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Perivascular demyelination and intramyelinic oedema in reperfusion nerve injury

HITOSHI NUKADA AND P. DENISE McMORRAN

Department of Medicine, University of Otago Medical School, Dunedin, New Zealand

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ABSTRACT

Nerve ischaemia plays a major role in the development of pathological alterations in various neuropathies, and the effects of ischaemia are amplified by reperfusion in various tissues. While pathological alterations in acutely ischaemic nerve have been established, nerve pathology resulting from reperfusion injury has never been elucidated. To evaluate what cell type in peripheral nerve is affected by reoxygenation following a hypoxic episode, we developed an animal model of transient severe limb ischaemia. Near-complete ischaemia, confirmed by the measurement of nerve blood flow, was achieved by clamping multiple arteries of supply to rat hindlimb. After 3, 5 or 7 h of limb ischaemia, vascular clips were released to reperfuse blood flow. Pathology in sciatic, tibial and peroneal nerves at the lower thigh level was examined at 7 d after reperfusion. All reperfused nerves developed demyelinated nerve fibres, particularly in perivascular regions. Although 3 h of ischaemia followed by reperfusion caused demyelination, perivascular demyelination was more prominent after a longer period of ischaemia with reperfusion. Two types of nerve oedema were observed; endoneurial oedema especially in perivascular and subperineurial spaces, and intramyelinic oedema. Nerve fibres with intramyelinic oedema were not confined to the perivascular region. Swollen endothelial cells in endoneurial vessels were also invariably observed. Nerve ischaemia per se, without reperfusion, did not induce these pathological changes. Because myelin appears to be particularly susceptible to activated free radicals, oxidative stress, activated neutrophils, and cytokine formation seem to be important underlying mechanisms in the development of perivascular demyelination and intramyelinic oedema in ischaemic/reperfused nerves. This study demonstrated that reperfusion causes selective damage to the myelin sheath, and reperfusion nerve injury needs therefore to be included in the differential diagnosis of peripheral nerve demyelination.

Key words: Peripheral nerve; ischaemia; postischaemic injury; myelin; vascular endothelium.

INTRODUCTION

Ischaemic neuropathy is commonly seen among subjects with peripheral vascular disease, diabetes mellitus, vasculitides and trauma (Asbury, 1970). Ischaemic nerve reveals various pathological abnormalities, such as segmental demyelination and remyelination, axonal degeneration and regeneration, focal, multifocal, or diffuse loss of nerve fibres, and endoneurial oedema (Hutchinson & Liversedge, 1956; Thomas & Lascelles, 1966; Dyck et al. 1972). Although nerve ischaemia plays a key role in the development of these pathological abnormalities, decreased blood flow has the potential to induce cell injury both as a direct result of hypoxia and through the secondary effects of subsequent reoxygenation. While neuropathic symptoms such as pain or numbness, are often initial manifestations in an ischaemic limb, they may also appear after transient limb ischaemia, e.g. vascular therapeutic intervention (Poole, 1956; D'Amour et al. 1987). Such postischaemic symptoms may continue for many months despite an adequate return of blood flow to the limb (Wilbourn et al. 1983; Levin, 1989), and may well

Correspondence to Dr Hitoshi Nukada, Department of Medicine, University of Otago Medical School, P.O. Box 913, Dunedin, New Zealand.

result from reperfusion nerve injury that begins only when aerobic respiration resumes. There is a body of literature suggesting that reperfusion amplifies the effects of ischaemia in various tissues (McCord, 1985). Pathological changes in reperfusion nerve injury, however, have never been previously studied. This study assessed what cell type or types in peripheral nerve are subject to the effects of hypoxia and reoxygenation. We developed a rat model of transient severe hindlimb ischaemia and evaluated pathological alterations at the lower thigh level of ischaemic/reperfused sciatic, tibial and peroneal nerves.

MATERIALS AND METHODS

Sprague-Dawley male rats (300 – 350 g, 9–10 wk old) were anaesthetised with xylazine (0.4 mg/100 g b.w. i.p.) and ketamine (8.0 mg/100 g b.w. i.p.). Major arteries supplying the right hindlimb—abdominal aorta, right common iliac, femoral and superficial circumflex iliac arteries—were occluded by microvascular clips (TKS-1, 40 g, Kyowa, Chiba) for 3, 5 or 7 h. The duration of ischaemia was chosen because: (1) previous studies have demonstrated that ischaemia for up to 8 h without reperfusion fails to produce pathological abnormalities in rat sciatic nerves (Nukada & Dyck, 1984; Nukada, 1986), (2) in tourniquet-induced ischaemic models 8 h of ischaemia has been cited as a critical time beyond which prominent nerve oedema occurs (Lundborg, 1988), and (3) reperfusion after 3 h severe ischaemia, but not 1 h of ischaemia, results in poor recovery of compound muscle action potentials in the rat sciatic nerve (Schmelzer et al. 1989). Arteries were clamped at the following levels: abdominal aorta proximal to the right iliolumbar artery but distal to the renal arteries, right common iliac artery at its origin, right femoral artery just distal to the inguinal ligament, and right superficial circumflex iliac artery near its origin from the femoral artery. It is critical to occlude these 4 arteries at these levels to prevent the development of collateral circulation and the systemic effect of reperfusion injury in this paradigm. Nerve blood flow (NBF) was monitored simultaneously at the lower thigh level of sciatic nerves by a laser Doppler flowmeter (ALF-21, Advance, Tokyo) in 9 rats. Reperfusion was ensured by the release of vascular clips in the order of superficial circumflex iliac, femoral, common iliac, and abdominal aorta. After vascular clips were released, NBF was monitored for 15 min. After a pattern of blood flow alterations during the procedure was established, NBF monitoring was not made in the subsequent 9 rats. Blood pressure was monitored by cannulating the left common carotid artery before and during ischaemia and following reperfusion in 3 rats. There were no significant changes in mean blood pressure. Rats were killed with an anaesthetic overdose and cervical dislocation following approval by the Committee on Ethics in the Care and Use of Laboratory Animals, University of Otago.

Seven days after the ischaemic episode the sciatic nerve and its branches were dissected under anaesthesia and fixed in situ for 30 min with 4% glutaraldehyde in 0.025 M cacodylate buffer, pH 7.40 (Nukada & Dyck, 1984). Although the entire length (approximately 65 mm) of the right sciatic nerve and its 3 major branches were taken in continuity, pathological evaluation in the present study was confined to the lower thigh level of sciatic nerves. The nerve was fixed by overnight immersion in 2.5% cacodylate-buffered glutaraldehyde, and cut into 10 mm consecutive segments, postfixed in 1% osmium tetroxide for 6 h, dehydrated in ethanol and propylene oxide. It was then cut into 1 mm segments consecutively and embedded in agar resin. Semithin sections (1 μm) were cut at 1 mm intervals and were stained with methylene blue or thionin and acridine orange for light microscopy. Ultrathin sections were also obtained from selected blocks and stained with uranyl acetate and lead citrate prior to examination under a Philips 410LS electron microscope. Control nerves for pathological investigations were obtained from 4 different groups of rats: (1) following 3, 5 or 7 h of ischaemia without reperfusion, (2) identical arterial ligations with NBF measurements but no reperfusion (7 d ischaemia), (3) identical surgical exposure of arteries and NBF measurements without arterial ligation, and (4) contralateral nerves in experimental rats. It should be emphasised that it is impossible to evaluate the effects of reperfusion, per se, without an ischaemic injury.

RESULTS

Immediately after arterial occlusions, NBF perfusion units fell steeply from 16.5 ± 4.8 (mean $\pm$ S.D. of preischaemic baseline values) to less than 2, a value that is indistinguishable from a biological zero. NBF perfusion units were maintained at this low level during the ischaemic period. After vascular clips were released, NBF perfusion units rose within 30 seconds to 20.6 ± 4.4 (mean $\pm$ S.D. of the highest values during reactive hyperaemia). NBF then quickly declined and settled at 14.8 ± 3.9 perfusion units (mean $\pm$ S.D.). Measurement of skin blood flow also confirmed near-

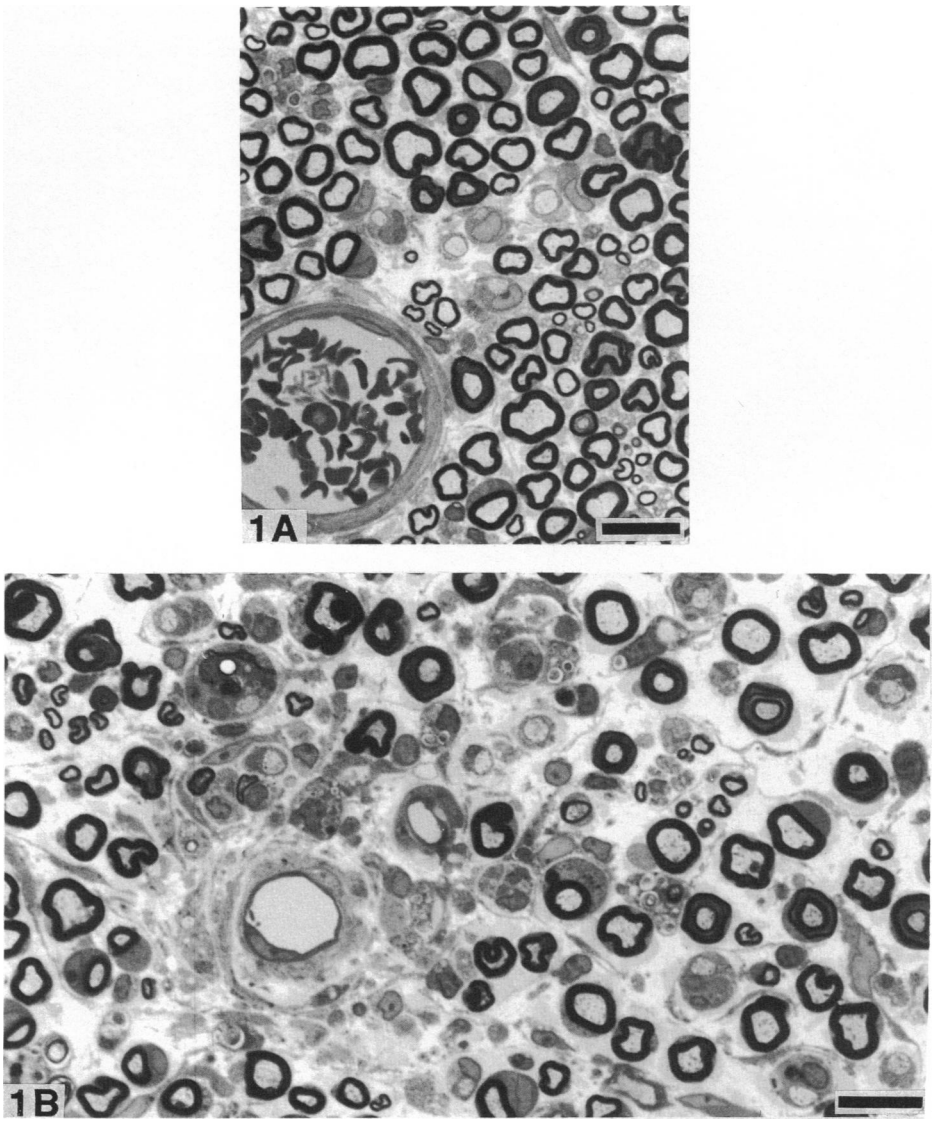

Fig. 1. Pathological appearance of rat proximal tibial nerve at the lower thigh level 7 d after reperfusion following 3 h (*A*) and 7 h (*B*) of near-complete limb ischaemia. (*A*) Perivascular demyelination can be observed and is characterised by a group of naked axons. (*B*) In this severely affected nerve, demyelinated nerve fibres showing various stages of myelinolysis are visible together with phagocytic cells and endoneurial oedema. Bars, 15 μm. Plastic sections stained with methylene blue (*A*) and thionin and acridine orange (*B*).

complete ischaemia on the dorsal surface of the right hindlimb. Clinically, the reperfused limb became oedematous within 8 h after reperfusion. Limb oedema was most prominent at 12 to 48 h postsurgery and then gradually subsided. The mean body weights were not significantly different between experimental and control rats at the time of killing and there was no mortality during the experiment.

Morphological examination revealed demyelinated nerve fibres concentrated around endoneurial vessels in ischaemic/reperfused sciatic, tibial and peroneal nerves at the lower thigh level. Although ischaemia for 3 h followed by reperfusion resulted in perivascular demyelination (Fig. 1*A*), demyelination was more frequent after a longer period of ischaemia (5 or 7 h) with reperfusion (Figs 1*B*, 2). Demyelinated axons were confined to a 2–3 mm nerve segment with affected fibres assuming a normal appearance at the distal lower thigh level. Macrophages were often seen associated with these demyelinated fibres (Fig. 1*B*). Another striking feature was nerve oedema, especially following a longer period of ischaemia. Endoneurial oedema was conspicuous around the vessels and at the subperineurial space (Figs 2, 3). Light microscopic study also revealed nerve fibres with intramyelinic oedema which were not confined to perivascular regions (Fig. 3). Nerve fibres with intramyelinic oedema were more frequently observed at the proximal segment of the lower thigh level in the sciatic, tibial and peroneal nerves. Ultrastructurally, intra-

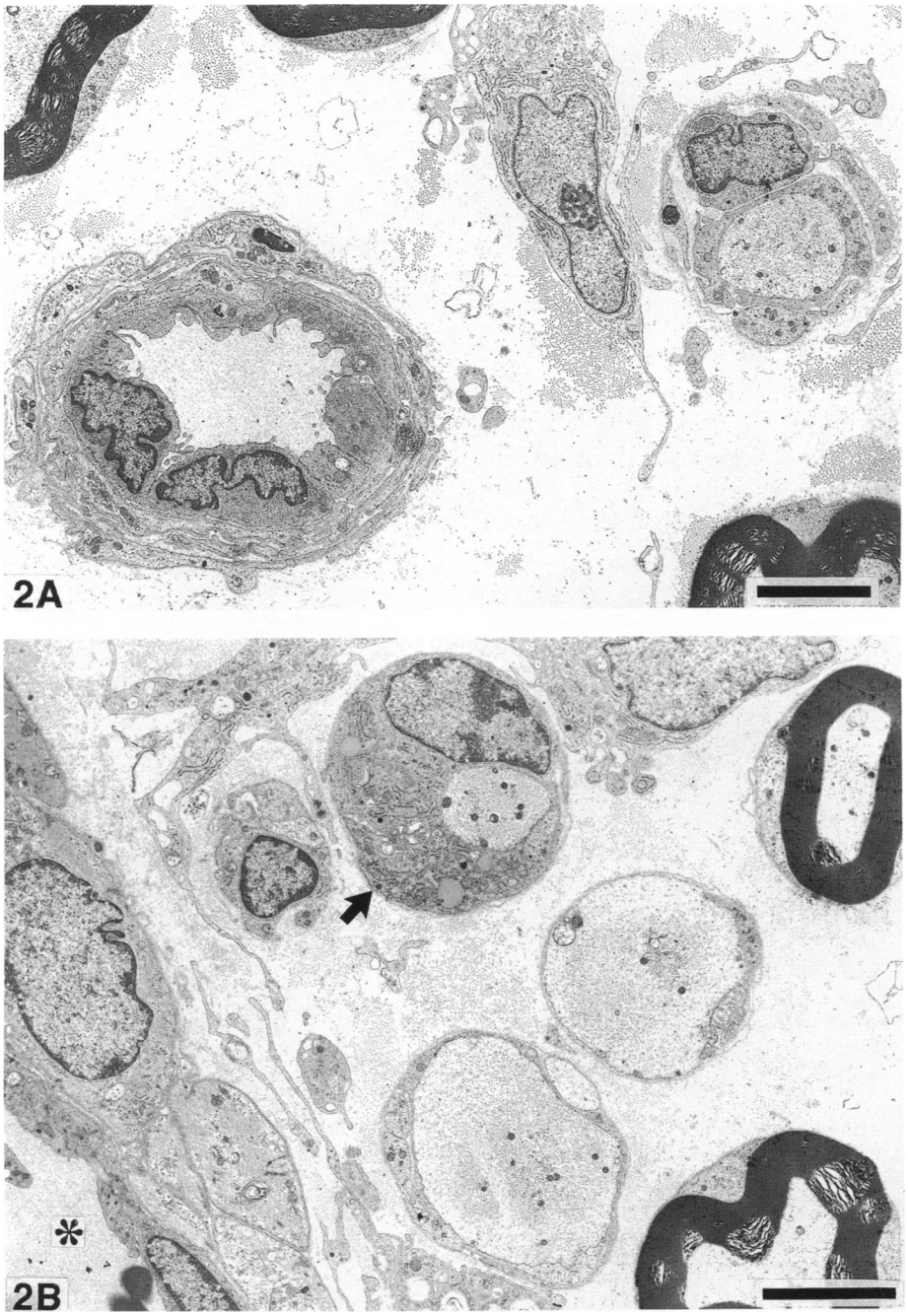

Fig. 2. Electron micrographs illustrating perivascular demyelination, endothelial swelling and endoneurial oedema in rat proximal tibial nerve 7 d after 7 h ischaemia with reperfusion. Fibroblasts can be seen in the expanded endoneurial space. (A) An endoneurial capillary with swollen endothelial cells, a demyelinated nerve fibre, and perivascular oedema are visualised. (B) Three demyelinated fibres are present close to a swollen postcapillary venule. Note reactive changes in Schwann cells, with increased number of mitochondria, endoplasmic reticulum and lysosomes (arrow). *, Lumen of endoneurial venule. Bars, 5 μm. Uranyl acetate and lead citrate.

myelinic oedema was due to splitting of the myelin lamellae at intraperiod lines (Fig. 4). No axonal degeneration was found at this level of sciatic nerves. Epineurial and endoneurial microvessels and venules had swollen endothelial cells and pericytes. Swollen endothelium and pericytes were more severe after a longer period of ischaemia with reperfusion (Fig. 2). Neutrophils and platelets attached to swollen endothelial cells were seen, although these findings were more noticeable during earlier stages after ischaemic/reperfusion injury. In control nerves, 3–7 h of ischaemia without reperfusion did not cause any pathological alteration in sciatic nerves. As expected sciatic nerves with 7 d ischaemia without reperfusion revealed central fascicular fibre degeneration at the midthigh level that was followed by panfascicular

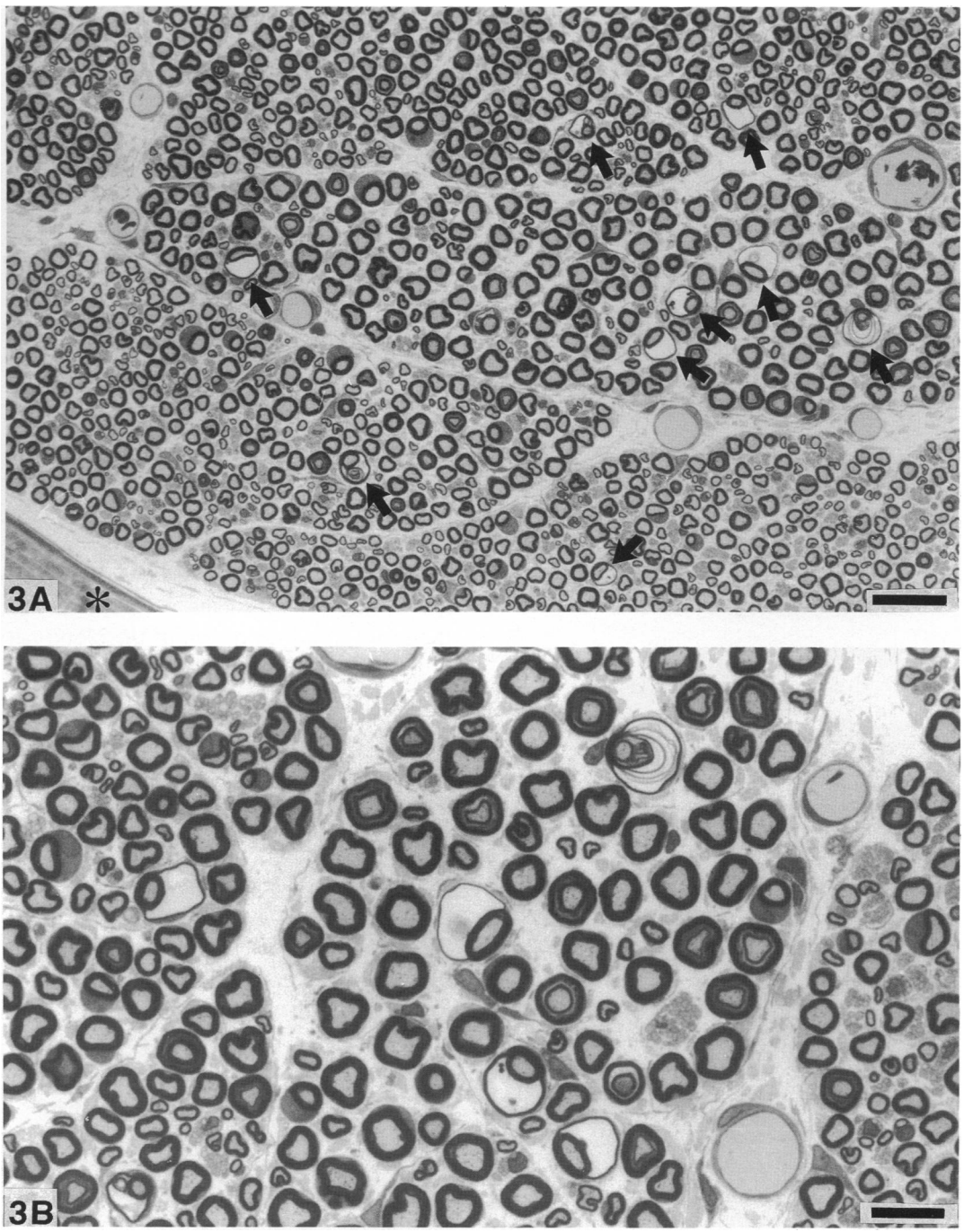

Fig. 3. Transverse section of rat proximal tibial nerve at the lower thigh level 7 d after reperfusion following 5 h of near-complete ischaemia. (*A*) Nerve fibres with intramyelinic oedema (arrows) and endoneurial oedema. Endoneurial oedema is more prominent around the endoneurial vessels and at the subperineurial space. *, Perineurium. Bar, 30 μm. (*B*) Nerve fibres with intramyelinic oedema shown at higher magnification. Bar, 15 μm. Plastic sections stained with methylene blue.

necrosis distally. No other control nerves exhibited pathological abnormalities.

DISCUSSION

In the present study there is clear evidence that reperfusion causes selective damage to the myelin sheath, predominantly around vessels. Reperfusion nerve injury needs therefore to be included in the aetiology of peripheral nerve demyelination. Whether nerve ischaemia per se causes selective damage to Schwann cells or the myelin sheath has long been a matter of debate. Several lines of evidence suggest that nerve ischaemia may cause demyelination. In nerves taken from chronically ischaemic limbs due to peripheral vascular disease, segmental demyelination

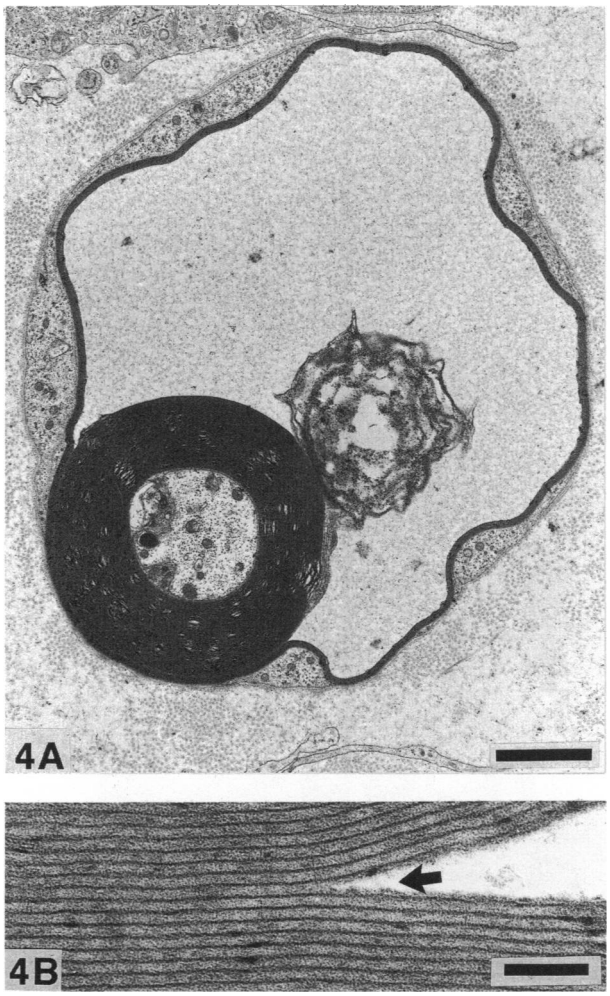

Fig. 4. (*A*) Electron micrographs from same specimen as Figure 3 illustrating typical appearance of intramyelinic oedema. Axon and corresponding Schwann cell cytoplasm remain unaffected. Bar, 2 μm. (*B*) Higher magnification of (*A*) showing intramyelinic oedema produced by separation of the myelin lamellae at intraperiod lines (arrow). Bar, 0.1 μm. Uranyl acetate and lead citrate.

is one of the common pathological changes (Chopra & Hurwitz, 1967; Eames & Lange, 1967). Diabetic nerves, which are ischaemic/hypoxic, often exhibit demyelination and remyelination (Thomas & Lascelles, 1965, 1966; Asbury et al. 1970; Ohnishi et al. 1983; Tuck et al. 1984; Newrick et al. 1986). Experimentally, rabbit sciatic nerve following arterial ligations showed paranodal demyelination proximal to axonal degeneration suggesting that mild ischaemia might induce demyelination (Hess et al. 1979). In a model of microsphere embolisation, secondary demyelination due to axonal swelling and attenuation was found at the border zone of the ischaemic lesion in rat sciatic nerves (Nukada & Dyck, 1987). A recent study where rat single arteries were ligated demonstrated that mild nerve ischaemia (40–60% reduction in NBF) caused endoneurial oedema, while moderate ischaemia induced demyelination and severe ischaemia (80–90% reduction in NBF) produced axonal degeneration (Nukada et al. 1993). Other experimental models that revealed demyelination associated with reduced NBF include galactose neuropathy, nerve compression, epineurial devascularisation, and perineurial rupture (Powell & Myers, 1983; Myers & Powell, 1984; Low et al. 1985; Myers et al. 1991; Nukada et al. 1992). In these neuropathies, however, the effects of reperfusion have not been elucidated, nor perivascular demyelination produced. Multiple foci of perivenular demyelination associated with endoneurial oedema are characteristic of experimental allergic neuritis (Wisniewski et al. 1974; Lampert, 1978), although the role of ischaemia/reperfusion in experimental allergic neuritis is unknown.

The present study demonstrated 2 types of nerve oedema: endoneurial oedema, most conspicuous in the perivascular spaces and the subperineurial space, and intramyelinic oedema in ischaemic/reperfused nerve. Vasogenic oedema occurs most commonly as the result of a breakdown of the blood-nerve barrier function which is formed by the capillary endothelium and by the perineurium. In contrast, cytotoxic oedema caused by some lipid-soluble neurotoxins tends to be restricted to the myelin sheath, producing intramyelinic oedema, without alteration of vascular and perineurial permeability. Morphologically, intramyelinic oedema in reperfusion nerve injury is similar to those in hexachlorophene neuropathy, i.e. oedema is found to arise as a result of splitting the myelin sheath between the intraperiod lines, and axonal degeneration is rarely seen at the level of nerve investigated (Towfighi et al. 1973). As we demonstrated both types of nerve oedema, it is likely that two different pathogenetic mechanisms are involved in the development of nerve oedema in ischaemic/reperfused nerves. Vessels, particularly microvessels and venules, in ischaemic/reperfused nerves showed swelling of endothelial cells and pericytes. These pathological abnormalities are similar to those seen in oxygen radical-induced damage in a variety of tissues. Endothelial swelling decreases the diameter of the vascular lumen and may thus cause increased resistance to blood flow when flow is restored. In addition, nerve oedema could cause reduced NBF and endoneurial hypoxia that have been identified in experimental models of oedematous neuropathy (Powell & Myers, 1989; McManis et al. 1993). These pathological alterations, vascular swelling and endoneurial oedema, may result in prolonged severe ischaemia ('no-reflow' phenomenon) in distal nerve segments below the knee level. Schmelzer et al (1989) reported that NBF was restored to only 45% of

resting values following 3 h ischaemia in rat sciatic nerve. We found, however, that the degree of NBF restoration with reperfusion after near-total ischaemia depends on the level along the length of sciatic nerves measured (Nukada, unpublished data).

Possible underlying mechanisms of reperfusion injury include the generation of free radical species, the release of chemoattractant mediators such as cytokines, and the activation of polymorphonuclear leucocytes (McCord, 1985; Zweier et al. 1988; Menger et al. 1991; Welbourn et al. 1991; Freischlag & Hanna, 1992). Reoxygenation stimulates the simultaneous production and release of nitric oxide and superoxide by ischaemia-damaged endothelial cells (Rosen & Freeman, 1984; Beckman, 1990; Beckman et al. 1990). Nitric oxide is synthesised enzymatically from the amino acid L-arginine (Palmer et al. 1988), and the L-arginine-nitric oxide pathway is causally involved in myocardial reperfusion injury in the hypoxic piglet (Zweier et al. 1987; Matheis et al. 1992). Neutrophils have also been identified as a mediator of lethal cell injury resulting from reperfusion (Hernandez et al. 1987; Weiss, 1989; Jaeschke et al. 1990; Welbourn et al. 1991; Freischlag & Hanna, 1992). Reactive oxygen species may be generated by activated neutrophils. Leucocyte adherence to injured endothelium seen in the present study has been shown in ischaemia/reperfusion injury (Granger et al. 1989; Engler et al. 1993).

The most conspicuous feature of myelin composition, relative to other cell-surface or intercellular membranes, is its high proportion of lipids. Isolated myelin from peripheral nerves contains 70–85% lipids and 15–30% protein (Norton, 1981; Yao, 1984). The myelin sheath contains a greater variety of bipolar lipids, double bonds of unsaturated fatty acid chains. Because polyunsaturated fatty acids are the primary target for activated free radicals, myelin may be particularly susceptible to the effects of lipid peroxidation in reperfused nerve. Early endothelial nitric oxide and superoxide generation may be of special pathophysiological importance since a variety of pathological cascades could be readily initiated by such endothelial activation. Thus endothelial production of free radicals may be a key triggering mechanism for the initiation of perivascular demyelination and nerve oedema, especially intramyelinic oedema, in ischaemic/reperfused nerve.

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