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Review*

Axonal regeneration through acellular muscle grafts

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ABSTRACT

The management of peripheral nerve injury remains a major clinical problem. Progress in this field will almost certainly depend upon manipulating the pathophysiological processes which are triggered by traumatic injuries. One of the most important determinants of functional outcome after the reconstruction of a transected peripheral nerve is the length of the gap between proximal and distal nerve stumps. Long defects (> 2 cm) must be bridged by a suitable conduit in order to support axonal regrowth. This review examines the cellular and acellular elements which facilitate axonal regrowth and the use of acellular muscle grafts in the repair of injuries in the peripheral nervous system.

Key words: Nerve injury; basal lamina; Schwann cells.

INTRODUCTION

Peripheral nerve damage is a relatively common consequence of trauma (when lesions often involve nerve roots or plexuses), tumour surgery or disease: its management remains a major challenge for the clinical team. In this brief review 2 different interfaces will be examined. One, the interface between regenerating axons and their environment, is familiar territory; the other interface, between neurobiologists and clinicians, is less commonly visited. Clinicians frequently question the validity of extrapolating from the laboratory to the clinic, and remain sceptical that work on rat nerve can be applied to the treatment of nerve injuries in man. Certainly it is true that the great majority of experimental studies on nerve regeneration, whether morphological, biochemical or physiological, have been carried out on young rodents. Moreover, crushing or transecting a peripheral nerve in an anaesthetised rat produces a standardised lesion with minimal disturbance to surrounding tissues and a relatively short distal stump: under these circumstances, axonal regrowth (although not necessarily functional recovery) is frequently good. In contrast, nerve injuries are almost always seen in the trauma clinic some considerable time after their production, rarely involve a nerve in isolation, often produce distal stumps many centimetres long and may happen at any age. Since these factors (delay before surgery, additional soft tissue damage, length of distal stump, age) are recognised as adverse predictors of the outcome of nerve repair it is hardly surprising that functional recovery under these circumstances is frequently unsatisfactory (Birch & Raji, 1991; see also Fu & Gordon, 1995*a*, *b*). However, it would be short-sighted to dismiss experimental models as irrelevant in the clinical context. The injury response within the mammalian peripheral nervous system appears to be a highly conserved phenomenon: there is, for example, no reason to think that human and rodent Schwann cells would respond in fundamentally different ways to acute axotomy (e.g. expression of the low affinity neurotrophin receptor p75 is upregulated in axotomised chick, mouse, rat and human Schwann cells; Sobue et al. 1988; Raivich & Kreutzberg, 1994). Moreover, it has recently been reported that purified cultured human Schwann cells survive and enhance axonal regeneration when grafted into interstump gaps in the peripheral nerves of immunodeficient rats (Levi et al. 1994). It is therefore reasonable to assume

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that experimental studies can usefully inform the design of new approaches to the clinical repair of nerve injuries, particularly where these involve the manipulation of the participating cells, the use of bioartificial materials as grafts, etc.

WALLERIAN DEGENERATION—A RESUME

When a peripheral nerve is transected or crushed, the injury initiates molecular and cellular changes within every part of the nerve, from the centrally located cell bodies (McAllister & Calder, 1995) to the periphery. A cascade of cellular responses collectively termed wallerian degeneration occurs at the extreme distal tip of the proximal stump and throughout the distal stump. Some of these stages would be constitutively expressed in any wound irrespective of location, whereas others reflect the unique problems associated with the repair of neurons, 'cells which are neither replaceable nor interchangeable' (de Medinaceli & Merle, 1991).

During the first 2 or 3 wk after injury, all axons and myelin sheaths within the distal stump disintegrate and the debris so generated is removed, predominantly by recruited myelomonocytic cells. Schwann cells divide and become aligned in cellular cordons within each persisting basal lamina tube, forming the bands of Büngner (Griffin et al. 1993). This burst of activity is matched by an exuberant cellular response within the proximal stump: the damaged axons sprout and Schwann cells and endoneurial fibroblasts proliferate. If physical continuity between the stumps has been retained, as would occur if the nerve has been crushed, the probability that axon sprouts will grow across the lesion site and into bands of Büngner is high (de Medinaceli & Merle, 1991). If, however, a gap exists between the stumps, regenerating axons have to negotiate unfamiliar territory before gaining the 'safe haven' of a band of Büngner. The probability that axons will grow successfully across a gap diminishes as the length of the interstump gap increases.

What happens in a gap?

The spatiotemporal sequence of cellular events which occur in an interstump gap has been exhaustively catalogued using an experimental paradigm developed from the old surgical technique of *entubulation*, in which the proximal and distal stumps of a transected peripheral nerve are inserted into the ends of a preformed tube ('regeneration chamber') and the contents of the tube subsequently analysed qualitatively or quantitatively. Numerous modifications of the basic model have been described: the tube may be cylindrical or Y-shaped; the material of which it is composed may be indiscriminately permeable, semipermeable or nonpermeable; the distal stump can be replaced with either nonneural or inert tissues; tubes can be preloaded with exogenous agents intended to enhance rates of axonal regeneration (see Hall, 1989 for references). Initially the chamber fills with wound fluid rich in neurotrophic molecules and matrix precursors. During the first week, the fluid is replaced by an acellular fibronectin positive, laminin negative, fibrinous matrix which is subsequently invaded by cells (perineurial cells, fibroblasts, Schwann cells and endothelial cells) growing out from both proximal and distal stumps to form a tissue cable in the centre of the tube. Axons are reported to be the last elements to enter the tubes, some days after the appearance of the nonneuronal cells.

The issue of how far axons will grow 'unaided' has been addressed in studies in which the interstump gap has been progressively lengthened. In rats, axons will grow approximately 5 mm into the lumen of a regeneration chamber in the absence of any 'target'; successful elongation beyond this distance requires the presence of a vital distal stump. Although axons can regenerate across longer interstump gaps in larger animals it is widely accepted that they will not grow more than a few centimetres in the absence of a facilitatory microenvironment. What imposes this apparent limit on axonal elongation is not known, but it may be a consequence of an inadequate supply of associated cells. Thus stump-derived cells may simply stop dividing, or their rate of division may slow to a point at which supply may cease to keep pace with demand, or they may lose their migratory phenotype.

The importance of the distal stump

The distal nerve stump plays 2 quite distinct roles in the repair process: it initially attracts regrowing axons and subsequently supports their elongation within persisting bands of Büngner. Forssman (1900) was probably the first to articulate the concept of neurotropism (chemotaxis). Cajal (1928) subsequently demonstrated that the distal stump of a transected nerve exerted a powerful attractant effect on axons growing out from a neighbouring proximal stump, even when the 2 stumps were placed out of alignment. Since the 1980s, numerous studies using Y-shaped tubes and various tissues as 'lures', have confirmed the hypothesis, and shown that a distal stump can attract axons over distances of $\sim$ 1 cm (e.g. Politis et al. 1982). It is assumed that axons respond to and

grow along a chemical gradient of tropic molecules secreted by cells within the stump. Recent work suggests that these factors are unlikely to include NGF (Doubleday & Robinson, 1995) and that they are not exclusively *neuro*tropic, since they may well act upon the Schwann cells and endoneurial fibroblasts which accompany the regenerating axons (Williams et al. 1993; Abernethy et al. 1994).

Some of the most compelling evidence demonstrating that the distal nerve stump plays an important role in nerve repair comes from studies of axonal regeneration in the CNS. The conventional wisdom that mammalian central axons are refractory to regeneration has been overturned in the face of numerous demonstrations that axons within CNS tracts will grow into and through implanted peripheral nerves (Richardson et al. 1980). Significantly, these axons cease growing as soon as they leave the transplanted endoneurium and re-enter CNS neuropil.

Which elements within a distal stump are most likely to facilitate axonal regrowth? Two candidates head the list: Schwann cells and their basal laminae.

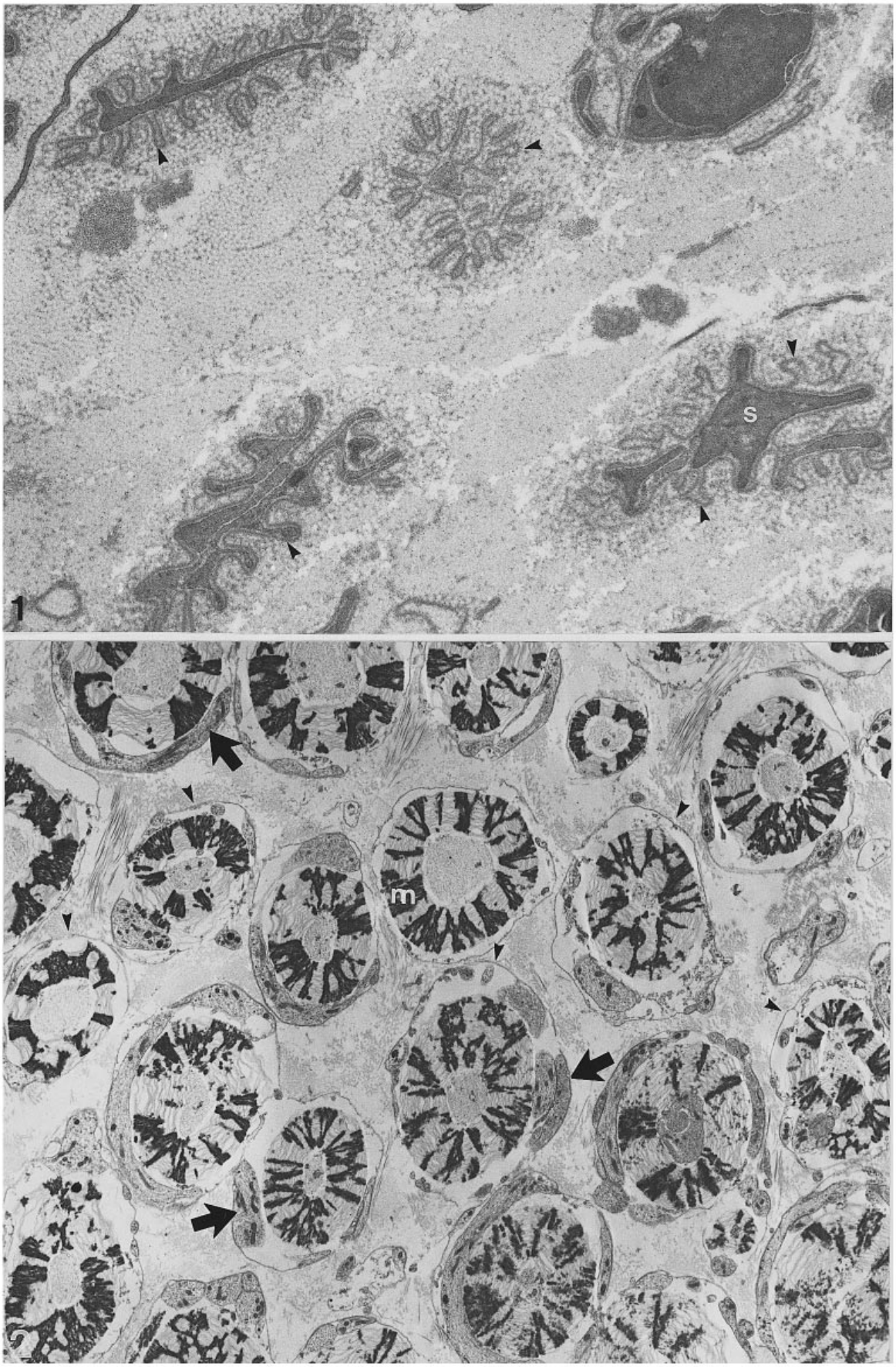
The role of the Schwann cell

'We may say that the cell of Schwann has multiple functions. Normally it protects and regulates the nutritive exchanges of the axon and myelin with their surroundings. Under pathological conditions it digests the remnants of the axons and myelin and it elaborates substances capable of stimulating the assimilation and amoeboidism of the nerve sprouts' (Cajal, 1928). Surprisingly little has changed in terms of our ideas about the basic functions of the Schwann cell since Cajal wrote these words, and few would question the pivotal role(s) that this cell plays in the response to injury. Indeed, the participation of appropriately responsive Schwann cells is central to the popular view that axonal regeneration in the PNS involves the recapitulation of (at least some) morphogenetic programs.

At 2–3 d after crush or transection, Schwann cells within the distal stump proliferate. The undifferentiated daughter cells remain within the original basal lamina tubes and, if deprived of axonal contact, soon stop dividing. A 2nd transient phase of proliferation occurs if axons penetrate the Schwann cell tubes (Pellegrino & Spencer, 1985). Phenotypically, axotomised Schwann cells resemble nonmyelinating cells: they downregulate expression of myelin protein genes (such as myelin-associated glycoprotein, myelin basic protein and P_0; Willison et al. 1988; Mitchell et al. 1990), whereas they upregulate gene expression of the low affinity neurotrophin receptor p75 (Heumann et al. 1986, 1987; Taniuchi et al. 1986); GAP-43 (Curtis et al. 1992; Scherer et al. 1994*a*): GFAP (Thomson et al. 1993); neurotrophins NGF (Matsuoka et al. 1991) and BDNF (Meyer et al. 1992; Korsching, 1993); the *neu* receptor c-erbB2 (Cohen et al. 1992; Hall, Li & Terenghi, unpublished observations); cell–cell adhesion molecules N-CAM and L1 (Martini & Schachner, 1988); cell-extracellular matrix molecules laminin (Kuecherer-Ehret et al. 1990); J1/tenascin (Martini et al. 1990; Martini, 1994) and fibronectin (Lefcort et al. 1992; Mathews & ffrench-Constant, 1995). Axon-derived signals, whether acting via direct contact or diffusible molecules, drive the expression of many Schwann cell genes during development (see Jessen et al. 1994 for references), and probably also during regeneration (Gupta et al. 1993). Much of this evidence is derived from tissue culture studies, and it is perhaps worth adding the caveat that 'axonal influence on Schwann cells may not be as clear in vivo as it is in vitro' (Siironen et al. 1994).

As functional Schwann cell–axon contact is established in the bands of Büngner, Schwann cells upregulate expression of $\beta 4$ integrin (Feltri et al. 1994); laminin B1 and B2 chains (Doyu et al. 1993); SCIP (Scherer et al. 1994*b*); L2/HNK-1 (but only in motor axon-associated Schwann cells, Martini et al. 1994); myelin-specific genes (Bolin & Shooter, 1993). Some genes which were upregulated after axotomy, e.g. the low affinity neurotrophin receptor p75 and the endopeptidase 24.11 (Kioussi et al. 1995) are downregulated following axonal contact. A series of elegant studies in vitro have demonstrated that axonal contact is usually necessary for Schwann cell extracellular matrix deposition (reviewed by Bunge, 1993): there is evidence that endoneurial fibroblasts also secrete factors which stimulate basal lamina deposition by Schwann cells in the absence of axons (Obremski et al. 1993, 1995).

Schwann cells promote regeneration of both CNS and PNS axons. Whether deliberately transplanted into lesions within the brain or spinal cord (Kromer & Cornbrooks, 1985; Montero-Menei et al. 1992; Neuberger et al. 1992; Bunge, 1994; Li & Raisman, 1994; Paino *et al*. 1994; Xu et al. 1994), or migrating 'naturally' through a temporarily compromised glia limitans after the induction of a demyelinating lesion such as that produced by the intraspinal injection of ethidium bromide (Yajima & Suzuki, 1979), Schwann cells will interact with central axons, often producing myelin sheaths and basal laminae which are apparently indistinguishable from their peripheral



Figs 1 and 2. For legend see opposite.

counterparts. Additional evidence that Schwann cells are powerful facilitators of axonal regeneration comes from studies on the mutant BW rat. In this animal axons in the proximal (retinal) 2 mm of the optic nerve are frequently associated with Schwann cells rather than oligodendrocytes (Berry et al. 1989). Transection of optic nerves in other rat strains produces transient but ultimately abortive sprouting of retinal ganglion cell axons at the lesion site, whereas when BW optic nerves are cut, those axons associated with Schwann cells regenerate until they reach the junctional zone with oligodendrocytes, at which point sprouting stops abruptly (Berry et al. 1992).

The role of basal lamina

Most basal laminae contain type IV collagen, an isoform of laminin, heparan sulphate proteoglycan, fibronectin and entactin/nidogen (Sanes, 1982; Eldridge et al. 1986; Timpl & Dziadek, 1986; Bosman et al. 1989; Timpl, 1989; Horwitz, 1991). In the context of axonal regeneration, a number of these molecules, notably laminin, fibronectin and heparan sulphate are known to promote axonal elongation in vitro and/or in vivo (Sandrock & Matthew, 1987; Giftochristos & David, 1988; Rogers et al. 1988; Tomaselli & Reichardt, 1988; Toyota et al. 1990; Wang et al. 1992*a*, *b*; Bryan et al. 1993; Kauppila et al. 1993). In a wider biological context, laminin/cell binding in vitro has been shown to affect phenomena as diverse as cell migration, spreading, division and the maintenance of the differentiated phenotype (Timpl & Brown, 1994).

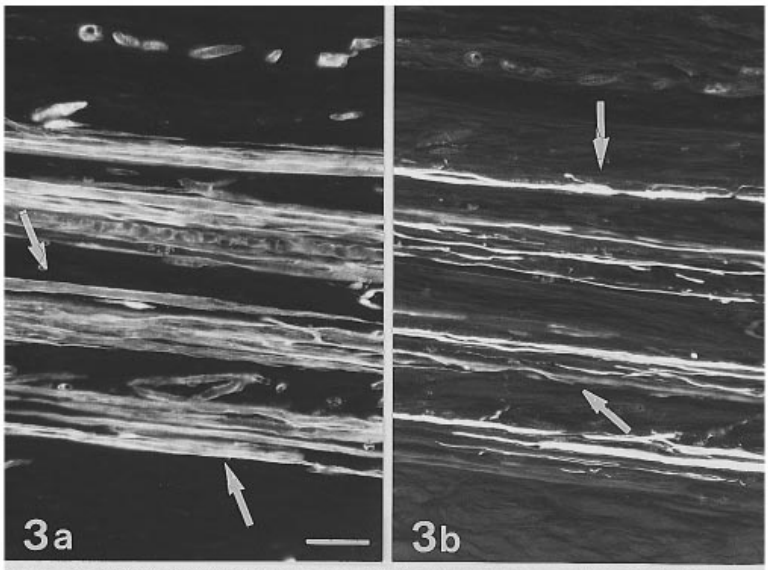
Schwann cell-derived basal laminae *appear* to be remarkably durable structures. They survive penetration by invading macrophages during the acute stages of wallerian degeneration or primary demyelination, and persist in chronically denervated distal stumps, where they surround the shrunken stacks of Schwann cell cytoplasm which constitute the surviving bands of Büngner (Fig. 1). Presumably the basal laminae undergo extensive remodelling with time, because the redundant loops of basal lamina associated with freshly denervated bands of Büngner are absent from the chronically denervated endoneurium: the mechanism underlying this modification has not been identified, but could reflect the activity of Schwann cell-derived tissue plasminogen activators (Clark et al. 1991). When axons grow into bands of Büngner they invariably do so in association with the inner aspect of the Schwann cell, i.e. the surface that is directed away from the basal lamina. Whether growth cones initially 'see' the inner aspect of the basal lamina, before they encounter the Schwann cell plasmalemma, is not known. For certain, both axons and Schwann cells grow preferentially along the inner aspect of the basal lamina rather than in association with its outer, extracellular, face. In a series of experiments which demonstrate this point unambiguously, we explanted embryonic rat dorsal root ganglia onto cryosections of normal or predegenerate rat sciatic nerve, and examined the subsequent pattern of neurite outgrowth: we found that GAP-43$^+$ neurites displayed an absolute predeliction for the inner surfaces of the laminin$^+$ basal laminae, irrespective of whether the sections had been prepared from normal or predegenerate nerve (Fig. 2) (Shewan, Cohen, Berry & Hall, unpublished observations).

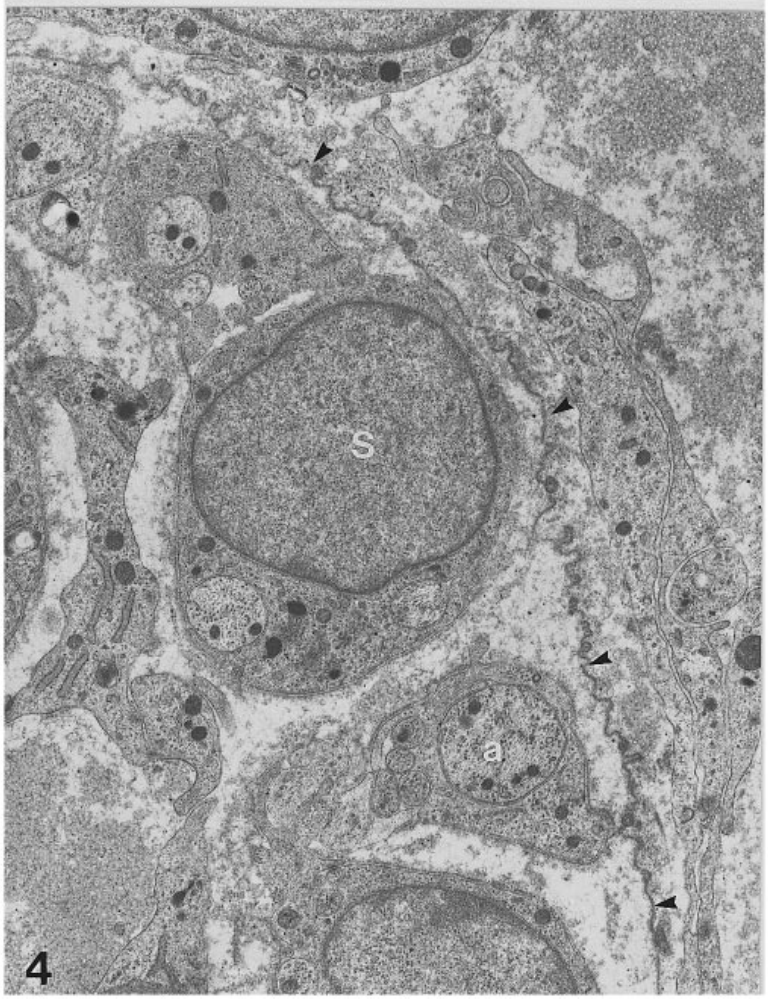
THE INTERSTUMP GAP

When a peripheral nerve is transected the proximal and distal stumps usually retract for a few millimetres. If, as is often the case clinically, it is also necessary to resect damaged nerve at the lesion site, either at the time of primary repair or at secondary exploration of a failed repair, a much longer interstump gap is inevitably produced. Under these circumstances, suturing the nerve ends together is no longer an option, indeed the tension this procedure induces at the suture line is recognised as a major reason for failure of nerve repair (Terzis et al. 1975). The preceding sections have documented the wealth of experimental evidence that the microenvironment of a peripheral nerve facilitates axonal regeneration. It

Fig. 1. Transverse section, chronically denervated distal stump, left median nerve. Specimen taken at the time of secondary repair, from a man who 9 mo earlier had sustained a lacerated wrist and transection of the median nerve. The endoneurium contains shrunken bands of Büngner, which consist of dense processes of Schwann cell cytoplasm (s) surrounded by convoluted basal laminae (arrowheads). ×7500 (Reproduced by kind permission of Dr Giorgio Terenghi, Blond McIndoe Centre, East Grinstead).

Fig. 2. Electron micrograph of a cryosection (7 μm) of normal adult rat sciatic nerve onto which embryonic rat dorsal root ganglia had been explanted 3 d earlier. Neurites (arrows) have extended from a dorsal root ganglion neuron onto the cryosection in vitro and have grown along the inner aspect of the Schwann cell-derived basal laminae (arrowheads). Immuno-electronmicroscopical staining of similar sections revealed that the neurites were GAP-43$^+$ and the basal laminae were laminin$^+$. m, myelin sheath. ×4000. (From unpublished work with Professor Martin Berry, Drs James Cohen and Derryck Shewan.)





Figs 3–8. All material has been prepared from muscle autografts (either frozen-thawed or heated to 60 °C prior to grafting) placed in 1 cm gaps in freshly transected adult rat sciatic nerve

Fig. 3. Longitudinal double-labelled immunostained section showing axonal regeneration within the midportion of a frozen-thawed muscle autograft 2 wk after grafting. Laminin$^+$ basal lamina tubes (*a*, arrows) contain RT97$^+$ axons (*b*, arrows). Polyester wax section; bar, 25 μm. (Reproduced from Enver & Hall, Neuropathology and Applied Neurobiology, 20, with permission.)

Fig. 4. Transverse section at the midpoint of a frozen-thawed muscle autograft, 2 wk after grafting. Schwann cells (s) and associated small unmyelinated axons (a) lie within a highly infolded sarcolemmal basal lamina tube (arrowheads). ×12000. (Reproduced from Hall and Enver, Journal of Hand Surgery 19B, with permission.)

therefore seems obvious to use another segment of nerve as a graft to bridge an interstump gap (Seddon, 1963). Sadly, the recovery obtained using cutaneous nerve autografts is often poor. There are many reasons for an unfavourable functional outcome, e.g. the length and/or diameter of expendable donor nerve may be insufficient, and the graft may become ischaemic or fibrosed. Moreover, harvesting a healthy functioning nerve to use as a graft is not without risk, and the patient may suffer sensory loss, scarring or even painful neuroma formation at the donor site. Identification of a graft material which can serve as a effective alternative to nerve therefore remains a priority in reconstructive surgery.

ACELLULAR MUSCLE GRAFTS—AN ALTERNATIVE TO NERVE GRAFTS

Striated muscle contains tubes of sarcolemmal basal laminae which persist even when the myocytes which lie within them have been destroyed chemically or thermally. Reports that these tubes supported axonal regeneration in the frog (Sanes et al. 1978) and mouse (Keynes et al. 1984) stimulated a series of further studies on the feasibility of using acellular muscle grafts instead of nerve grafts to repair defects in damaged peripheral nerves (Fawcett & Keynes, 1986; Glasby et al. 1986*a*, *b*; Feneley et al. 1991; Glasby, 1991).

Initially the method of denaturation was to freeze a piece of muscle in liquid nitrogen and then osmotically shock the tissue by immersing it in distilled water. Modifications of this basic protocol have included repeated episodes of freeze-thawing at -25 °C followed by immersion in distilled water (Enver & Hall, 1994); heating to 60 °C in distilled water (Hall & Enver, 1994); heating to 60 °C in a microwave source (Whitworth et al. 1995). (Technical note. Heat pretreatment was an experimental modification introduced with the specific aim of reducing the shrinkage and tissue handling problems that freezing induces: heating muscle in a water bath or in a microwave to 60 °C produces muscle grafts which exhibit minimal shrinkage, are easier to suture than their frozen-thawed counterparts and which still facilitate axonal regeneration).

All these pretreatments produce the same general changes, namely acute necrosis of myocytes, endothelial cells, intramuscular nerves and interstitial cells. The cellular debris thus generated is rapidly cleared by recruited macrophages. Evacuated, intensely laminin-positive, sarcolemmal basal lamina tubes are penetrated by Schwann cells, fibroblasts, perineurial cells and endothelial cells from both proximal and distal stumps and by axons from the proximal stumps (Figs 3, 4). Within 3 wk, the majority, if not all, sarcolemmal tubes contain minifascicles of regenerating axons and Schwann cells, each axon-Schwann cell unit being surrounded by a newly formed basal lamina (Figs 5–7). Individual minifascicles are encircled by 1 or 2 layers of perineurial-like cells, which are also enclosed in basal laminae. The grafted sarcolemmal basal laminae fragment and ultimately disappear (Fig. 6*a*).

A month after grafting, axons will have traversed a 1 cm graft and entered the distal stump. Comparisons of axonal regeneration through short acellular muscle grafts or nerve autografts of equal diameter in the rat (Glasby et al. 1986*c*) and marmoset (Glasby et al. 1986*b*) have shown that there is no difference between the types of graft in terms of either the numbers or maturity of axons which penetrated the distal stumps.

HOW DO MUSCLE GRAFTS WORK?

Internally a coaxially aligned acellular muscle graft consists of a bundle of empty cylinders which apparently offer little or no resistance to ingrowing cells. More importantly, the walls of the tubes contain molecules such as laminin and fibronectin which possess domains known to support the outgrowth of neurites and neural crest cells in vitro (Carbonetto, 1984; Humphries et al. 1988; Perris et al. 1989; de Curtis, 1991). Studies using experimentally manipulated grafts have provided circumstantial evidence that these molecules are equally important in vivo. Thus, axons do not regenerate through grafts which have been pretreated by heating to 80 °C, a temperature at which laminin no longer supports neurite outgrowth in vitro (Goodman et al. 1991) and at which the distribution of immunostaining for laminin along the sarcolemmal basal laminae as assessed electronmicroscopically is reduced to $\sim 30\%$ of normal (Hall & Enver, 1994; Kent & Hall, 1995; Hall & Kent, 1996). (Interestingly, acellular *nerve* grafts, which contain coaxially aligned bundles of empty Schwann cell basal laminae, fail to support axonal regeneration if they are first soaked in antilaminin or antifibronectin antisera (Wang et al. 1992*a*, *b*).)

Confocal microscopical and ultrastructural analyses of the early stages of innervation of acellular muscle grafts reveal that axon sprouts and fine cytoplasmic processes of nonneuronal cells appear to palpate the inner surface of the transplanted basal laminae (Hall & Kent, unpublished observations). Presumably the tubes offer a facilitatory substrate for

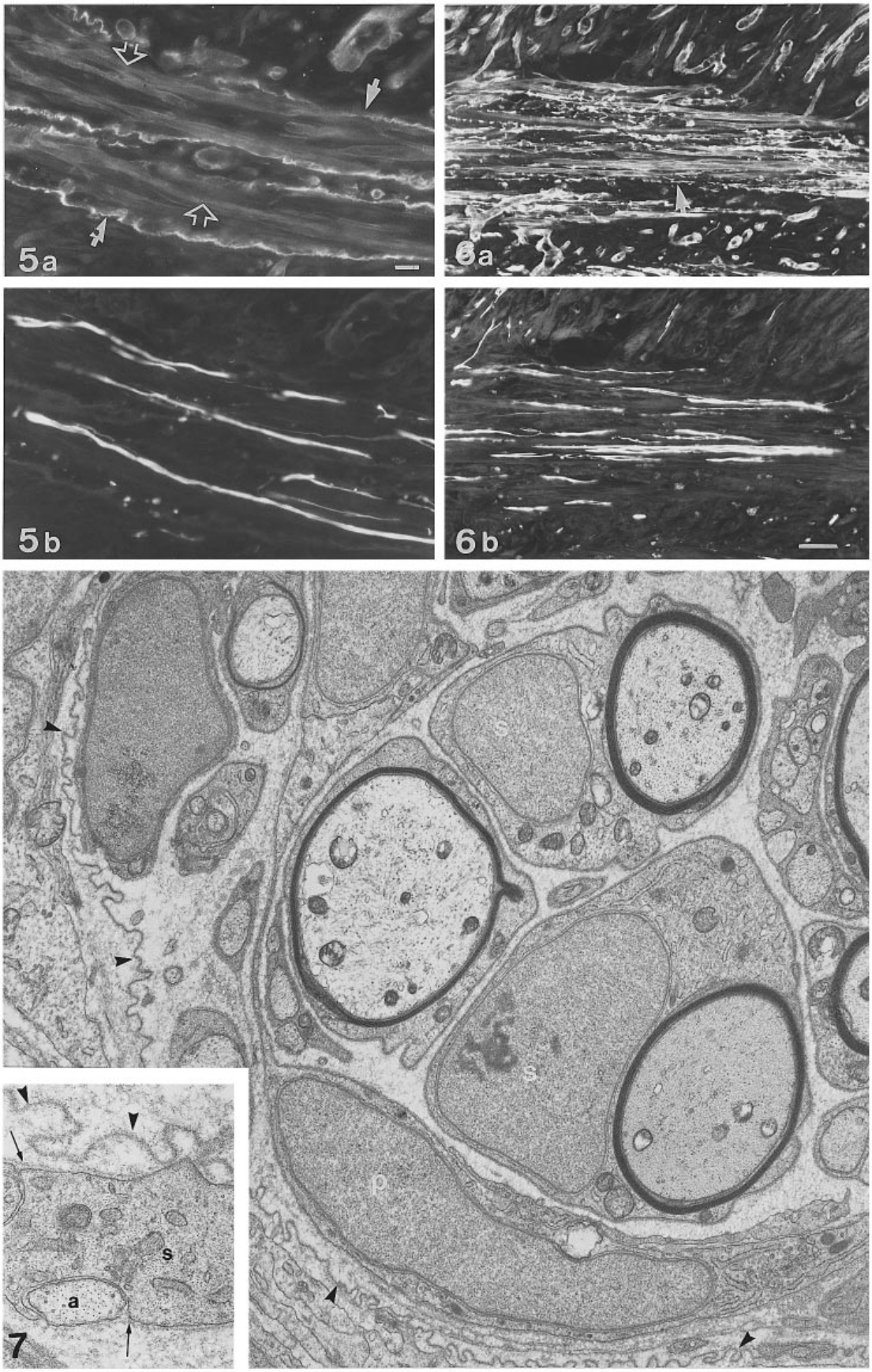


Fig. 5. Longitudinal section of a frozen-thawed muscle autograft, 3 wk after grafting. Section has been double stained with antilaminin antibodies (*a*) and RT97 to demonstrate regenerating axons (*b*). Laminin$^+$ cells (open arrows, *a*) lie inside the tubes of laminin$^+$ sarcolemmal

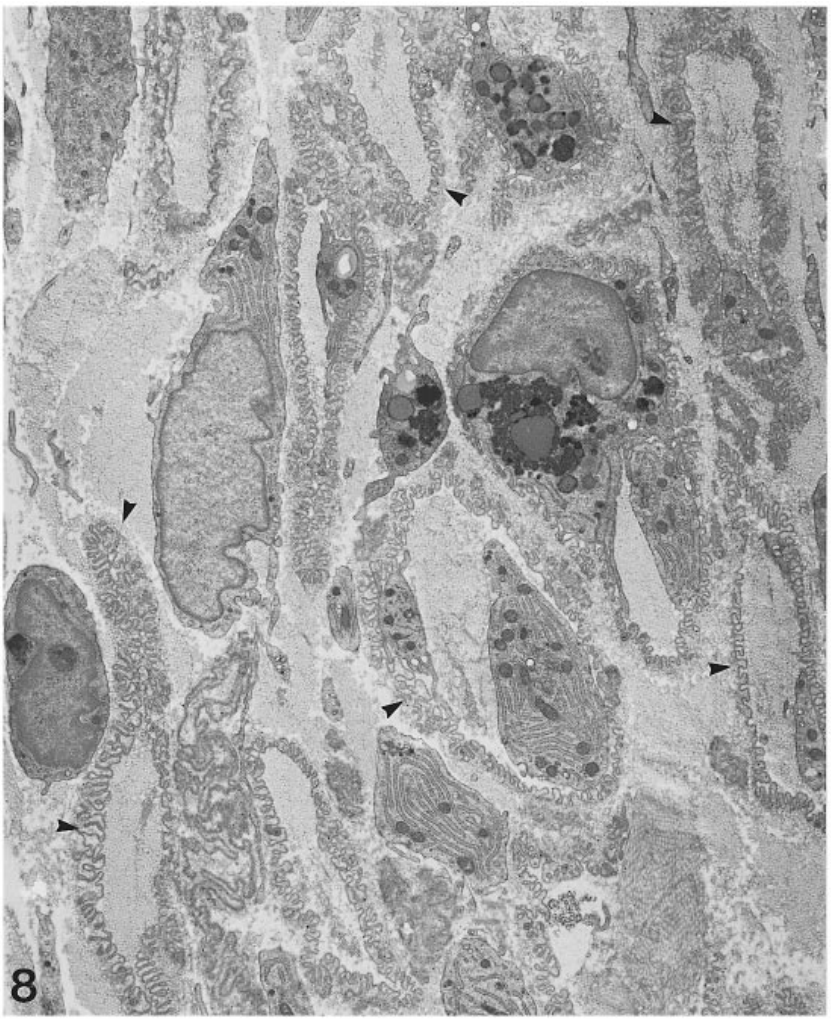


Fig. 8. Representative field of a transverse section from the midpoint of a frozen-thawed muscle autograft sutured to a mitomycin C-pretreated proximal nerve stump, 2 wk after grafting. Sarcolemmal basal lamina tubes (arrowheads) are either empty or contain only macrophages. The field from which this micrograph was selected contained 32 basal lamina tubes, none of which contained axons. ×6000.

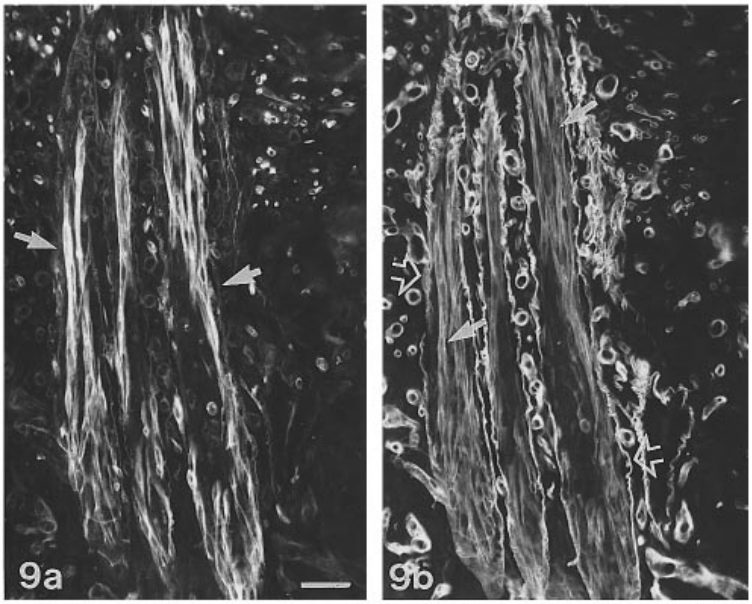
the advancing front of axonal growth cones and the filopodia of comigrating cells, but not for maturing axon-Schwann cell units which are rapidly sequestered from graft basal laminae by Schwann cell-derived basal laminae.

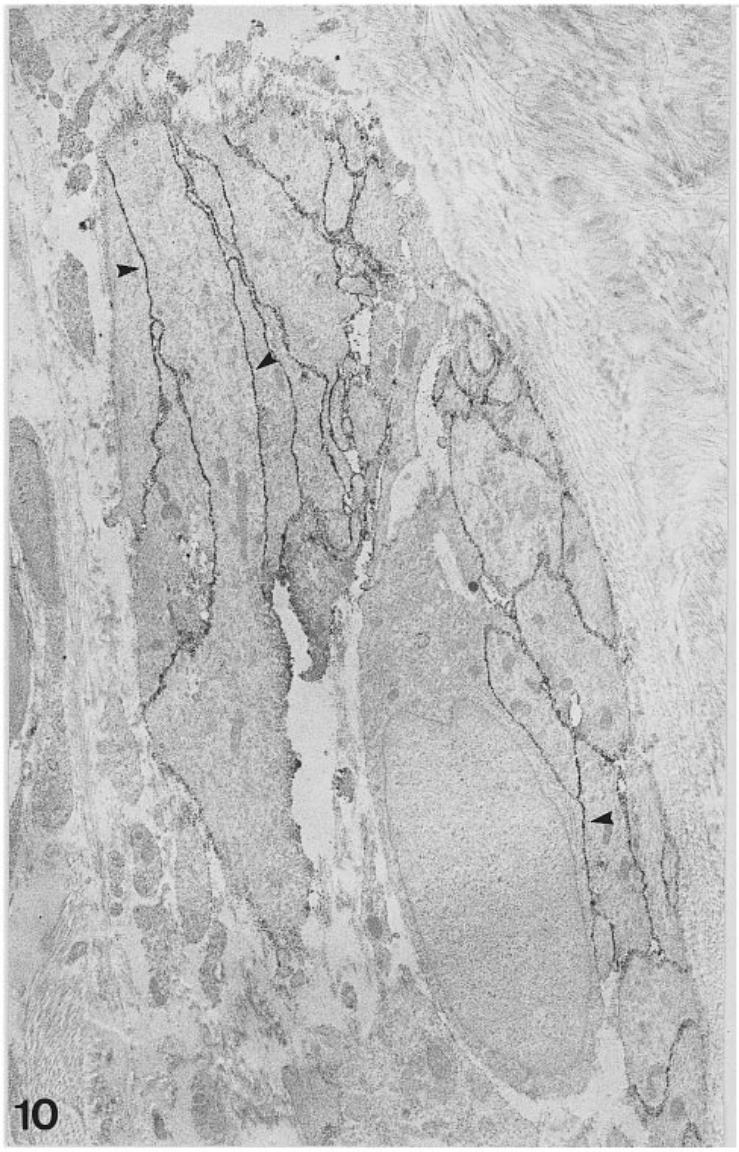
Axons growing into acellular muscle grafts are accompanied by cells which have been generated within the nerve stumps. It is reasonable to ask whether these cells are essential for axonal regeneration, especially when the graft supplies an abundance of surfaces rich in molecules capable of promoting axonal elongation. The number of cells available to precede and/or migrate with the axons into the grafts can be reduced by depressing post-

basal laminae (solid arrows). In adjacent sections, these cells were found to be S-100$^+$, i.e. they were Schwann cells. Additional laminin$^+$ profiles external to the sarcolemmal tubes are endothelial basal laminae. Polyester wax section; bar, 10 μm

Fig. 6. Longitudinal section of a muscle autograft pretreated by heating to 60 °C for 15 min before grafting and 3 wk after grafting. Section has been double stained with antilaminin antibodies (*a*) and RT97 (*b*). Sarcolemmal basal laminae (solid arrow, *a*) are fragmenting, whereas the Schwann cell-derived basal laminae associated with regenerating axons are continuous. Additional laminin$^+$ profiles external to the sarcolemmal tubes are endothelial basal laminae. Polyester wax section; bar, 5 μm. (Reproduced from Hall & Enver, Journal of Hand Surgery, 19B, with permission.)

Fig. 7. Transverse section at the midpoint of a frozen-thawed muscle autograft, 2 wk after grafting, showing a greater level of cellular organisation than is demonstrated in Figure 4. Some regenerating axons are already thinly myelinated. Most of the Schwann cells (s) and axons within this field are separated from the sarcolemmal basal lamina tube (arrowheads) by perineurial-like cells (p). ×8000 Inset: a small axon (*a*) lies on the surface of a process of Schwann cell cytoplasm (s) close to the inner aspect of a sarcolemmal basal lamina tube (arrowheads). The axon-Schwann cell unit is covered by Schwann cell-derived basal lamina (arrows). ×18000. (Reproduced from Enver & Hall, Neuropathology and Applied Neurobiology, 20, with permission.)





Figs 9 and 10. For legend see opposite.

traumatic cell proliferation in the stumps using an antimitotic agent such as mitomycin C (Hall & Gregson, 1977; Pellegrino & Spencer, 1985). Under these circumstances, nonneuronal cells do not emerge from the nerve stumps for several weeks, axons do not grow into the grafts during this time and consequently the basal lamina tubes remain empty (Fig. 8) (Hall, 1986; Enver & Hall, 1994).

Since approximately 95% of the minifascicles grow inside the basal lamina tubes, rather than in the connective tissue surrounding them, the sarcolemmal basal laminae clearly provide a preferential route between the nerve stumps. However, it is equally clear that axonal regrowth through short (< 2 cm) acellular muscle grafts also requires a sustained supply of Schwann cells, perineurial cells and fibroblasts.

LONG GAPS

The results obtained using acellular muscle grafts to repair transected nerves in rats were sufficiently encouraging to be translated into clinical trials (Norris et al. 1988). The grafts proved effective in the repair of small defects in digital nerves, but not in the repair of gaps > 5 cm in mixed peripheral nerves (Calder & Norris, 1993), a finding which is consistent with a report that 10 cm muscle grafts in the rabbit are totally ineffective (Hems & Glasby, 1991).

There is no a priori reason to believe that very long acellular grafts should support axonal regeneration, indeed the available experimental evidence suggests that these grafts are unlikely to succeed simply because of their length. Once axons have negotiated the proximal suture line, their subsequent growth appears to be critically dependent upon the length of the interstump gap (see above), almost certainly, an adequate supply of comigrating cells (Anderson et al. 1991), and the vascularity of the graft are also likely to be significant determinants of success. Even if species differences are taken into consideration, the general consensus is that there are limits to the distance that axons will grow in the absence of a distal stump or segment of vital nerve. Most studies have found that this distance is of the order of 5 cm even in the largest animals and probably < 2 cm in rodents. Since there appears to be nothing within *denatured*, as opposed to *denervated* muscle (Weis & Schröder, 1989), which can act as a surrogate distal stump, it follows that axons are unlikely to grow all the way through such a graft.

One way of increasing graft length is to adopt a 'mixed economy' tactic, and to use 'sandwich' or 'stepping-stone' grafts, in which a small segment of vital nerve is placed inside a long acellular conduit in the hope that the nerve will provide a depot of nonneuronal cells and/or a source of neurotrophic and neurotropic factors for ingrowing axons. Autogenous veins containing segments of nerve have been used experimentally to bridge defects of 1.4 cm in rat femoral nerve (Smahel & Jentsch, 1986) and clinically to reconstruct defects $> 2 < 5$ cm in digital nerves (Tang et al. 1993). In a recent development of this protocol, nerve has been combined with acellular muscle: axonal regeneration is reported to be enhanced across 1.5 cm sandwich grafts of muscle and nerve when compared with that across freeze-thawed muscle grafts (Calder & Green, 1995). The latter experimental model is an interesting one, not least because it presents an opportunity (1) to compare the axon-associated cellular outgrowth from the proximal nerve stump with the outgrowths from the distal nerve stump and from the 2 ends of the nerve graft; and (2) to examine the interaction of the various stump-derived cells with each other and with the sarcolemmal basal lamina $\pm$ axons.

We have recently completed a detailed ultrastructural and immunohistochemical study of axonal regrowth through 1–2 cm nerve/muscle sandwich grafts in the rat (Hall & Kent, unpublished). Within 2 wk of grafting, the basal lamina tubes within a 0.5 cm distal muscle graft contain cells derived from both the distal stump and the nerve graft. In the succeeding weeks, pioneering axon sprouts therefore meet the ideal substrate over which they can continue to grow, namely basal lamina tubes already seeded with stump-derived cells. Many of these cells are immunopositive for the low affinity neurotrophin receptor, p75, and are also laminin$^+$ and S-100$^+$, i.e., they are Schwann cells (Figs 9, 10). Schwann cells migrating from the ends of the nerve graft and the distal stump are intensely p75$^+$, whereas those growing

Figures 9 and 10 are from the distal frozen-thawed muscle graft in 1.5 cm sandwich grafts, 2 wk after grafting. In a sandwich graft, the grafted tissues are arranged in the following sequence: proximal muscle graft $\rightarrow$ nerve graft $\rightarrow$ distal muscle graft. Fig. 9. Longitudinal section, 2 wk after grafting. The section has been double stained with antibodies against the low affinity neurotrophin receptor p75 (*a*) and laminin (*b*). Note that the p75$^+$ cells (arrows, *a*) are also laminin$^+$ (solid arrows, *b*) and that they lie within laminin$^+$ sarcolemmal basal laminae (open arrows, *b*). Adjacent immunostained sections revealed that the cells were S-100$^+$, and that there were no regenerating axons within the muscle graft. Bar, 5 μm.

Fig. 10. Electron micrograph (uncontrasted), pre-embedding immunolabelling technique, anti-p75 antibody. Adjacent surfaces of a bundle of Schwann cells are immunostained (arrowheads). The bundle is surrounded by a basal lamina. $\times 6000$.

out from the proximal nerve stump in association with regenerating axons are weakly p75^{+}. This finding is in agreement with a recent report that actively migrating, axon-independent, Schwann cells stain intensely for p75 (Madison & Archibald, 1994), and with the suggestion that p75 may be one of the candidate molecules which control Schwann cell migration (Anton et al. 1994). Moreover, in agreement with Madison & Archibald (1994) we found that p75 immunostaining was exhibited by newly generated Schwann cells, so its expression in these cells could not have been related to loss of previous axonal contact.

ALTERNATIVE STRATEGIES

The sandwich graft is an additional experimental model in which to study the cellular and molecular events which occur during axonal regeneration. However, although the concept is based on sound theoretical principles, its implementation may not appear so attractive from a clinical perspective. Sandwich grafts take longer to prepare than either nerve grafts or frozen-thawed muscle grafts, (and are thus likely to add complications to what may already be complex surgery) and, perhaps more importantly, they also contain multiple suture lines, each of which is an additional obstacle to axonal growth.

There are alternative strategies. One eschews 'natural' conduits in favour of bioartificial materials which can be manipulated prior to grafting, e.g. fibronectin mats preloaded with neurotrophic factors (Whitworth et al. 1995) or porous hydrophilic sponges infiltrated with Schwann cells (Plant et al. 1995). The other approach comes full circle back to the nerve graft, but this time using *allo*grafts not *auto*grafts. A peripheral nerve allograft is regarded as a 'nonessential' tissue transplant. Such a graft therefore only becomes an attractive prospect if its antigenicity can be reduced so that long term systemic immunosuppression is unnecessary. A recent report suggests that this may be possible (Strasberg et al. 1996).

CONCLUSION

Bridging the gap between the 2 ends of a transected nerve remains an important clinical problem. However, it is a sobering thought that it is but one of the problems that must be overcome if the functional outcome of nerve repair is to improve. Closer collaboration between basic scientists and clinicians is essential if significant progress is to be made in this field.

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