) that showed delayed but persistent NF changes. The fact that \( M. \text{leprae} \) infection in the foot pad can bring about alterations in the sciatic nerve NFs and activity of the KSPXK kinase; suggest that diffusible products derived from \( M. \text{leprae} \) the host play a role in this cascade. The biochemical alterations in the NF proteins appear to precede axonal atrophic changes in this mouse model of \( M. \text{leprae} \) infection and offer a biochemical explanation for axonal atrophy in \( M. \text{leprae} \) infection.

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Neurofilament phosphorylation and KSPXK kinase activity