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Regeneration of the perineurium across a surgically induced gap in a nerve encased in a plastic tube

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INTRODUCTION

In normal nerves the perineurium consists of layers of flat cells joined by zonulae occludentes, both surfaces of each layer being covered by a basal lamina (Thomas, 1963; Reale, Luciano & Spitznas, 1975). The nature of perineurial cells has been investigated using a variety of experimental models. Denny-Brown (1946) suggested that perineurial cells were of endothelial origin. Morris, Hudson & Weddell (1972) suggested that damaged perineurium regenerates from multipotential cells from which Schwann cells and endoneurial fibroblasts may also differentiate. Thomas & Bhagat (1978) repeated Denny-Brown's experiment and concluded that perineurial cells originate from cells which have 'morphological features of fibroblasts'. This conclusion is in keeping with the developmental studies of Gamble & Breathnach (1965) and confirms previous results by Thomas & Jones (1967).

The stages of regeneration of peripheral nerves have been studied recently using a new model in which the severed proximal and distal stumps of a nerve, separated by a gap, are enclosed within silicone chambers (Lundborg *et al.* 1982*a*, *b*), polyglactin (Molander *et al.* 1982) or semi-permeable acrylic tubes (Uzman & Villegas, 1983). After various intervals of time the gaps are bridged by regenerating axons and Schwann cells which eventually become arranged in bundles surrounded by elongated processes of perineurial cells.

The purpose of this paper, which describes the early stages of regeneration of cut sciatic nerves in mice, is to investigate the process of the formation and structural organisation of the perineurium in severed nerves enclosed within a tube.

MATERIALS AND METHODS

Balb/c mice of both sexes and from three to five months of age, weighing 22–25 g were anaesthetised by intraperitoneal injection of pentobarbitone. The skin and the muscles of the posterolateral aspect of the right thigh were incised longitudinally and the sciatic nerve exposed, mobilised and cut at the mid-point between the sciatic notch and the popliteal fossa. The two stumps were allowed to retract for two minutes and the gap was subsequently increased to 5 mm by trimming the ends of the stumps or by retracting them. A ribbon of plastic film was wrapped around the stumps creating a tube which contained a short segment of the severed nerve at each end (Fig. 1). Taking care to maintain the gap between the stumps, the muscles and the skin were sutured and the animals allowed to survive for varying lengths of time.

After 7, 12, 15, 20, 30 and 60 days operated animals were anaesthetised with ether

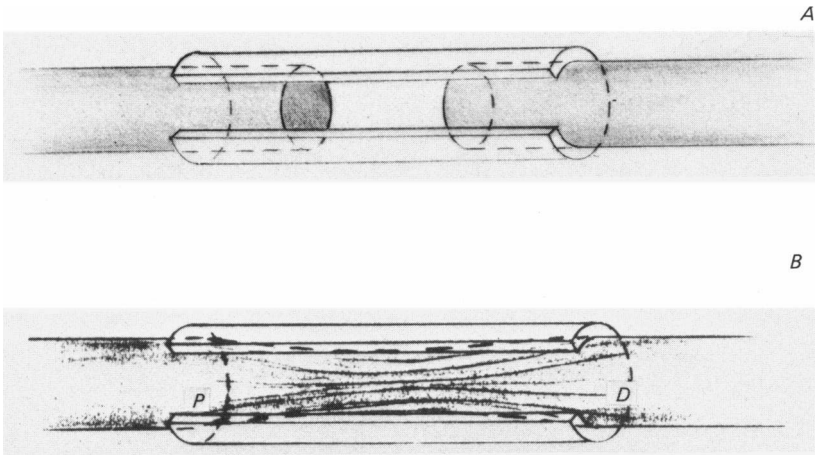

Fig. 1 (*A–B*). Illustrations showing the proximal (*P*) and distal (*D*) stumps of the sciatic nerve within the tube of plastic film immediately after the surgical procedure (*A*) and one month after (*B*) when the two stumps are joined by the regenerated nerve.

and perfused with Karnovsky's fluid (3 % glutaraldehyde and 1 % paraformaldehyde in 0·1 M sodium cacodylate buffer, pH 7·3). After removing the plastic film, fragments of the proximal and distal stumps and the newly regenerated segment between them were removed, post-fixed in osmium tetroxide, dehydrated and embedded in Araldite. Transverse sections, 1 μm thick, were stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate.

A total of 35 mice was used in this study, four in the 7 and 60 day group, six in the group at 30 days and seven in each of the other three groups.

RESULTS

Light microscopy

At *7 days* the tube of plastic film was filled with fluid material in which some macrophages and cells which were probably fibroblasts could be seen.

At *12 days*, the gap between the stumps was bridged by a narrow column of tissue. The segment near the proximal stump contained scattered remnants of myelin, capillaries and axonal sprouts of varying calibre arranged in small clusters, each cluster associated with a Schwann cell. Surrounding this bundle were layers of elongated cells. Distally, the tissue inside the tube was surrounded by more loosely arranged cells and consisted of capillaries and isolated cells with elongated processes which formed a meshwork separating apparently empty spaces.

The appearances at *15 days* were similar to those at 12 days with the addition of occasional myelinated axons in the proximal segment.

At *20 days* myelinated fibres were seen at all levels of the tissue between the stumps. This is now referred to as regenerated nerve. Fibres also appeared in the distal stump.

After *30 days* the process of myelination within the regenerated nerve was well advanced and appeared to be of the same degree throughout the whole segment as well as in the distal stump. Layers of flattened cells and collagen surrounded the newly formed nerve.

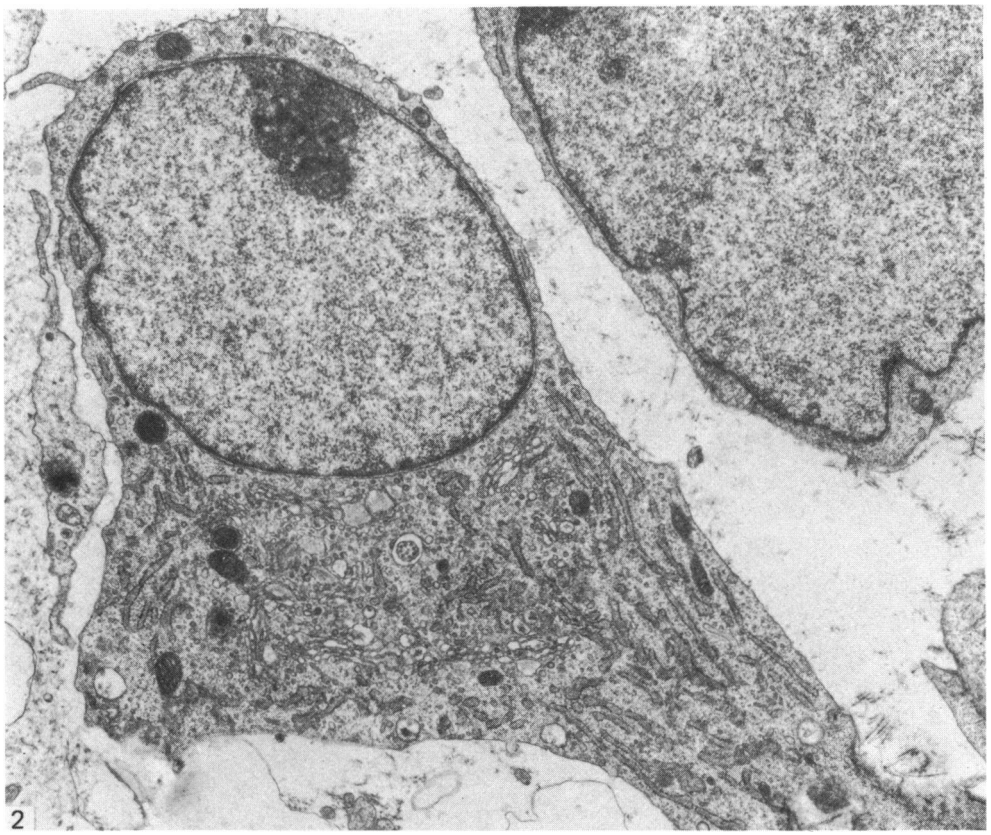

Fig. 2. Cells, probably fibroblasts, are present in the fluid material inside the tube. Their cytoplasm is electron-dense and contains abundant organelles. × 10500.

No further changes were observed at *60 days* other than a considerable increase in the size of the axons and in myelin thickness.

Electron microscopy

At *7 days*, macrophages were present; their cytoplasm was somewhat electron-dense and contained electron-lucent vacuoles. Cells considered as probable fibroblasts were rich in organelles (tubules, granular endoplasmic reticulum, mitochondria, Golgi apparatus) and did not appear to make contact with each other (Fig. 2). Basal lamina was present only around capillaries.

At *12 days* the more proximal part of the segment of tissue between the stumps contained numerous clusters of axons. Their calibre was variable, with a maximum diameter of 5–6 μm, and they were occasionally widely separated from adjacent axons and surrounded by a thin myelin sheath. Thinner axons were either closely packed together or separated from the others by thin tongues of Schwann cell cytoplasm. The axoplasm contained abundant filaments, 10 nm thick, less numerous microtubules and mitochondria. Although the orientation of the filaments was mainly parallel to the long axis of the axons, bundles of filaments arranged obliquely or transversely were also seen, particularly in the larger axons. Axons were occasionally distended by large collections of organelles which included autophagic vacuoles,

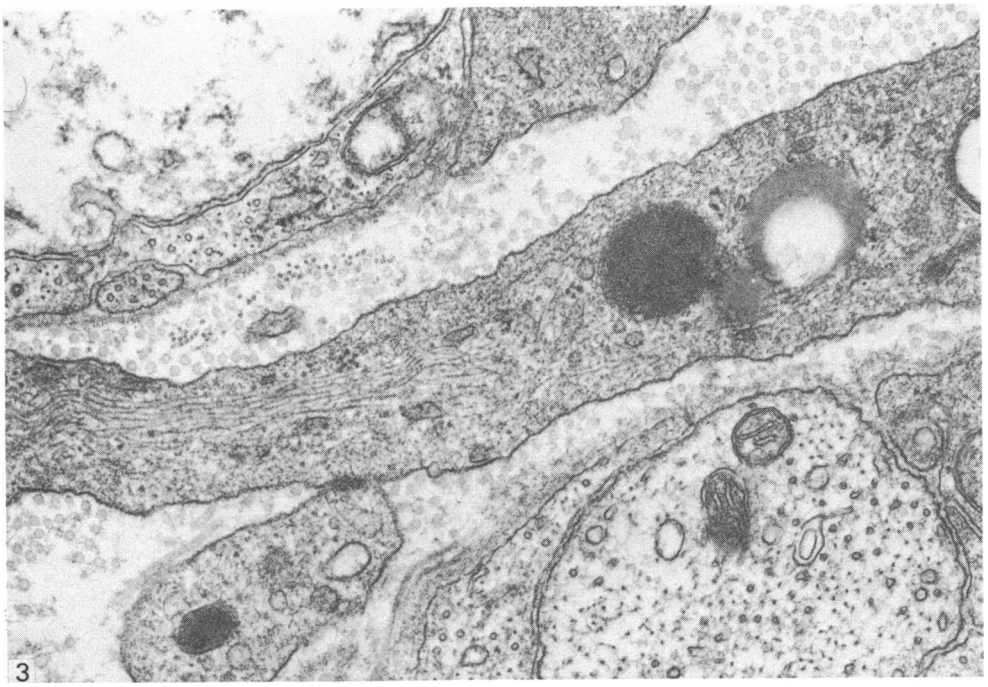

Fig. 3. Thin processes of interstitial cells devoid of basal lamina contain bundles of filaments. ×45000.

mitochondria, filaments and neurotubules. Less frequently, a degenerate myelin sheath was surrounded by one Schwann cell, the outer edge of which was bordered by axon sprouts. An uninterrupted basal lamina surrounded each of the axon–Schwann cell complexes, following their irregularities and extending into deep indentations in the Schwann cell surface.

The interstitial space was occupied by abundant collagen and finger-like cytoplasmic processes which in some areas completely surrounded the Schwann cell–axon complexes. The collagen consisted of fibrils with a diameter of about 40 nm, the greater majority of which shared the longitudinal orientation of the growing axons as recognised by their round shape in transverse sections. Small clusters of fine tubular structures were also dispersed among the fibrils and these were frequently in close contact with a cell process which occasionally contained aggregations of fibrillary material. The same fibrillary material was also present within the cytoplasm of some Schwann cells.

The strips of cytoplasm within the interstitium were found to originate from elongated cells with nuclei which contained clumps of chromatin adherent to an indented nuclear membrane. The cytoplasm contained abundant granular endoplasmic reticulum and a well developed Golgi apparatus, mitochondria and, in some cells, bundles of filaments 5–7 nm thick (Fig. 3). Processes that extended for long distances from the cell body were seen to establish contacts with similar extensions of other cells, forming zonulae occludentes (Fig. 4). Most of these processes and their cell bodies were devoid of basal lamina. In some areas, however, shorter or longer segments of basal lamina lined the cell (Fig. 4) and showed irregular thickenings or separation from the underlying cell membrane. Several layers of these

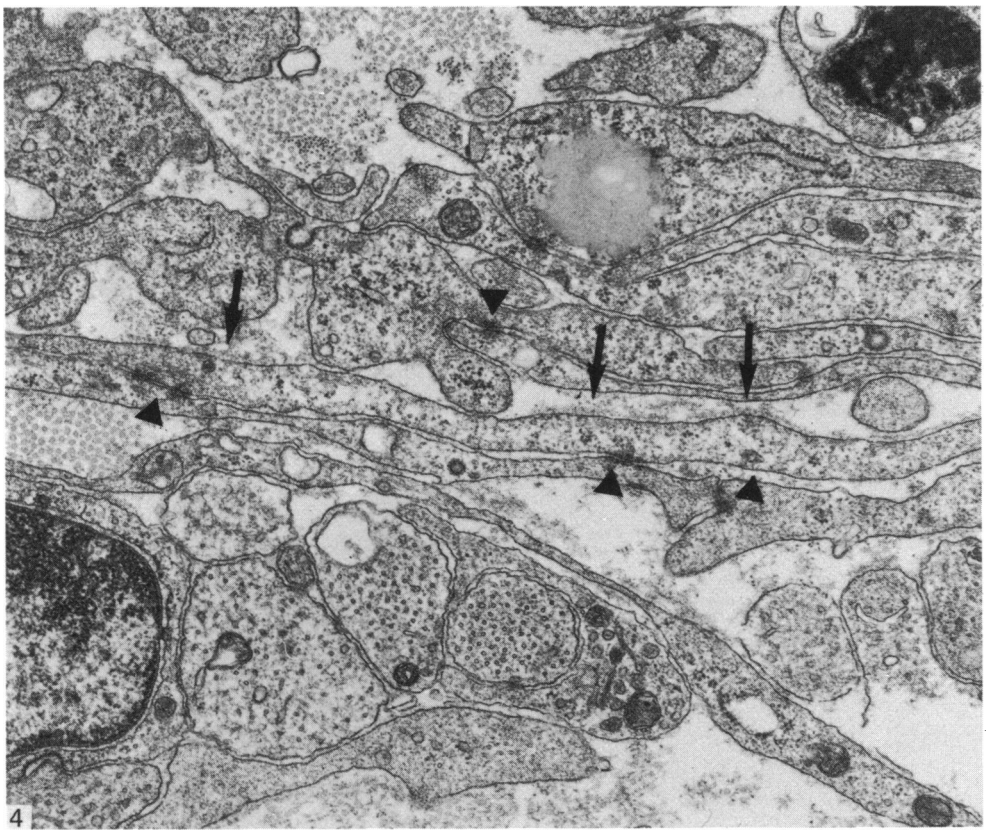

Fig. 4. Numerous cell processes extend for long distances from their cell bodies and make contact with similar processes. The picture shows numerous zonulae occludentes (arrowheads) and basal lamina covering short segments of cell membranes (arrows). ×22500.

elongated cells surrounded the regenerated nerve bundle and the layers were separated by spaces containing collagen fibrils aligned predominantly in the long axis of the plastic tube. Some of the cells contained large vacuoles and occasional pinocytotic vesicles and were surrounded by patchy segments of basal lamina.

At the mid-point between the proximal and distal stumps, the appearance varied slightly from that described in the more proximal regions. Axons showed a great variability in size and some were extremely swollen and distended by organelles (Fig. 5); few were myelinated and the number of myelin lamellae never exceeded ten (Fig. 6). Large complexes consisting of Schwann cells and axons and completely surrounded by basal lamina were also seen; some of these included remnants of myelin and occasional nuclei in mitosis. Collagen fibrils and aggregates of tubular material were abundant and were aligned parallel to the long axis of the axons. In addition to collagen, the interstitial space between the clusters of nerve fibres and their associated Schwann cells was filled with flattened cells similar to those described above. The basal lamina covering these cells was patchy and involved only short segments of the cell membrane. In areas covered by basal lamina the underlying cytoplasm occasionally contained finely granular or floccular material.

The layers surrounding the regenerated nerve consisted of cells similar in appearance to those described above. No basal lamina was seen.

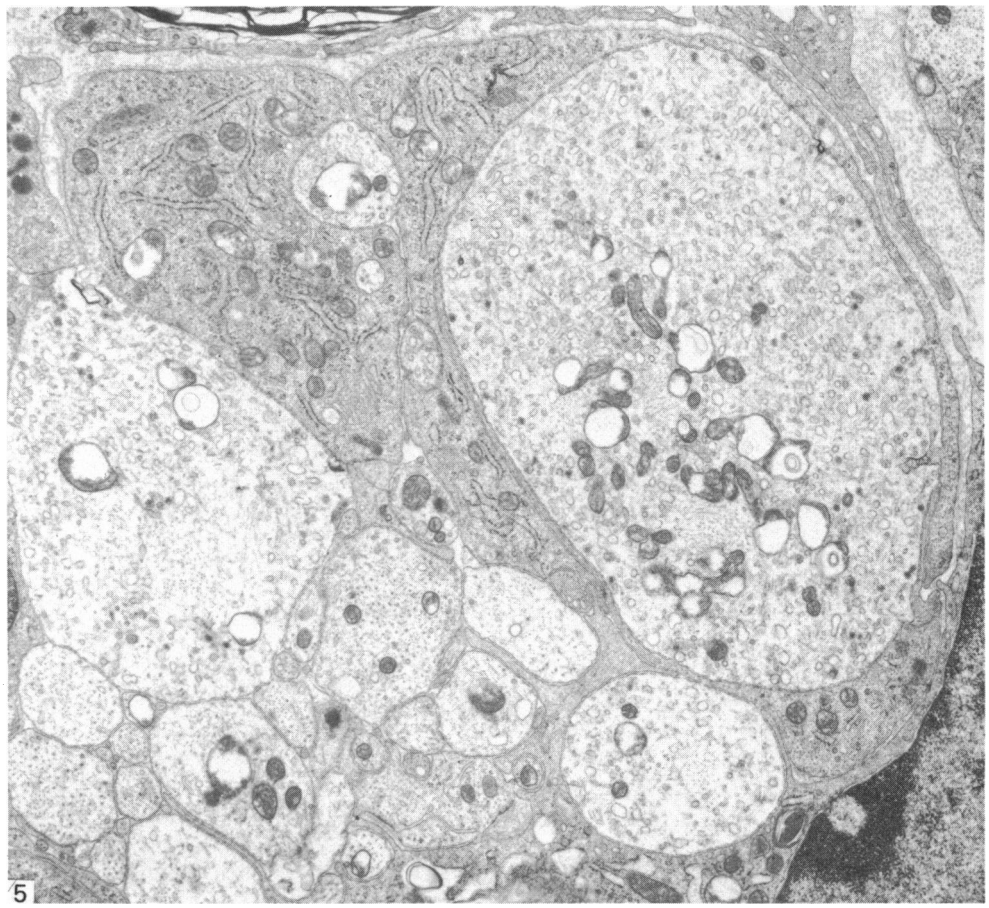

Fig. 5. Regenerating axons are characterised by considerable variation in diameter. One of the axons shown in the picture appears extremely swollen and filled with organelles. × 10500.

At this stage (12 days), the most distal part of the segment between the stumps consisted of a network of cell processes, the spaces between which were filled with bundles of collagen arranged longitudinally (Fig. 7). The cells were covered by only short segments of basal lamina (Fig. 8) and their cytoplasm was rich in organelles and in some regions included aggregates of fibrillary material occasionally related to a thickening of cell membrane (Fig. 8).

In addition to the bundles of collagen, the spaces between cell processes contained aggregates of tubular material and clusters of cytoplasmic profiles surrounded by an almost continuous basal lamina (Fig. 9). Some of the profiles, which clearly belonged to Schwann cells identifiable by their characteristic nuclei, contained fibrillary material similar to that previously described in interstitial cells. No axons were seen.

At *15 days* the structures in the tube did not differ significantly from those described at 12 days, except for the presence of axons at all levels and for a moderate increase in the number of myelinated axons.

By *20 days*, however, the supporting structures of the regenerated nerve had undergone considerable changes. The cells which formed the layers surrounding the bundle or the smaller groups of fibres had become extremely thin. Their cytoplasm,

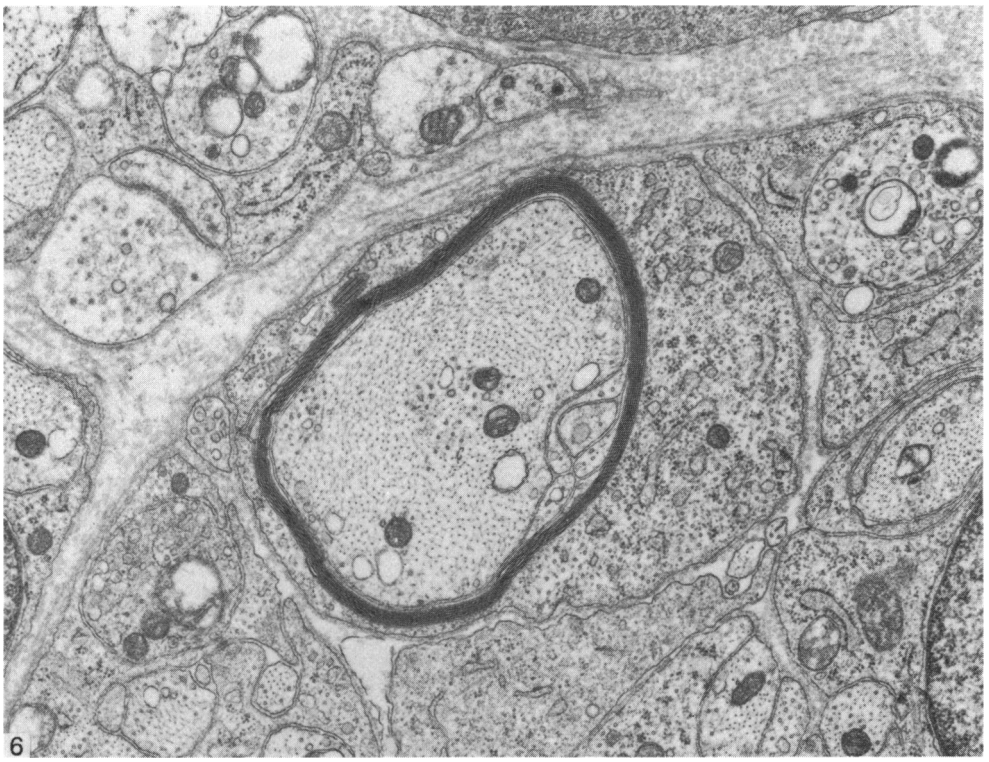

Fig. 6. At this stage of regeneration only a few axons are surrounded by thin myelin sheaths. ×19000.

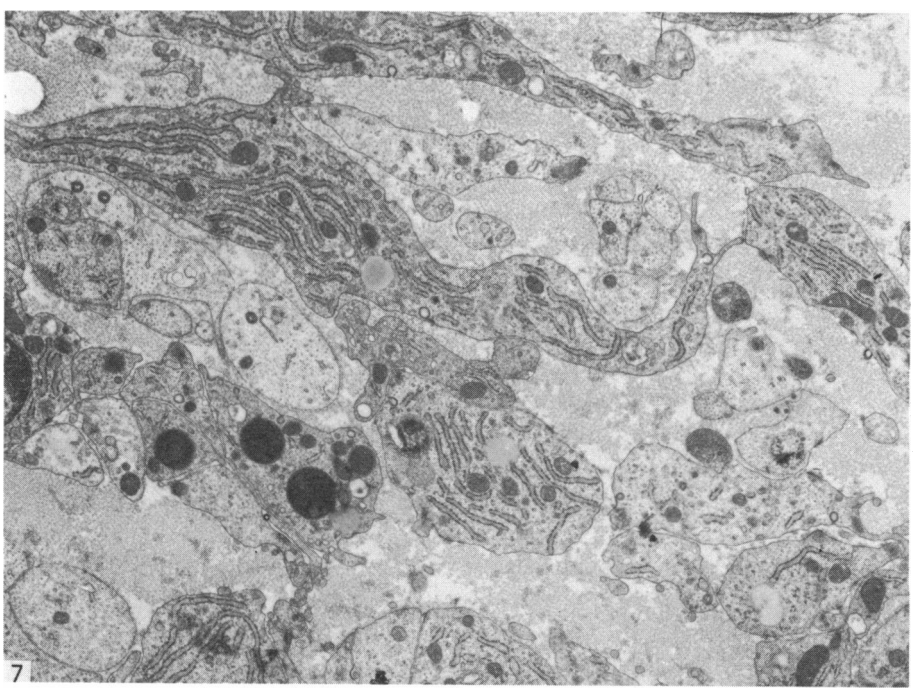

Fig. 7. In the distal segment between the stumps, cell processes devoid of basal lamina establish mutual contact and delineate large irregular spaces. ×8000.

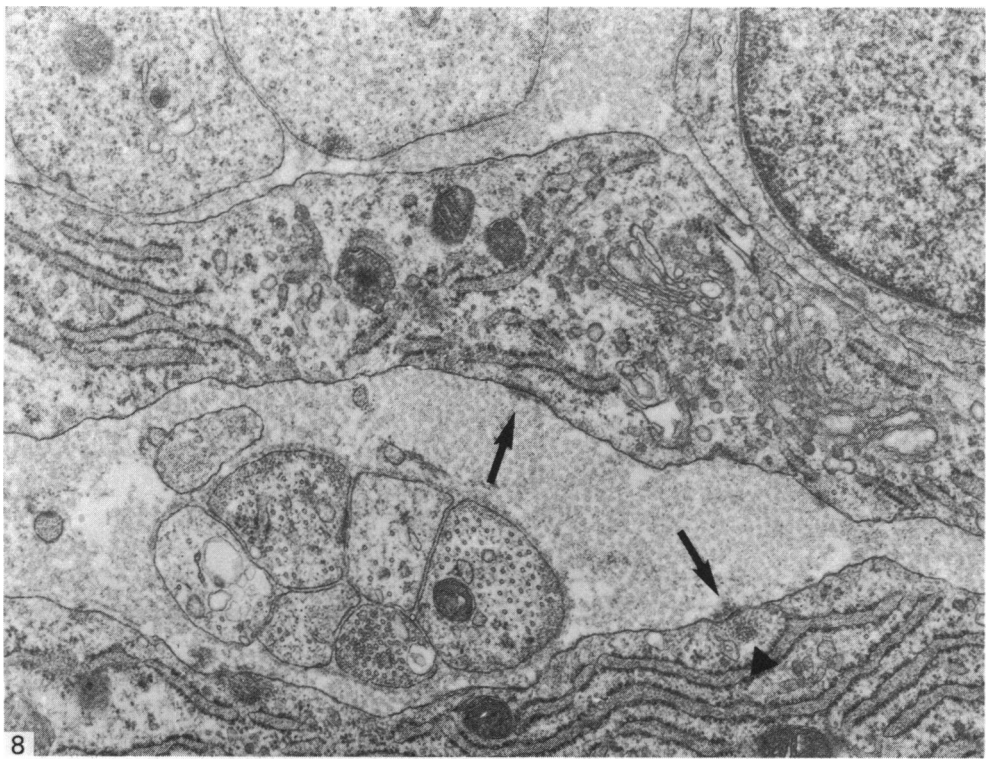

Fig. 8. Detail of two cell processes. Basal lamina covers only a short segment of cell membrane which, at those levels, appears thickened (arrows). Note aggregates of fibrillary structures in relation to the thickenings (arrowhead). ×22500.

which was less rich in organelles, contained occasional bundles of filaments and tubules as well as pinocytotic vesicles, and fewer nuclei were now visible. The thin processes of these cells were in contact with one another at their edges by means of zonulae occludentes. At this stage, basal lamina covered wider areas of the cell surfaces but still appeared to be incomplete (Fig. 10).

The most distal part of the regenerated segment still differed from the more proximal regions in its smaller content of myelinated axons and the thinness of the myelin sheaths. Collagen was abundant but, unlike the earlier stages, thin tubular structures were seen less frequently (Fig. 10).

At *30 days*, all components of the regenerated nerve had an almost mature pattern (Fig. 11). The slender cell processes which surrounded the bundles of nerve fibres had acquired all the characteristics of mature perineurial cells; they were lined almost completely by basal lamina and their cytoplasm was less rich in organelles and contained numerous pinocytotic vesicles. Collagen fibrils were abundant both inside and outside the perineurium and despite a great variability of their diameter showed a predominance of larger diameter fibrils in the extraperineurial compartment. On the other hand, the tubular structures noted at earlier stages of repair had completely disappeared. Furthermore, the diameter of the axons had increased considerably and their myelin sheaths had become thicker. The increase in fibre calibre and myelin sheath thickness continued during the second month, but no

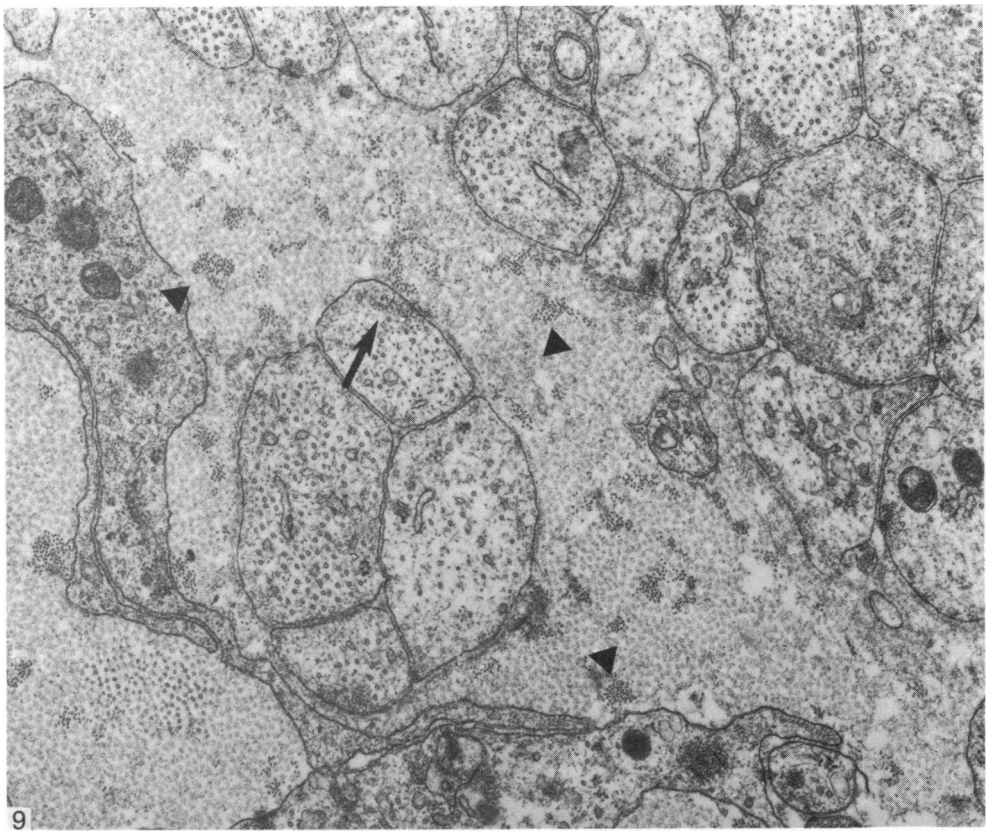

Fig. 9. Spaces surrounded by the cell processes are filled with bundles of collagen aligned in the long axis of the plastic tube, aggregates of tubular material (arrowheads) and cell profiles forming clusters. An almost complete covering by basal lamina is seen. Note the presence of fibrillary material underneath the cell membrane similar to that described in interstitial cells (arrow). ×22000.

further changes were observed in the structure of the nerve or in the relationship between its various components.

DISCUSSION

The process of regeneration of cut peripheral nerves across a gap has been described by several authors (Lundborg *et al.* 1982*a*, *b*; Molander *et al.* 1982; Uzman & Villegas, 1983) as progressing at a fast rate so that, by the end of the first month following injury, the gap is bridged by fibres which penetrate deeply into the distal stump.

With the pace of regeneration progressing so rapidly, the histological appearances at various levels change considerably during the early stages of regeneration and provide an opportunity to follow the chronological sequence of events leading to the re-establishment of continuity between the stumps.

The florid axonal proliferation seen in the proximal segments at 12 days after nerve section, at a stage when no axons are present more distally, shows striking similarities to the appearances of the five isolated fibres described by Friede & Bischhausen (1980). In the present study, most of the axons seem to sprout abund-

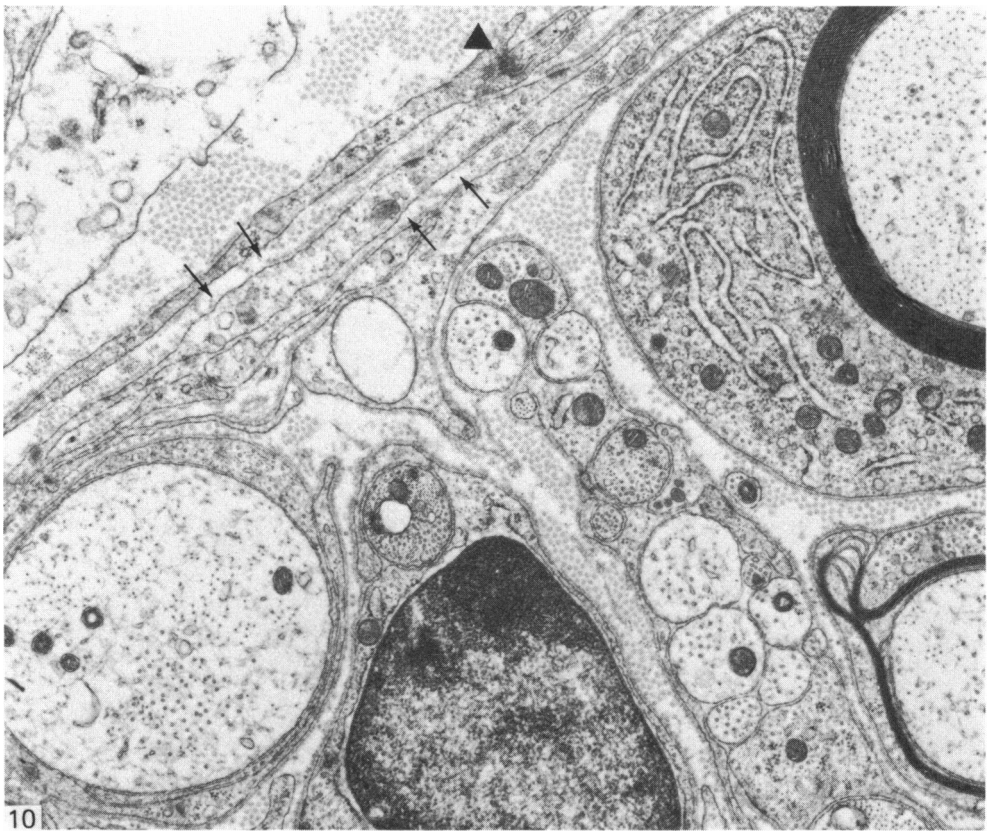

Fig. 10. Appearances of the regenerated nerve at 20 days. Thin layers of perineurial cells are in contact with each other through zonulae occludentes (arrowhead). Their covering by basal lamina is extensive but still incomplete (arrows). ×16000.

antly, like fibres A, B and C of these authors, while less numerous axons are swollen and resemble those labelled D and E in their study. The functional interpretation of these findings is not easy to determine but the swollen axons may have the same significance as those described by Lampert (1967) as being reactive and regenerative. In addition to the axons, there are at this level Schwann cell nuclei, occasional collapsed myelin sheaths and cells surrounded by basal lamina. The latter cells, which are also found at some distance from the proximal stump, are reminiscent of the migratory cells of Friede & Bischhausen (1980), and the presence of a basal lamina around them supports an origin from the Schwann cell.

Observation of the regenerating segments at proximal and distal levels shows that myelination is well advanced in the former before axons have reached the distal side of the gap. A proximodistal gradient in the process of remyelination is observed in regenerating nerve fibres after crush (Quilliam, 1958) and the same trend is also known to be followed during development (Strong, 1906), although in these studies it is not stated whether the axons which are surrounded proximally by myelin had already reached their target before the process of myelination had begun. However, the observation that myelination is independent of the contact of the axon with its target is further supported by the work of Feasby, Pullen & Sears (1981) who have observed remyelination in regenerated roots that fail to reach their central target.

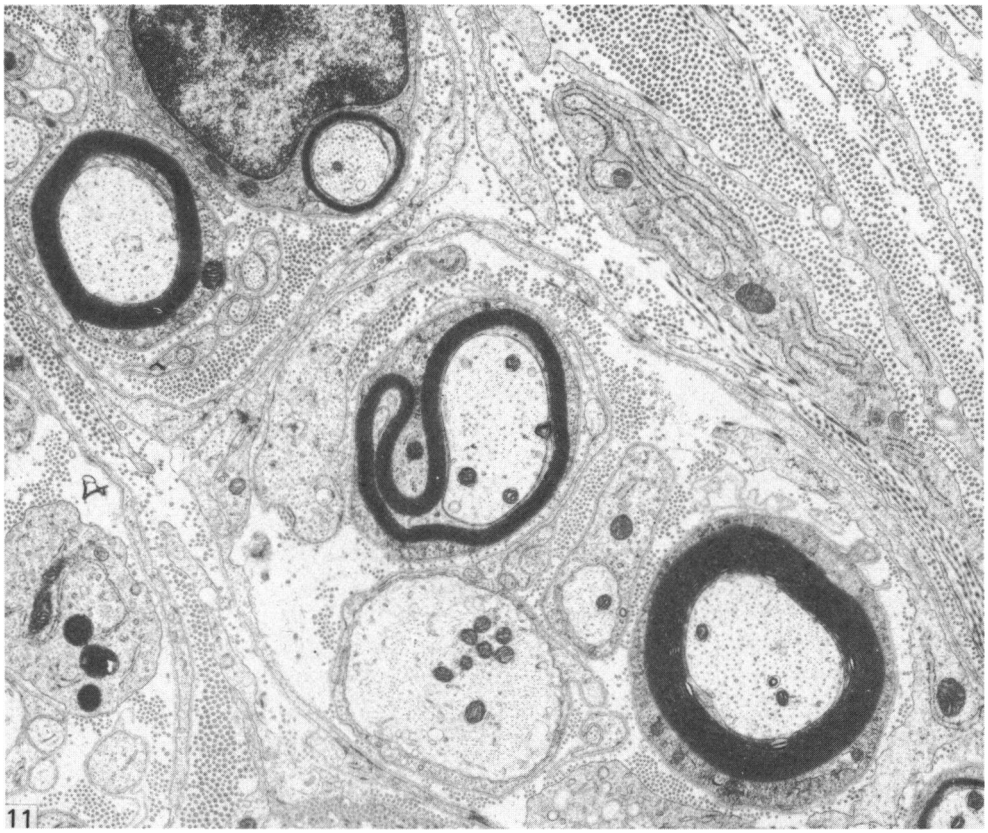

Fig. 11. Electron micrograph of an area of regenerated nerve at 1 month showing small groups of fibres surrounded by perineurial cells. Covering by basal lamina is virtually complete. × 10000.

The appearances of a fully developed perineurium at the site of regeneration have been described by Morris *et al.* (1972) and by Thomas & Bhagat (1978) as consisting of one or two layers of flat cells surrounding small nerve fascicles. These features are confirmed by Lundborg *et al.* (1982*b*) and are also observed in regenerated nerves in this study.

The nature of perineurial cells has been investigated by numerous authors. Key & Retzius (1876) and Shanthaveerappa & Bourne (1962) concluded that they originate from the leptomeninges, while Harvey & Burr (1926) thought that they are derived from the neural crest. Denny-Brown (1946) and Thomas & Bhagat (1978) used an experimental approach to this problem, studying the regeneration of the perineurium after extraction of the intrafascicular contents. Morphological evidence which they obtained suggests that perineurial cells originate from cells with the appearances of fibroblasts. These cells are first seen adjacent to remnants of basal lamina and later become organised into layers around small nerve bundles. In an earlier experiment using transected nerves, Thomas & Jones (1967) had arrived at similar conclusions: bundles of Schwann cells and axons are surrounded by cells with the appearance of fibroblasts. The results of the present study support the same conclusions but show that in regenerating segments the only cells present at 7 days are endothelial cells and cells with the appearance of either macrophages or fibroblasts. In the distal

regenerating segment at 12 days, Schwann cells are also present but they are outnumbered by fibroblasts and macrophages. Fibroblasts are already in contact with each other and in the process of enclosing spaces, some of which contain Schwann cells. It appears, therefore, that the first cells to arrive at the site of interruption of the nerves are cells devoid of basal lamina and that these cells precede Schwann cells, although it is not possible to ascertain whether they arrive via blood vessels or by migration from the stumps.

At later stages of regeneration, features characteristic of established perineurial cells become progressively more apparent in these cells. They include the loss of the abundant endoplasmic reticulum and of the Golgi apparatus and the presence of a basal lamina which appears in the distal segment at 12 days as short segments covering parts of the cell membrane. By 1 or 2 months, the basal lamina covers the surfaces of perineurial cells almost completely. Furthermore, pinocytotic vesicles are only occasionally seen at 12 days and become more numerous at 20 days. Other structures characteristic of developing perineurial cells are thickenings of the cell membrane and increasing density of the subjacent cytoplasm. These are fairly numerous at 12 days, most of them corresponding to hemidesmosomes (Kelly, 1966), and have been described previously by Thomas & Jones (1967) in regenerating nerves.

The appearances and arrangement of the collagen fibrils is of interest. While no collagen is seen at 7 days, at 12 days it is abundant at all levels indicating that a massive deposition of fibrils has taken place. Among the several types of cells recognised to produce collagen, fibroblasts are the most active using up to 10% of their total protein (Green & Goldberg, 1964). Large macrophages and fibroblasts are the most common types of cells present in the early stages of regeneration through the gap. Some of the fibroblasts contain bundles of 5–7 nm filaments and have an appearance reminiscent of the myofibroblasts found in granulation tissue (Gabbiani, Ryan & Majno, 1971).

The participation of other cells, notably Schwann cells, in the production of collagen in peripheral nerves has been suggested by various authors (Murray & Stout, 1942; Nathaniel & Pease, 1963; Thomas, 1964). More recently Cohen & Hay (1971) have provided morphological evidence suggesting synthesis and secretion of collagen by chick embryonic neuroepithelium. Furthermore, Church, Tanzer & Pfeiffer (1973) and Bunge *et al.* (1980) have demonstrated biochemically that Schwann cells can synthesise collagen. The results of the present paper, showing that the cytoplasm of both fibroblasts and Schwann cells contains the same type of dense material in contact with the cell membrane and that this material is related to hemidesmosomes and to short segments of basal lamina, support the view that Schwann cells take part in the regeneration of interstitial material, as postulated by Thomas (1964) and shown by Bunge *et al.* (1982) in tissue culture.

In the early stages of the regenerative process, interstitial tissue includes two types of structure, fibrils measuring 40 nm in diameter and smaller tubules. The tubular structures decrease considerably in the late stages and have disappeared almost completely by 1 month. It is very likely that they represent elastin and/or its associated microfibrils, and are similar to the structures present in epineurial connective tissue and in the endoneurium of normal nerves. As for the orderly longitudinal arrangement of the collagen in the regenerated segment, Thomas' (1964) conclusions leave the question open about the conditions which induce it. The present investigation shows that several factors might contribute to this arrangement: at an early

stage of regeneration, the large number of fibroblasts with long branching processes form a framework which circumscribes longitudinally oriented tubular columns of matrix; in addition, the tube of plastic film helps to maintain all newly formed structures within a confined cylindrical space; finally, the constant traction which the segment undergoes after the two stumps have been joined might encourage the collagen to become aligned preferentially in the direction of the tube.

SUMMARY

Sciatic nerves of mice were cut and the early regenerative stages were studied after the stumps had been encased within plastic tubes and kept separate by a gap of 5 mm.

Only isolated cells were seen inside the tube after 7 days; after 12 days active regeneration and myelination were seen proximally; more distally, cells with long processes formed large spaces filled with collagen and less numerous Schwann cells. Zonulae occludentes and segments of basal lamina became more evident at a later stage. One month after the operation an almost complete regeneration of the nerve had taken place and perineurial cells were lined by a continuous basal lamina. The regeneration of the perineurium seemed to take place from fibroblasts; their cytoplasm as well as that of Schwann cells contained fibrillary material at this stage, sometimes in relation to segments of basal lamina.

The results of this study indicate that both types of cells take part in the formation of endoneurial structures and that the early arrangement of fibroblasts contributes to the orderly longitudinal alignment of collagen fibrils.

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