Denervation of skin in neuropathies: the sequence of axonal and Schwann cell changes in skin biopsies

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We compared the pathological changes in cutaneous axons and Schwann cells of individuals with nerve transection to the changes in patients with chronic neuropathies. Following axotomy there was segmentation of axons in the epidermis and dermis on the first day, and loss of axons from the skin was virtually complete by Day 11. Epidermal and small superficial dermal axons were lost before larger caliber and deeper dermal axons. Within the first 50 days following nerve transection, the denervated Schwann cells in the dermis were easily identified by their markers p75 and S100, but by 8 months they had largely disappeared.

The chronic neuropathy patients had distally predominant fibre loss, with greater loss of epidermal and dermal fibres in the distal regions of the leg than proximal regions. Several patients had large axonal swellings, often alternating with axonal attenuation, even in regions with normal or nearly normal fibre densities. By electron microscopy the swellings contained accumulations of mitochondria and other particulate organelles as well as neurofilaments. These swellings are likely to represent predegenerative changes in sites of impaired axonal transport, and previous data indicate that the swellings presage fibre loss in the subsequent months.

Some of the severely denervated regions had remaining Schwann cells, as judged by immunocytochemistry and by electron microscopy, but others lacked Schwann cells. By analogy with animal experiments, these regions are likely to have had more prolonged denervation. The distribution of axonal loss, the axonal swellings and the changes in Schwann cells all have implications for the design of clinical trials of agents intended to protect cutaneous innervation and to promote regeneration of cutaneous axons in peripheral neuropathies.

Keywords: epidermis; dermis; unmyelinated axon; Schwann cell; skin biopsy

Abbreviations: IENF = intraepidermal nerve fibre; EM = electron microscopy; ATN = antiretroviral toxic neuropathy; SSN = silent sensory polyneuropathy; ISN = idiopathic sensory neuropathy; IR = immunoreactivity

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Introduction

Loss of small sensory fibres is characteristic of polyneuropathies associated with diabetes, HIV/AIDS, amyloidosis, some heritable neuropathies and leprosy (Cornblath and McArthur, 1988; Shetty et al., 1988; Kennedy et al., 1996; Reilly and Staunton, 1996; Periquet et al., 1999; Polydefkis et al., 2002), and can be seen in inflammatory demyelinating (Chiang et al., 2002) and vasculitic neuropathies (Lee et al., 2005). The advent of immunocytochemical staining of skin biopsies to identify epidermal and dermal nerve fibres has provided a useful tool for assessing these small sensory fibres (McArthur and Griffin, 2005). Within the epidermis the individual axons are separated from each other as they pass between the keratinocytes, so at the light microscopic level immunostained fibres can be counted. The density of epidermal axons is now used to diagnose neuropathies and to assess the distribution and severity of denervation. Skin biopsies can be sampled from several anatomical sites simultaneously and can also be performed serially over time (McCarthy et al., 1995; Kennedy et al., 1996; Holland et al., 1997; McArthur et al., 1998; Periquet et al., 1999; Gibbons et al., 2006) to generate a spatial and temporal picture of progression or reversal of axonal loss.

In this study, we used skin biopsies for two related goals: first, to time the axonal and Schwann cell changes in nerves undergoing Wallerian degeneration after nerve transection,
Table 1 Clinical features, intraepidermal nerve fiber (IENF) densities and appearance of swellings in chronic neuropathies

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Normal value of IENF density = Lower fifth percentile value is 8 fibres/mm in PT and 5 fibres/mm in DL; AJ = ankle jerk; PT = proximal thigh; DL = distal leg; Asterisk indicates the patients are diabetic; ATN = antiretroviral toxic neuropathy; SN = sensory polyneuropathy; ISN = idiopathic sensory neuropathy; N = normal; ++ = large epidermal axonal swellings; + = small axonal swellings; nil = no prominent swellings; ↓ = reduced.

and second, to compare these changes to those in patients with chronic peripheral neuropathies involving small sensory fibres. Both sets of skin biopsies were compared to normal biopsies. For the studies of Wallerian degeneration the skin samples were taken from the territory innervated by the sural nerve at various times after diagnostic sural nerve biopsies. The neuropathy patients had mild sensory neuropathies, and most were recruited from a cohort of individuals with HIV infection (Cherry et al., 2006). We used immunocytochemistry and electron microscopy to study biopsies in which the cutaneous fibre density was only mildly reduced and in which axonal swellings, axonal attenuation, or both were seen at the light microscopic level. The results indicate that there are sequential changes in both axons and Schwann cells after axotomy and in chronic neuropathies. These sequences share common features but have important differences, including the prominence of axonal swellings in some chronic neuropathy patients.

We sought especially to identify changes in the biopsies that might be useful in defining inclusion criteria or outcome measures that could be used to design clinical trials of new approaches to axonal protection and/or regeneration in small fibre neuropathies. Trial design of potential axonoprotective or proregenerative strategies in patients, even at a proof-of-concept level, is often vexed by the scale of the effort required. If the patients are changing too slowly or if the disease is too far advanced, detection of protection or regeneration may require prohibitive numbers of patients and trial duration. The data from this and other recent studies suggest strategies that might be used to enrich study populations in patients in whom a response would be biologically feasible, were the therapy effective, and in whom a response could be detected.

Materials and methods

Wallerian degeneration

A group of ten patients, six with HIV and neuropathy and 4 with HIV-seronegative neuropathies, were examined for changes that occurred after nerve transection from diagnostic sural nerve biopsy on a specific date. The transection of the sural nerve was performed in the calf. Skin from the lateral aspect of the heel, within the sural territory as confirmed by sensory testing, was sampled at intervals after biopsy. In two patients with AIDS who died 2 and 48 months after previous sural biopsies, we removed the distal stump of the sural nerve at autopsy and examined it by light and electron microscopy.

Skin biopsies from patients with neuropathies

Punch skin biopsies (3 mm) from the lateral aspect of the distal leg and proximal thigh were obtained from 9 patients, four female and 5 male. Seven were from HIV-positive patients, six of whom had mild neuropathy manifested by neuropathic pain and changes in sensibility in the feet, reduced or absent tendon reflex at the ankle, and/or reduced intraepidermal nerve fibre densities. The seventh patient had no symptoms, a normal examination, and normal IENF densities. To insure that findings were not exclusive to HIV-associated neuropathies, we also studied two HIV-seronegative individuals clinically diagnosed to have idiopathic sensory neuropathy (ISN) (Table 1). Thigh biopsies from four healthy neuropathy-free adults aged 40–50 were used as controls. Informed consent was obtained from each participant, and the study was approved by the Institutional Review Board.

Immunocytochemistry

Each skin biopsy was divided into two halves; one half was used for immunocytochemistry and nerve counting while the other half was studied under electron microscopy (EM). The first half was fixed for 12–18 h in 2% paraformaldehyde/lysine/periodate fixative followed by cryoprotection. The biopsies were sectioned with a sliding microtome into 50-μM thick free-floating sections.
Four sections were randomly selected for intraepidermal nerve fibre (IENF) density determination. Sections were stained with a polyclonal antibody to neuron-specific ubiquitin hydrolase, anti-protein gene product, PGP 9.5 (rabbit 1:2000; Chemicon, Temecula, CA, USA), and developed with chromogens as previously described (McCarthy et al., 1995; Holland et al., 1995). Samples for immunofluorescence were stained with anti-protein gene product, PGP 9.5 (rabbit 1:2000; Chemicon) and with anti-p75 nerve growth factor receptor, (mouse 1: 500; Chemicon) and anti S-100 (B-subunit, mouse 1:500; Sigma). The secondary antibodies Cy3 goat anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc) were used at 1:150 dilution and Alexa-Fluor goat anti-mouse IgG (Molecular probes) at 1:500 dilutions. The fluorescence-labeled sections were mounted in Mowiol 4-88 (Calbiochem, San Diego, CA, USA) to prevent fluorescent quenching. Fluorescent samples were viewed and scanned using a Zeiss LSM510 confocal imaging system.

The intraepidermal nerve fibres were counted along the length of the 3 mm punch biopsies. In each biopsy the counts were enumerated in four sections. The length of the epidermis along the upper margin of the stratum corneum was measured with Bioquant software (R&M Biometrics, Nashville, TN). The IENF density was derived and expressed as the number of fibres per millimeter of epidermal length (fibres/mm) using counting rules previously described (Stocks et al., 1996; McArthur et al., 1998). Individual nerve fibres were counted as they crossed the dermal–epidermal junction, whereas secondary branching within the epidermis and epidermal nerve fragments that do not cross the basement membrane were excluded from the quantification (Kennedy et al., 2005; Lauria et al., 2005).

**EM technique**

The halves of the skin biopsy and the sural nerves that were taken for EM were fixed overnight in 4% paraformaldehyde–3% glutaraldehyde in 0.1M Sorenson’s phosphate buffer, rinsed, osmium fixed, embedded in plastic and sectioned according to standard procedures. The samples were embedded vertically so that the sections went through epidermis and dermis. One micron thick sections were stained with 1% toluidine blue. Sections were screened and the blocks were trimmed to visualize superficial dermis and epidermis. Three thin 60—70 nm sections from each block were stained with uranyl acetate (2.5% in 50% ethanol) and lead citrate (3%). Both the dermis and epidermis were photographed at 3000—50 000 magnifications using a Hitachi H-600 electron microscope. Photos were scanned at 600 dpi and minimal contrast and brightness levels were adjusted for optimal quality without altering images. Remak bundles were identified in the dermis of biopsies by using previously specified criteria (Gibbels, 1989; Murinson and Griffin, 2004).

**Morphometry of axons**

The film images were captured by using a Sony CCD-IRIS/RUB camera, the live images viewed under Bioquant 2.50.4 image analysis system, and the magnifications calibrated. Individual distance array was used to measure the axonal diameters (the shortest distance). The total number of unmyelinated axons per Remak bundle, number of axons abutting the basal lamina, polygonal pockets, perineurial and collagen arrangements and several other pathological aberrations were recorded. Statistical analysis was carried out by Fisher’s exact test and Chi-square test.

The ultrastructural findings of Remak bundles were recorded at two levels of the skin sections; dermis and epidermis.

### Results

#### Normal skin innervation

In controls the axonal findings at the immunocytochemical level corresponded to those previously described (Kennedy and Wendelschafer-Crabb, 1993; McCarthy et al., 1995; Lauria et al., 2003; Wendelschafer-Crabb et al., 2006). Normally PGP9.5, a panaxonal marker, and both p75 and S100, markers prominently expressed on Remak Schwann cells, were co-localized in small (unmyelinated) nerve fibres (Fig. 1A). P75 can normally be seen in a proportion of epidermal axons as well as Schwann cells, but under the fixation condition used in this study p75 was a marker of Schwann cells (Hsieh et al., 1996). P75 staining ended at the dermal–epidermal junction and was absent in the epidermis, reflecting the loss of Schwann cell ensheathment at the dermal–epidermal junction (Fig. 1A inset). Myelinated fibres had no p75 staining, so that in these fibres only PGP9.5 positive axons were seen in dually
stained preparations (Fig. 1B). P75 also prominently stained the perineurium (Fig. 1B).

The IENF densities in all the control biopsies were within normal range (Table 1) (Kennedy et al., 2005; Lauria et al., 2005). The lower limit of normal in our laboratory (defined as the 5th percentile) is 5 fibres/mm at the distal leg site and 8 fibres/mm at the proximal thigh. No prominent morphological changes were identified in the control biopsies. At the EM level in controls, the unmyelinated axons were seen as single fibres without the Schwann cells residing in the intercellular spaces between the keratinocytes. The mitochondria within the epidermal nerve fibres were aligned longitudinally along the axoplasm (Fig. 1C). In addition epidermal Langerhans cells were seen.

At the EM level 90% of all the nerve fibres within the dermis were unmyelinated. Larger and deeper nerves had complete perineurium with their basal lamina and intercellular junctional complexes. Many smaller and more superficial nerves had no perineurium; often there were partially encircling fibroblast-like cells that lacked a basal lamina. However, even the superficial Remak bundles, devoid of perineurium, were surrounded by collagen bundles oriented in the long axis of the nerve fibres, resembling endoneurial collagen. The individual Remak bundles typically contained from one to several axons (Table 2 and Fig. 2). The basal lamina around each Remak bundle was tightly applied (Fig. 1D); loose, serpentine or redundant basal laminae were rare, and free basal lamina (not associated with cells) in the endoneurium of dermal nerves was not seen. Polyaxonal pockets, with one axon directly in contact with one or more others without intervening Schwann cell processes, occurred in only less than 2% of the 59 Remak bundles (Table 2). In most instances the lips of the external mesaxon surrounding an axon met, enclosing the axons. However, 7% of the time a portion of the axon directly abutted the basal lamina; in such instances no true mesaxon was present (Table 2). The interface between Schwann cell plasma membrane and the apposed axon was a very thin regular gap and occasional coated pits were noted.

### Wallerian degeneration after nerve transection

The skin biopsies from the lateral heel demonstrated, as expected, rapid disappearance of the axonal marker PGP9.5. By 24 h axons within the epidermis were undergoing segmentation (Fig. 3A). Within the dermis this process began in small fibres by 24–48 h, and affected larger fibres later, with the formation of ovoids that progressively shortened over time (5–7 days) (Fig. 3B). By 11 days all PGP9.5 staining had disappeared (Fig. 4A). At no time in this sequence were large epidermal or dermal axonal swellings seen.

The Schwann cell marker p75 became more prominent within the first 2 weeks after denervation, both in the deep and the superficial dermis. This prominence reflected more intense immunoreactivity on individual Schwann cells as well as more stained cells, resulting from the new expression in the Schwann cells of myelinated fibres that had undergone axonal degeneration. P75-IR was still prominent in denervated Schwann cells at 51 days (Fig. 3C), but by 8 months it had essentially disappeared from the superficial dermis and was markedly reduced in the deep dermis (Figs 3D, 4A and B). Similar patterns were seen with the other Schwann cell marker S100 (The perineurium remained p75 positive throughout this sequence).

This process of atrophy and disappearance of Schwann cells was confirmed at the EM level in sural nerve biopsies.
At 2 months axonal and most myelin debris had been cleared (Fig. 5A), but foamy macrophages (Supplementary Fig. 2A) and neutral lipid droplets within macrophages, Schwann cells (Supplementary Fig. 2B) and perineurial cells remained. Many of the denervated Schwann cells formed stacks of lamellated processes (Fig. 5A). The Schwann cell nuclei remained euchromatic, and the perikaryal region of the Schwann cells contained abundant cytoplasm. By 4 years densely packed collagen occupied most of the endoneurial area, often demarcated into islands by long fibroblastic-like cells (Fig. 5B). Only a few Schwann cell nuclei remained, and these were darkly heterochromatic and surrounded by scanty cytoplasm (Fig. 5B). Strikingly, Schwann cell processes had largely regressed, so that there were numerous empty basal lamina tubes (Fig. 5B) and others containing only thin fingers of Schwann cell processes within serpentine loose basal lamina (Fig. 5B). Isolated basal lamina fragments were numerous (Fig. 5B).

**Chronic neuropathies**

**Axonal measures**

The epidermal nerve fibre densities were at or below the lower limit of normal range in subjects with chronic neuropathies. Axonal measures

**Graphs showing IENF density and Schwann cells in lateral heel after sural nerve transection in 10 patients.** (A) Baseline IENF and the reduction of epidermal nerve fibres (IENF density) in heel after sural nerve transection. (B) Semi log graph showing the disappearance (shaded areas) of stained epidermal (epi) and dermal (der) nerve fibres and p75 stained Schwann (Sch) cells from heel skin after sural nerve transection.

**Electron micrographs of distal stump of sural nerve.** (A) At 2 months the distal stump of sural nerve showing many denervated Schwann cells forming stacks of lamellated processes (arrows). The Schwann cell contains euchromatic nuclei and the perikaryal region with abundant cytoplasm (broken arrow). (B) At 48 months this sural nerve shows islands of densely packed collagen demarcated by long fibroblastic-like cells (arrow heads). The single Schwann cell nuclei in the field is densely heterochromatic. Numerous attenuated Schwann cell processes (arrow), empty basal lamina tubes (broken arrows) and basal lamina fragments are seen. Scale bars = All 1 μm.
neuropathies, and in all but one were higher at the level of the thigh (Table 1). In 4 patients the biopsies showed many large axonal swellings, >5 times the diameter of the non-swollen segments, in the thigh as well as in the distal leg. Smaller swellings with a 2- to 3-fold increase were also seen (Fig. 7D). In dermal nerves there were prominent abnormalities. Eighteen percent were denervated, and 8% had empty collagen pockets (Table 2). There was an increased proportion of units with 1 axon in a Schwann cell (Table 2 and Fig. 2). Axonal caliber varied widely within nerve bundles, from small attenuated and atrophic fibres (Fig. 7A and B) to enlarged axons (Fig. 7C). Axon diameter ranged from 0.4 to 11.67 μm (Fig. 8).

Degenerative changes at varying stages were noted in the fusiform axonal swellings. The swollen axons often contained watery axoplasm, dilated vesicular profiles, granular debris and a paucity of particulate organelles (Fig. 7C). These changes were not a consequence of fixation artefact, as neighbouring axons were well fixed and they were rarely seen in control nerves. In such swellings distinct microtubules and neurofilaments were often hard to identify, and some axons of these axons had no identifiable cytoskeletal elements (Fig. 7C). Some axons contained abnormal and large bizarre-shaped mitochondria (Table 2). Fusiform axons containing large abnormal mitochondria enveloped by flattened elongated Schwann cell process orienting in stacks of parallel array were also seen (Fig. 7D).

**Schwann cell changes**

The Schwann cells of Remak bundles exhibited a variety of changes. The cytoplasm adjacent to dilated axons was often attenuated and 10% of axons abutted and were covered only by basal lamina. As noted, in the neuropathy biopsies the larger dermal nerves contained varying numbers of small, unusually round, empty Schwann cell tubes reflecting complete denervation (Fig. 7E and inset). Other Schwann cell changes (Table 2) included large reactive nuclei (Fig. 7F), unusually electron-dense cytoplasm (Fig. 7B) or watery cytoplasm (Fig. 9A and B), abnormally fine collagen fibrils (Fig. 9A), and, in the papillary dermal Remak bundles, large abnormal clusters of mitochondria (Fig. 9C). Loose serpentine or empty basal lamina was seen frequently lying in the endoneurium adjacent to degenerating Remak bundles (Fig. 9D). Empty Schwann cell tubes, attenuated atrophic fibres, and axonal swellings could all be seen within the same nerve fascicles.

Occasionally the Schwann cells and axons displayed unusual interactions. Long tongues or ingrowths of the Schwann cell plasmalemma deeply indented the axoplasm, pushing the axolemma into the resulting invagination and producing a ‘four membrane’ profile, so that in
cross-section these ingrowths have four membranes (traver-
sing an ingrowth from within the axoplasm one would
cross axolemma, Schwann cell plasmalemma, Schwann cell
plasmalemma and axolemma) is previously described in
experimental neuropathies (Spencer and Thomas, 1974;
Griffin and Price, 1981). The ingrowths partitioned the
axoplasm in compartments (Fig. 10A and B), and
incompletely encircled abnormal mitochondria (arrow). (E) Dermal nerve with many denervated
Schwann cell complexes (arrows). Inset showing a Schwann cell
complex with no unmyelinated axons. (F) Higher power showing
a persisting denervated Schwann cell (arrow) with a large nucleus,
adjacent to a Remak bundle. Scale bars = All 1 μm.

Importantly, in some of the biopsies that had a severe
loss of axons we could not identify remnants of Schwann
cells, either by immunocytochemistry or by EM. These
samples resembled those seen months after Wallerian
degeneration. In the same patient there could be extensive
Schwann cell loss in the distal leg and relatively preserved
Schwann cells in the face of denervation at the thigh level. Other patients had severe Schwann cell loss at both levels. Similarly, Schwann cells in the superficial dermis could be entirely absent while denervated Schwann cells in the deep dermis persisted.

Axonal and Schwann cell changes identified were similar in both HIV-positive and idiopathic sensory neuropathic cases with no recognizable differentiating features.

**Discussion**

A major challenge, fundamental to the development of axonoprotective or regenerative treatments, lies in the long time required to assess progression of neuropathies or regeneration. This study set out to identify the sequence of axonal and Schwann cell changes in peripheral neuropathies affecting small sensory fibres, with the goal of contributing to the design of neuroprotective and regenerative trials. We first established the time course of changes in simple Wallerian degeneration in small sensory fibres in man. Within the first day after sural transection, the epidermal axons began to segment into rows of PGP9.5-immunoreactive dots, and these axonal remnants lost immunoreactivity by 3–5 days. The deeper and larger dermal fibres began to segment into the classical ovoids at 3–7 days. Only a few small ovoids remained at 11 days, and no axonal residue could be identified at later times. Importantly, by 8 months the Schwann cells had largely disappeared from the deep and superficial dermis, as confirmed by p75 immunocytochemistry and confirmed at the EM level.

These results provided a context for the studies of neuropathies. Our observations confirmed previous studies in finding that the loss of unmyelinated cutaneous nerves in most patients was length dependent, so that the IENF densities in the distal leg were markedly lower than in the thigh. The findings in some of the neuropathy patients differed from any of those in the Wallerian degeneration group in one key aspect—the prominence of axonal swellings in the epidermis and dermis. Frequent axonal swellings could be found in regions in which the nerve fibre density was within normal limits. These swellings could be divided into globular and fusiform types. At the EM level, the globular swellings contained accumulations of mitochondria and other particulate organelles, whereas other swellings, often fusiform, had watery cytoplasm with a loss of cytoskeletal elements.

In several neuropathy patients Schwann cells were absent from the distal leg when they were still present in the thigh, even though there was severe axonal loss at both sites. In comparing completely denervated sites among different patients, we found that in some denervated Schwann cell bands could easily be identified, but in many both p75/S100 staining and identifiable Schwann cells by EM were absent. As discussed below, we suggest that these regions have been denervated for longer periods. The superficial dermal Schwann cells were lost before deeper dermal Schwann cells. This sequence has implications for subject selection for inclusion in treatment studies intended to promote axonal regeneration. We suggest that the absence of Schwann cells may indicate less reversible stage in which regeneration is unlikely.

**Axonal changes**

**Wallerian degeneration**

The sequence of Wallerian degeneration has been studied extensively in experimental animals after nerve section at the level of peripheral nerves such as the sciatic (Ballin and Thomas, 1969; Lunn et al., 1989; Stoll et al., 1989) and phrenic (Lubinska, 1982) for review (Griffin et al., 1996, 2006). In all settings there is a latent period after axotomy during which the axons in the distal stump appear relatively stable.
normal and during which conduction of nerve impulses continues. This latent phase was seen in the present study, and varied with fibre type and location. The first fibres to degenerate were the intraepidermal axons. The small fibres of the superficial dermis degenerated next, with the large myelinated fibres being the last to be cleared. This hierarchy of fibre involvement has also been seen in cross-sectional studies among patients with IGT and de novo diabetes (Sumner et al., 2003). The Schwann cells participated in segmentation of the axon into segments containing axonal debris, which initially retained its immunoreactivity. These segments were separated longitudinally by Schwann cell processes. These changes form the characteristic ovoids recognized in longitudinally sectioned nerve fibres. The lengths of the ovoids decreased over time.

Neuropathies
The patients chosen for this study all had mild neuropathies. In eight there was low normal or reduced fibre density in the distal leg, but fibre density was within the normal range at the level of the thigh. This pattern confirms many previous reports identifying the greater loss of nerve fibres distally in most axonal neuropathies (Lauria et al., 1999; Scott et al., 1999; Smith et al., 2001; Omdal et al., 2002; Polydefkis et al., 2002; Pan et al., 2003). In contrast, in patients with known sensory ganglion neuronal degeneration (sensory ganglionopathies) fibre loss is typically seen both proximally and distally in a non-length dependent pattern (Lauria et al., 2001).

In four individuals there were large axonal swellings at the thigh site even when there was no fibre loss. This suggests that the small skin fibres can go through a phase of axonal swelling before fibre loss supervenes. This phenomenon has been supported by two recent reports (Lauria et al., 2003; Gibbons et al., 2006), demonstrating that the presence of large epidermal swellings is a predictor of fibre loss over the next 0.5–2 years. The frequency of the swellings in these individuals suggests that the swellings must persist for weeks or months after their formation; if they were more transient, persisting for only a few days before the axonal loss, numerous swellings would not be seen at one point in time in mildly affected individuals. The swellings must be also relatively non-specific as to etiology because they have been seen in a variety of neuropathies, and they have also been produced experimentally by Hsieh and colleagues in the toxic disorder, acrylamide neuropathy, in the mouse (Ko et al., 2002).

Many of the axonal swellings in the present series could be classified as either globular or varicose. Normally the unmyelinated nerve fibres in the dermis of hairy skin contain scanty axoplasmic organelles (Cauna, 1973). The globular axonal swellings represented accumulation of mitochondria and other particulate organelles including dense bodies as well as intermediate filaments. These accumulations are likely to reflect focal or multifocal breakdown in fast bidirectional transport within the preterminal regions of these axons. Similar accumulations of particulate organelles can be produced experimentally by focal reduction in axonal transport by cooling (Tsukita and Ishikawa, 1980) or pharmacologic agents. These models have demonstrated that the lysosomes and dense bodies are carried retrogradely. Thus the presence of dense bodies in the globular swellings suggests that retrograde transport is at least one component of the transport system that is impaired. Defects in retrograde transport could amplify axonal injury, because growth factors are normally carried to the neuronal perikaryon through the retrograde transport system.

By contrast, the watery axoplasm seen in many of the fusiform swellings resembles that of fibres in advanced stages of Wallerian-like degeneration. How long individual fibres might retain this appearance is unknown, but it is noteworthy that Trapp and colleagues have recently observed such changes in a high proportion of fibres in the caudal corticospinal tract in multiple sclerosis, and they have argued that this appearance can be maintained for relatively long periods (Trapp, B.D., personal communication).

Schwann cells responses
Wallerian degeneration
In the first month after axotomy p75 immunostaining by Schwann cells was more prominent than normal. This undoubtedly reflects in part the fact that following degeneration of their axons the Schwann cells of the myelinated fibres in the dermis, usually p75-negative, expressed p75 after denervation (Byers et al., 1992; Song et al., 2006). However, it probably also reflects increased immunostaining of the protein in the Remak Schwann cells, which normally express some p75. This conclusion comes from the fact that more prominent p75-immunoreactivity was seen in the superficial dermis, which normally contains few myelinated fibres.

At later times the Schwann cells disappeared. This was not just a loss of immunoreactivity for the various Schwann cell markers, because Schwann cells were not found at the EM level. They were lost first from the superficial dermis and only later from the deeper dermis. The Schwann cell changes in the skin were also reproduced in the distal stump of transected sural nerves obtained at autopsy. Our observations resemble closely with the Schwann cell changes seen after a few months in rat sciatic nerves (Politis et al., 1980; Giannini and Dyck, 1990; Sulaiman and Gordon, 2000), they occur more slowly in man. Thus even in the sural nerve samples obtained at autopsy four years after transection a few Schwann cells remained, although their numbers were markedly depleted. The Schwann cells that remained were characterized by dark heterochromatic nuclei, scanty cytoplasm and loose basal laminae. Fragments of serpentine basal laminae in the endoneurial space

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identified the location of fibres that had disappeared. Rodent studies indicated that such profiles gradually shorten and eventually disappear (Giannini et al., 1990). Whether these remaining Schwann cells could respond to regenerating axons with proliferation and production of growth factors is unknown.

Neuropathies

Electron microscopy identified a similar Schwann cell changes in the neuropathy samples. In addition, we saw ingrowths of Schwann cell processes into the axon. Similar changes have been described in experimental toxic neuropathies (Spencer and Schaumburg, 1977; Spencer and Thomas, 1974; Griffin and Price, 1981). As in the experimental studies, we saw these ingrowths in regions of accumulation of particulate axonal organelles. Finger-like Schwann cell processes indent the axolemma. They have been postulated to reach into the axon and remove degenerating organelles (Spencer and Thomas, 1974).

Other non-specific Schwann cell responses have been well described in studies of experimental and human peripheral nerve pathology, including collagen pockets, stacked lamellated Schwann cell processes, and redundant basal lamina. In neuropathy patients in our study these findings could all be identified in Remak bundles in which some unmyelinated axons remained, but they were rare in normal control nerves, and thus serve as a useful marker for neuropathy at the EM level.

Implications for trial design

In experimental animals and tissue culture a variety of agents have been shown to have axonal protective properties and/or to promote regeneration, including immunophylins, growth factors and agents targeting mitochondria, glycolytic metabolism and oxidative stress. In addition to drugs, there is interest in the potential value of diet, exercise and tight glycemic control in diabetes (DCCT, 1995; Singleton et al., 2005), as well as the effects of reducing viral load in HIV. To date only a few approaches, such as chemotherapy in leprosy (Miko et al., 1993) and acetyl-L-carnitine in HIV neuropathy (Hart et al., 2004), have been demonstrated to have efficacy in man. There is interest in developing such treatments, based on the high prevalence of diabetic, HIV-associated, and other neuropathies, the looming diabetes epidemic associated with obesity, and the morbidity of the resulting neuropathic pain and painless injuries.

Trials of therapies for small fibre neuropathies are generally considered to require large numbers of patients and long duration. These considerations are barriers to testing new approaches. The need for large numbers reflects interpatient variability and the different stages of involvement at entry. The long duration of trials reflects the time inherent in regeneration at 1 inch/month when long distances are involved. The farther a fibre dies back from the target involved in the outcome measure, the longer a period will be required for regeneration. For example, if the outcome measure requires regeneration to the feet and nerve fibres have degenerated to a level above the calf, more than two years would be required to detect benefit.

Proof-of-concept studies might be done more efficiently by enriching the study population in patients who would be likely to respond rapidly to an intervention. The present data suggests criteria for identifying such patients, based on skin biopsies (Supplementary Fig. 3). It would be desirable to study anatomical areas where denervation was known to be ongoing, where Schwann cells were known to persist, and where only short-distance regeneration would be needed to reach an outcome (Supplementary Fig. 3). These criteria are based on three reasonable assumptions: first, the pre-Wallerian swellings of axons presage axonal degeneration over relatively short periods. Previous studies have shown that regions containing normal IENF but prominent pre-Wallerian axonal swellings in epidermal nerve fibres show progression of fibre loss over 12–24 months (Lauria et al., 2003; Gibbons et al., 2006), a rate faster than in regions lacking swellings. Second, the presence of Schwann cells in the nerves and targets to be reinnervated improves the prospects of successful reinnervation. Abundant experimental data suggests that nerves in which the Schwann cells have degenerated do not support regeneration well (Sulaiman et al., 2002; Hoke et al., 2002, 2006; Heine et al., 2004; Midha et al., 2005; Furey et al., 2007). Third, for regeneration to a given target, the extent of innervation of the next site up the limb will influence the time to reinnervation (Supplementary Fig. 3). If that site is well innervated the nerves passing that site are likely to contain abundant fibres, and thus the time to regenerate to a target only a short ways distally should be less. Conversely, if the sites above the target lack fibres, longer distance for regeneration and consequently more time will be required. For all three criteria investigators can take advantage of the capacity of skin biopsies to identify the spatial distribution and cellular pathology of small cutaneous nerve fibres.

Supplementary material

Supplementary material is available at Brain online.

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