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TISSUE CULTURE OBSERVATIONS RELEVANT TO THE STUDY OF AXON–SCHWANN CELL INTERACTIONS DURING PERIPHERAL NERVE DEVELOPMENT AND REPAIR

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SUMMARY

During peripheral nerve development the Schwann cell population is expanded so that adequate numbers are available for ensheathment of both nonmyelinated and myelinated nerve fibres. As ensheathment of these fibres progresses each axon–Schwann cell unit becomes surrounded by a basal lamina, providing a unique microtubular framework within the peripheral nerve trunk. Tissue culture studies of pure populations of neurones and Schwann cells cultured separately and in combination indicate that a surface component on the axon provides a mitogenic signal to Schwann cells requiring cell–cell contact. Biochemical, electron microscopic and immunocytochemical analyses of these cultures indicate that Schwann cells in contact with axons are able to generate a basal lamina (containing type IV collagen, laminin and heparan sulphate proteoglycan) and fibrous collagen, without the aid of other cells, and that axonal contact is required for deposition of the basal lamina. The role of Schwann cells and the extracellular matrix they synthesize and organize, as well as the role of the other known products of the Schwann cells in the process of peripheral nerve regeneration, are discussed. It is suggested that the large numbers and advantageous position of the Schwann cells, as well as their ability to provide their own surfaces, a basal lamina and multiple secretory products, may account for their extraordinary ability to foster nerve fibre regeneration.

INTRODUCTION

At first sight the peripheral nerve trunk would not appear to provide an environment well suited for the regeneration of nerve fibres. About one-half of the substance of the trunk is connective tissue, including substantial amounts of fibrous collagen. These collagen fibres are located not only in the external sleeve of the epineurium (which has a dominant fibrous collagen component) and among the flattened cells of the perineurium (which surrounds each fascicle of nerve fibres) but

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throughout the innermost region, the endoneurium, containing individual nerve fibres (Thomas & Olsson, 1984). On closer examination, however, it is apparent that this connective tissue matrix is systematically penetrated by tubular spaces which house the individual axon–Schwann cell units. This tissue pattern is retained in portions of the nerve distal to a site of transection, and provides there multiple tubular channels which house the Schwann cells orphaned by degeneration of severed axons. These Schwann cells are thus retained in a linear cordon, positioned to assist the growth of regenerating axons. As regenerating axons enter the peripheral nerve trunk, each axon grows within these tubular spaces, between the innermost aspect of the surrounding connective tissue and the Schwann cell surface (Scherer & Easter, 1984).

It is the purpose of this paper to review some aspects of how the cells and tissues of the peripheral nerve trunk become organized during embryonic and perinatal development and the roles these components are known to play during peripheral nerve regeneration. Many of the pertinent aspects of Schwann cell biology have been studied in most detail in tissue culture preparations. Thus, observations in tissue culture will be emphasized.

FACTORS INFLUENCING SCHWANN CELL NUMBERS

In the trunk region of the developing embryo, Schwann cells and neurones of the peripheral nervous system are generated together from neural crest cells. The point at which the neurone–Schwann cell lineages diverge is unclear; it seems likely that this occurs early, as the ventralward migration of neural crest cells begins (Le Douarin, 1982). As sensory and motor axons grow into the peripheral body tissues the number of Schwann cells is increased so that adequate numbers are available to provide ensheathment for both unmyelinated and myelinated nerve fibres. When the number of nerve cells (and consequently the number of nerve fibres) in a region of the peripheral nervous system is experimentally reduced, the number of Schwann cells populating that region is correspondingly reduced (Aguayo *et al.* 1976). Experimental results from *in vivo* studies suggest that Schwann cell numbers are regulated by neuronal signalling. Detailed observations on how this regulation may be mediated are available from tissue culture studies. These studies, which offer new insights into how cells may exert influences by direct membrane–membrane contact, will be reviewed here in some detail.

Tissue culture preparations are now available in which sensory neurones and Schwann cells are established separately as pure populations and then recombined (for a review see M. Bunge *et al.* 1983). This technique allows the direct demonstration that axon contact influences Schwann cell proliferation, and, moreover, that direct contact between axon and Schwann cell is required to cause Schwann cell division. The requirement for axonal contact in this Schwann cell response has now been demonstrated in a number of culture systems (see Ratner,

Bunge & Glaser, 1986*a*, for a review). Evidence that the mitogenic agent is a component of the axon surface comes from observations that axons treated with trypsin lose mitogenic potency, and homogenized axonal preparations retain the activity in the particulate rather than the soluble fraction (Salzer & Bunge, 1980; Salzer, Bunge & Glaser, 1980*a*; Salzer, Williams, Glaser & Bunge, 1980*b*). Axolemmal preparations derived from central nervous system white matter are also mitogenic (Cassel, Wood, Bunge & Glaser, 1982; DeVries, Salzer & Bunge, 1982; Sobue, Krieder, Asbury & Pleasure, 1983).

Recent efforts to characterize the axonal component responsible for this mitogenic signal have employed antimetabolites with specific effects on glycoprotein and proteoglycan synthesis. The experiments are designed to determine if failure by the neurone to complete synthesis of these surface components would alter the axon's capability to deliver the mitogenic signal to Schwann cells. Use of castanospermine, which specifically blocks essential steps in the processing of asparagine-linked oligosaccharides, has allowed observations on the influence of modified glycoprotein synthesis on the ability of axons to stimulate Schwann cell proliferation (Ratner *et al.* 1986*b*). Whereas basal lamina production by Schwann cells (a subject discussed in detail below) and myelination were substantially disturbed in these cultures, Schwann cell proliferation was not. In contrast, β-D-xylosides, drugs which inhibit proteoglycan biosynthesis, dramatically inhibited Schwann cell proliferation in this culture system (Ratner, Bunge & Glaser, 1985; Ratner, Eldridge, Bunge & Glaser, 1986*c*). Further evidence that a proteoglycan may be involved in the mitogenic signal present on the surface of cultured axons came from observations that digestion of the neuronal cell surface with the glycosaminoglycan degrading enzyme heparitinase (but not chondroidinase ABC) modified mitogenic capacity (Ratner *et al.* 1985). Most recently, a purification procedure for axonal components designed to enrich for proteoglycans has achieved substantial purification (Ratner, Hong, Bunge & Glaser, 1986*d*).

It is not yet clear how an axonal membrane-associated proteoglycan might function in regulating Schwann cell proliferation. These distinctive proteins bear lengthy, unbranched glycosaminoglycan side chains attached to a core peptide *via* *–O–* linkages to serine (or threonine). Certain glycosaminoglycans are known in other cell systems to be involved in binding mitogenic proteins (such as fibroblast growth factor) (Lobb, Harper & Fett, 1986). Thus, it is possible that the axolemmal proteoglycan is not itself a mitogen but is instrumental in presenting (or acting in concert with) a second protein mitogenic for the Schwann cell.

Recently, evidence has been presented that Schwann cells may release substances into the culture medium which can be mitogenic for Schwann cells under certain circumstances (Porter, Glaser & Bunge, 1986; S. Porter, L. Glaser & R. P. Bunge, in preparation; Eldridge & Bunge, 1987). It is possible that Schwann cells release material capable of driving their own proliferation, but that they are not responsive to this agent unless it is presented to them by another cell type – in this case by a specific proteoglycan on the surface of the axon. Alternatively, the function of the

axolemmal proteoglycan may be to influence (increase) the Schwann cell responsiveness to growth factors. Similarities to the mechanism of proliferation response in other cell systems are worthy of note. Autocrine growth regulation has been described in other normal cell populations, such as in the action of interleukin-2 on activated lymphocytes (Smith, 1985). In this case, contact with specific antigen influences the response of lymphocytes to this autocrine agent. As in the neurone–Schwann cell system, proliferation appears to be mediated or regulated *via* cell–cell contact.

Certainly there is relevance in the study of these mechanisms, believed to be utilized during normal development, to the issue of neural repair. The extraordinary number of Schwann cells generated during early development ensures that each group of unmyelinated axons will be harboured within the cytoplasm of a series of ensheathing Schwann cells, and each myelinated fibre will be provided with a series of Schwann cells over its length (Fig. 1). Later, with body and axonal growth, the spacing of Schwann cells along the myelinating axons will increase (Webster & Favilla, 1984). Thus, while the peripheral nerve trunk is interlaced with copious connective tissue, as noted above, it also has a substantial stock of cellular components; these are retained and expanded after nerve injury.

The increase in Schwann cell numbers distal to the site of nerve transection has been well documented and apparently occurs by several mechanisms (see discussion by Spencer, Politis, Pellegrino & Weinberg, 1981). These authors have shown that in the phase of myelin breakdown during Wallerian degeneration, there occurs a first wave of Schwann cell proliferation; this can be shown, under appropriate experimental conditions, to be augmented by Schwann cell proliferation driven by renewed contact with regenerating axons entering the distal nerve stump. It seems reasonable to assume that this latter effect results from contact with the axolemmal mitogen discussed above. The former effect – Schwann cell proliferation during myelin breakdown – appears to be related to the degradation of myelin by Schwann cells (assisted by invading macrophages). A series of studies by DeVries and his colleagues (for review see Meador-Woodruff *et al.* 1985) has suggested cellular mechanisms for this Schwann cell proliferation which accompanies Wallerian degeneration in myelinated nerves. One consequence of this post-injury proliferation is that cells egress from the cut ends of the nerve stumps: Cajal (1928) has emphasized that this

Fig. 1. A sensory nerve fibre undergoing myelination branches as it crosses this light microscopic field. Four Schwann cells have spaced themselves along this fibre and three (*a, b, d*) are beginning the process of myelination. The forming compacted myelin segments are stained black with Sudan black. Each segment of myelin will be deposited by one Schwann cell; the nuclei of these Schwann cells (unstained in this preparation) are indicated. The Schwann cell at *c* will compete for the space between the branching point and the area occupied by the Schwann cell at *d*. It may form a short myelin segment near the branch point or be pushed off the axon as the cell at *d* extends its territory. As ensheathment progresses a basal lamina is deposited as a continuous tube along the axon–Schwann cell unit (see Fig. 2). In the background many Schwann cells are attending to the communal ensheathment of smaller nerve fibres which will not myelinate. Light micrograph of a neurone–Schwann cell culture free of other cell types. Scale bar, 10 μm.

expanding and migrating population contributes significantly to providing cellular bridges in the gap area. Williams *et al.* (1983) have undertaken a systematic study of the migratory capacity of Schwann cells (and other cellular constituents) into tubular encasements surrounding gaps of controlled length in rat peripheral nerves. Their study documents the substantial migratory capacity of Schwann cells after injury. Nerve fibre growth in their experimental system occurred only after migration of cells (including Schwann cells) to bridge the interposed gap.

PRODUCTION AND REGULATION OF EXTRACELLULAR MATRIX WITHIN PERIPHERAL NERVE TRUNKS

A second aspect of nerve development known to have crucial implications for subsequent nerve regeneration is the deposition of basal lamina along each axon–Schwann cell unit. The source of this basal lamina and the way in which its production is regulated have also been elucidated by observations in tissue culture. In this instance it was of particular interest to prepare cultures of Schwann cells alone and of Schwann cells co-cultured with neurones in the absence of fibroblasts, perineurial cells and endothelial cells. In this way the amount of extracellular matrix deposited as basal lamina and endoneurial collagen could be assessed when only neurones and Schwann cells were available for its production and organization (for review see Bunge & Bunge, 1981; McGarvey, Baron-Van Evercooren, Kleinman & Dubois-Dalcq, 1984; Eldridge *et al.* 1986). When sensory neurones and Schwann cells (free of fibroblasts) are established in co-culture on a reconstituted type I collagen substratum and provided over several weeks with appropriate medium, Schwann cell function can progress to axonal ensheathment (of the nerve fibres less than 1 μm in diameter) and myelination (of axons over 1 μm in diameter). Concomitant with this ensheathment, basal lamina is formed around each axon–Schwann cell unit and collagenous fibrils are deposited external to these basal lamina tubes (Fig. 2). The amount of fibrous collagen deposited between the axon–Schwann cell units is considerably augmented if fibroblasts are added to the culture. Neither an epinerium nor perineurial sheaths are formed in cultures containing only neurones and Schwann cells; perineurium is formed if fibroblasts have been added (Williams, Bunge & Wood, 1982).

These neurone–Schwann cell cultures can be established in a configuration which allows subsequent excision of the neurones; after axonal degeneration these cultures contain only Schwann cells and the extracellular matrix constructed during the previous association between neurone and Schwann cell. If neurones are excised from a neurone–Schwann cell culture after a substantial basal lamina has been deposited the basal lamina is retained, housing linear arrays of Schwann cells. This preparation provides an *in vitro* model of Wallerian degeneration. If the basal lamina in these cultures is removed by trypsin digestion the orphaned Schwann cells will not form a new basal lamina (Figs 3, 4). If neurones are added back to these cultures and their axons grow among and contact the Schwann cells, basal lamina will again be formed (M. Bunge, Williams & Wood, 1982). These observations on the requirement for axon contact for basal lamina deposition around the ensheathing Schwann cell correlate with observations made *in vivo* (Billings-Gagliardi, Webster & O'Connell, 1974). It seems reasonable to conclude from these observations that (1) axonal contact regulates the deposition of basal lamina by Schwann cells, (2) Schwann cells contacting axons are capable of forming basal lamina without the aid of fibroblasts, (3) fibroblasts and Schwann cells both contribute to the fibrous collagen present in the endoneurium, and (4) fibroblasts are required for formation of the cellular sheath

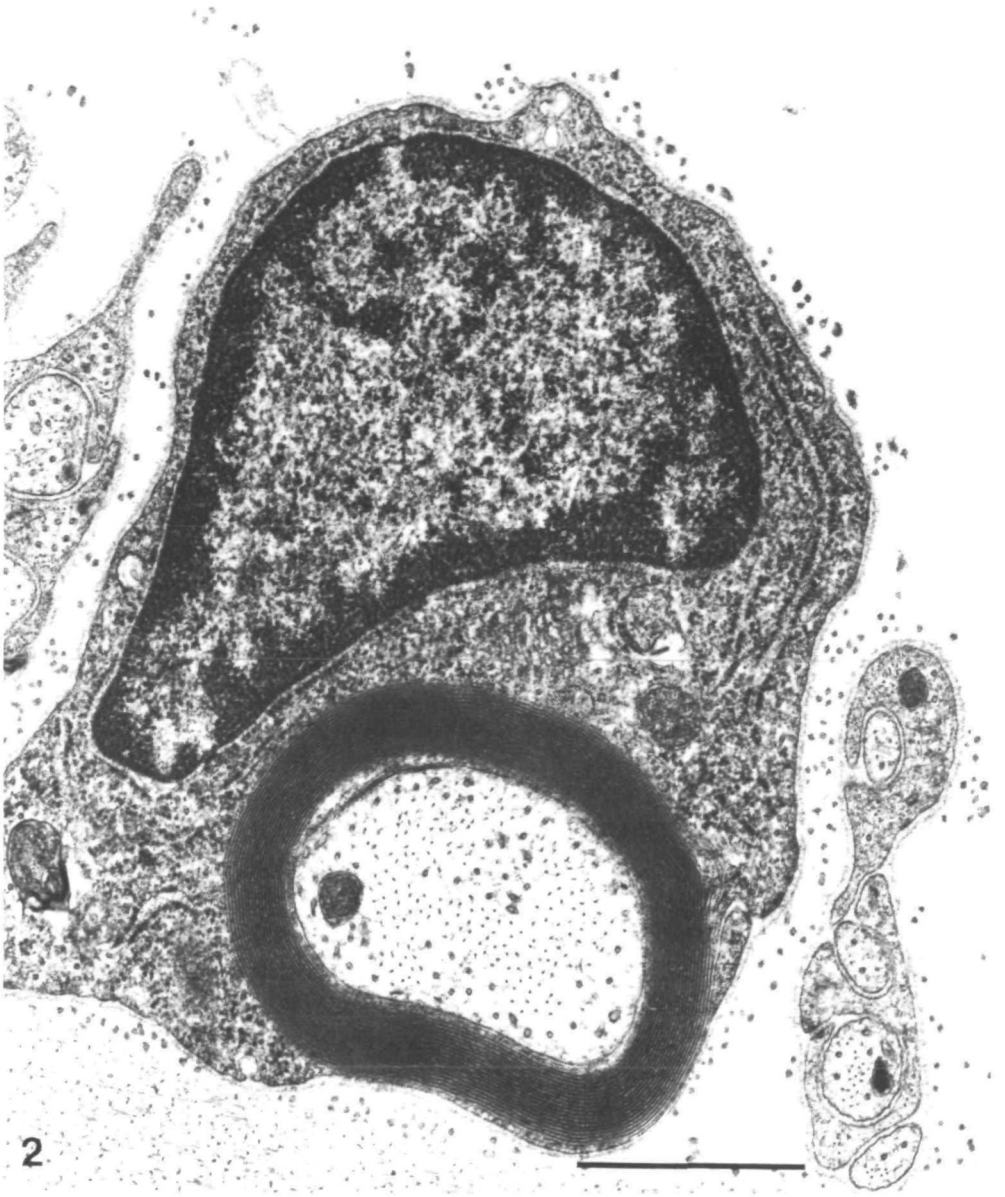
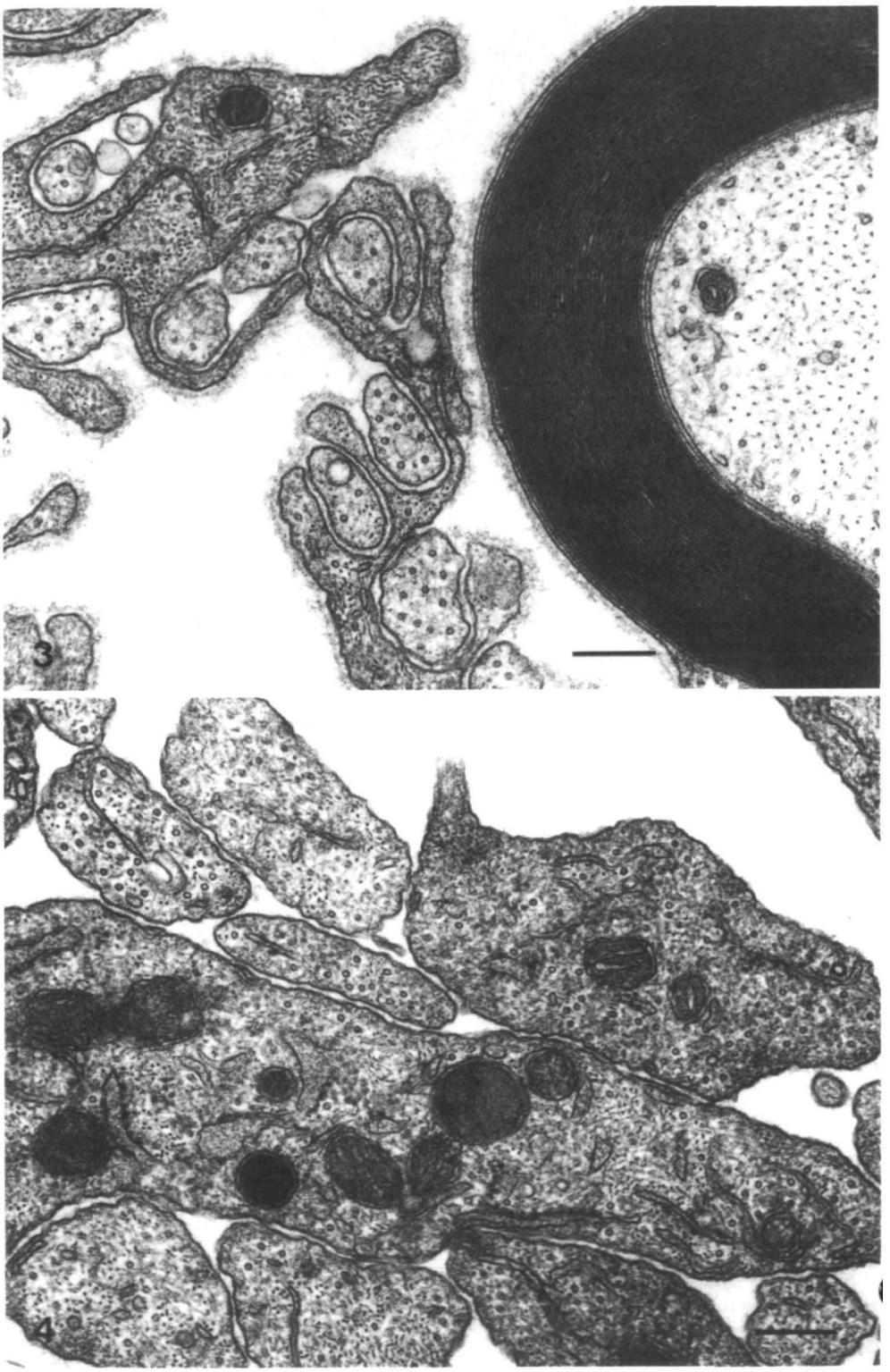


Fig. 2. An electron micrograph from a culture containing neurones and Schwann cells to illustrate the amount of extracellular matrix material deposited when only these cells contribute to its deposition. A complete surround of basal lamina encircles the myelinating axon–Schwann cell unit in the centre of the field. External to this basal lamina are cross-sections of collagen fibres. Adjacent Schwann cells surrounding much smaller nonmyelinated nerve fibres also have a complete basal lamina surround. The fibrous material in the lower left corner is the reconstituted rat tail collagen on which these tissue culture preparations were grown. (Electron micrograph provided by Dr M. Bunge.) Scale bar, 1 μm.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– the perineurium – that defines individual nerve fascicles within the peripheral nerve trunk.

Biochemical and immunocytochemical analyses of similar types of culture preparation have added to these morphological observations (for a review see M. Bunge *et al.* 1983; Eldridge *et al.* 1986). Radiolabelled peptides released into culture medium from Schwann cells have been analysed after separation on gels. These experiments established the substantial secretory capacity of Schwann cells. Among products released from Schwann cells in tissue culture are the major basal lamina components, type IV collagen, laminin, entactin and a proteoglycan. Also noted is the release of types I, III and V collagens, and several proteases (Clark & Bunge, 1986; Krystosek & Seeds, 1984). When Schwann cells influenced by neuronal contact were compared to orphaned Schwann cells it was observed that axonal contact increases type IV collagen release into the culture medium and its deposition in basal lamina (Carey *et al.* 1983). Whereas laminin production by Schwann cells proceeds in the presence or absence of neurones (Cornbrooks *et al.* 1983), much more laminin is found over the Schwann cell surface after axonal contact. It is now known, from studies with probes for mRNA for laminin in Schwann cells, that axonal contact dramatically increases the amount of laminin message in cultured Schwann cells (Dean, Milbrandt & Bunge, 1986).

Extracellular matrix deposition in the peripheral nerve trunk and the nature of its regulation have major implications for peripheral nerve regeneration. In a sense, in the development of peripheral nerve trunks, the system delays establishment of connective tissue components until axonal growth patterns are established and Schwann cell numbers are increased to provide the necessary capabilities for the carpentry of extracellular matrix materials. Then each axon–Schwann cell unit is provided with a tubular sleeve which will stay in place with its enclosed Schwann cells even if the axon (around which this organization originally centred) is lost, as in Wallerian degeneration. These Schwann-cell-filled, tubular channels are continuous to the target organs of the nerve fibres, and are available to guide regrowing axons towards these distal targets; this linear cellular construction is termed the band of Büngner.

There is no evidence that axon–Schwann cell units in these bands retain specific characteristics which allow, for example, regrowing motor axons to identify bands previously occupied by axons to a specific muscle (Scherer, 1986). This lack of available specificity in the bands of Büngner can be compensated by individual nerve fibres branching extensively at the site of nerve injury so that growing branches may enter several distal bands (for discussion see Scherer, 1986).

The foregoing provides an explanation of how the Schwann cell–basal lamina construction in the peripheral nerve becomes organized during development, and

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Figs 3, 4. Demonstration of the requirement for the presence of axons for basal lamina deposition by Schwann cells. In this experiment mature Schwann cell–neurone and Schwann cell only cultures (which had previously contained neurones) were trypsinized to remove basal lamina. One month later both types of culture were examined electron microscopically. Basal lamina was found to be present in Schwann cell–neurone cultures (Fig. 3) but not in Schwann cell only cultures (Fig. 4). Scale bars, 0·25 μm.

how it is retained after injury. There has recently developed a sometimes contentious discussion regarding the relative importance for nerve fibre regeneration of the basal lamina surface *vis-à-vis* the living Schwann cell. In a recent study Ide *et al.* (1983) used freezing and thawing to kill the cellular elements in the peripheral nerve stump but to retain the extracellular matrix materials. Nerve fibre growth occurred into these cell-depleted nerve segments, and these workers interpreted electron micrographs of the growth front as indicating that nerve fibre growth cones were progressing by direct contact with the internal aspect of the basal lamina of the bands of Büngner, growing ahead of a Schwann cell front which subsequently migrated into the nerve segments. These observations correlate with reports that purified extracellular matrix materials, particularly laminin, are very effective promoters of neurite growth when tested in tissue culture (e.g. Rogers *et al.* 1983; Manthorpe *et al.* 1983). There has been a recent tendency to ascribe to extracellular matrix materials much of the neurite growth-promoting properties of the peripheral nerve stump. For example, an antigenic site present on laminin–heparan sulphate proteoglycan complexes released from several cell types in culture and present in basal lamina in peripheral tissue regions known to promote axonal regeneration has been proposed as an important component in fostering neurite regeneration (Chiu, Matthew & Patterson, 1986).

Fortunately, this is now being balanced by a series of reports demonstrating the efficacy of cell surfaces as promoters of neurite growth. Fallon (1985*a*,*b*) has shown the efficacy of astrocyte and Schwann cell surfaces in promoting growth of certain neuronal types. More recently, Tomaselli, Reichardt & Bixby (1986) have compared neurite growth on extracellular matrix constituents with growth on neuroglial and muscle cell surfaces. Chick ciliary ganglion neurones provided neurite outgrowth on several types of extracellular matrices studied; in each case this substrate activity was blocked by monoclonal antibodies to the CSHT and JG22 receptors known to be involved in many forms of cell–matrix interactions. The surface of living cultured astrocytes, Schwann cells and myotubes was found to promote neurite growth by another mechanism, mediated through detergent-extractable macromolecules on the cell surface and interacting with uncharacterized receptors on neurones. It is thus becoming evident that a variety of influences on neurite growth are available to neurones, and that these include agents presented both by extracellular matrix components and by cell surfaces.

Very recently an additional cell surface component has been noted as a possible significant influence on regenerative neurite growth. It has been observed that section of the rat sciatic nerve induces Schwann cells distal to the lesion to express greatly increased levels of nerve growth factor receptors (Taniuchi, Clark & Johnson, 1986). Seven days post-transection, receptor density had increased 50-fold distal to the lesion and immunocytochemical examination revealed prominent (and exclusive) staining of Schwann cells distal (but not proximal) to the lesion. The authors suggest that this dramatic increase in nerve growth factor receptors on Schwann cells may be a mechanism to facilitate the regeneration of nerve growth factor-responsive neurones in injured peripheral nerve trunks.

To test directly the relative effectiveness of Schwann cells and basal lamina components in promoting neurite growth Ard, Bunge & Bunge (1987) established separate cultures of Schwann cells without extracellular matrix and cultures containing extracellular matrix deposited by Schwann cells (from which Schwann cells had been extracted). These studies showed that both Schwann cells alone (without organized extracellular matrix) and extracellular matrix organized by Schwann cells were effective promoters of neurite growth from sensory neurones challenged to grow under these culture conditions. These studies suggested that for sensory neurite growth both components of the band of Büngner – the Schwann cell surface and the basal lamina interface – were effective in prompting regenerative growth.

However, our most recent studies, utilizing neurite growth from embryonic rat retinal ganglion cells to test growth requirements for a central neurone in tissue culture, indicate substantially different requirements for this neuronal species (N. Kleitman, P. Wood, M. I. Johnson & R. B. Bunge, in preparation). When tested in cultures of the type described above, providing either Schwann cell surfaces or extracellular matrix materials deposited as basal lamina by Schwann cells, it was observed that neurites from retinal explants taken from 15-day-old rat embryos were extended on Schwann cell surfaces, but not on extracellular matrix components presented alone. For an alternative central neuronal type, the neurones of the olfactory bulb, Schwann cell extracellular matrix provided an effective promoter of neurite growth; individual extracellular matrix components, such as laminin, were less effective (Kleitman & Johnson, 1986).

SECRETORY ACTIVITIES IN INJURED PERIPHERAL NERVES

The discussion above has emphasized the agencies *in situ* within the injured nerve trunk which are available to aid neurite growth. There are several lines of evidence suggesting that injured nerves release components which may act at some distance from the injured tissue. This would seem the most reasonable explanation for the observation that isolated peripheral nerve segments have the capacity to attract the growth of axons regenerating from intact proximal nerve trunks (Politis, Ederle & Spencer, 1982). This type of attraction has also been observed in several tissue culture models (see, for example, Richardson & Ebendal, 1982). It is not clear which cell types within the peripheral nerve segment may be releasing components of interest. In one instance a protein, believed to be of Schwann cell origin (Skene & Shooter, 1983), was later found to originate from macrophages active in the myelin breakdown phase of Wallerian degeneration (Ignatius *et al.* 1986). Nonetheless, the manifest secretory capacity of Schwann cells (Carey *et al.* 1983) and their known capacity to release extracellular matrix components in soluble form into culture media (see, for example, Davis, Varon, Engvall & Manthorpe, 1985) make the Schwann cell a prime candidate as the source of these released agents. The ability of a distal nerve stump to attract growing neurites may be mediated by soluble extracellular matrix materials which are deposited as a gradient onto cellular and

extracellular constituents near the site of injury, thus providing a progressively more conducive substratum for neurite growth as the site of transection is approached.

It seems reasonable to conclude, from these experiments, that neurite growth is influenced by a variety of external factors and the growth requirements can be expected to differ among neuronal types. The particular potency of the peripheral nerve trunk in promoting neurite growth in nerve regeneration appears to be related to the fact that the composition of the distal nerve trunk provides a large number of retained Schwann cells which have a particular versatility in providing growth support *via* agents expressed on their surfaces, as well as agents released into their environment. Among these released agents are extracellular matrix components organized into basal lamina tubes. These not only provide retained pathways for growth, but also matrix components of demonstrable value in promoting growth of many types of nerve fibres. It seems entirely reasonable that the peripheral nerve trunk should serve as a suitable Rosetta stone in the effort to understand which trophic factors provide the requirements for each neurone's regenerative capacity.

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