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THE ROLE OF NEOVASCULARIZATION IN TISSUE BRIDGE FORMATION PRIOR TO AXONAL REGENERATION.

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TABLE OF CONTENTS

A. REVIEW OF THE LITERATURE

Peripheral nerve injury has been reported in medical texts for many years. Documented in Hippocratic writings "Division of bone, cartilage, nerve, the delicate part of the jaw . . . is not followed by growing and joining together again." (14). During the Second World War, armed conflict tested neurosurgical skills, pushing the technology of peripheral nerve repair forward. With the advent of the operating microscope, and an improved understanding of peripheral nerve anatomy, the results of surgical repair improved. Since that time, some functional recovery is expected after peripheral nerve injury and repair. The current focus of research in peripheral nerve regeneration could be broadly divided into the cellular response of the nerve, surgical technique and tropic and trophic influences on regeneration. Because the peripheral nervous system is capable of regeneration and accessible to many aspects of investigation, results from this work may improve the understanding of the mammalian central nervous system and its limited ability to regenerate.

a. The Cellular Response of a Peripheral Nerve to Injury.

The immediate response of a mammalian peripheral nerve to transection is the elastic retraction of both nerve stumps from the lesion site, a cascade of chemical reactions and an outspilling of blood, lymph, axoplasm and its constituents into the interstump gap (26,132).

During the development of the peripheral nervous system, Schwann cells divide and migrate peripherally to enwrap and myelinate the elongating axons (69,119). In the adult state, after axotomy, Schwann cells near to the transection site, undergo transformation entering a mitotic phase sufficiently intense to produce a bulbous swelling at the severed nerve tip (69,89,102,132). The response of the Schwann cell to axotomy and its role in regeneration is the subject of study. It has been suggested that myelin breakdown after nerve transection is the trigger for Schwann cell proliferation (110-112). However, Schwann cell mitosis has been induced *in vitro* by regenerating nerves, implying a stimulus from the axons (94).

After peripheral nerve transection Wallerian degeneration is observed. The axons in the distal segment

of a transected peripheral nerve degenerate and die from the point of transection to the target tissue (103,132). Within ten weeks of transection, all existing myelin is digested (156). In an ultrastructural study of degenerating and regenerating rat sciatic nerve, it was reported that Schwann cells transformed into macrophages and digested the myelin (83). A trigger for myelin breakdown may be the degranulation of mast cells (49). Mast cells are present in peripheral nerve in large numbers, and further degranulation may be triggered by the presence of myelin protein, a possible model for demyelinating disease (49). After Wallerian degeneration, Schwann cell basal lamina, or so-called bands of Bungner, remain and may have an important role in axonal regeneration (132). Electron microscopy revealed growth cones adhering to Schwann cell basal lamina, supporting the view that the bands of Bungner help to guide axons back to their target by providing a substrate for elongation (102,113).

After peripheral nerve transection, retrograde degeneration also occurs, but it is not as extensive as Wallerian degeneration (103). Due to the initial outpouring of axoplasm after transection, axons close to

the lesion site tend to collapse. Axons are found to shrink back from the proximal cut end approximately 1-2 mm. (51). Within one day of axotomy, swellings at the cut ends of the axons containing axoplasmic organelles are observed. These swollen ends will sprout growth cones, which form the leading edge of axonal elongation (51,98,102). A growth cone has filopodia, which extend into the immediate environment and move continuously. By phagocytosing particles in their path, growth cones sample the environment and appear to follow particular stimuli (65,93,99,105).

The regenerating axons which leave the proximal stump, may encounter inhospitable substances including: scar produced by excessive tissue reaction, spilled blood, ischemic regions, spaces left unoccupied by cells or extracellular matrix (33). If successful in crossing this area into the distal stump, some axons will find their way into the bands of Bungner which may provide the framework for elongation, myelination and eventual reinnervation (69). Many axons do not find a supportive substrate and instead retract and die or create a tangled neuroma.

The nerve cell body also undergoes change, two to three days after the nerve has been transected. The nerve

cell body and nucleus swell, the nucleus migrates eccentrically and about a week after transection, the Nissl substance undergoes chromatolysis (50). The nerve cell body begins to produce larger amounts of cytoskeletal material and proteins unique to the regenerative process (7,8,121). One example of the nerve cell body's response is the observation that levels of mRNA encoding actin and tubulin increase, while those for neurofilament decrease (43).

Axonal transport has long been thought to have an important role during regeneration. Axonal transport continues to function after nerve transection, but it carries different proteins and substances which are necessary for regeneration (8,12,29,60,69,120,121). One possible role for axonal transport after nerve injury is that of signalling the nerve cell body. The cell body could be alerted to injury and triggered to respond to it by: the appearance of endocytosed material from the lesion site, the change in the turnaround time of anterogradely transported substances, or the lack of trophic factors retrogradely transported from the target tissue (8).

Particular fast-transported proteins are synthesized

in dorsal root ganglion neurons after mammalian peripheral nerve transection (43,85,121). The function of these proteins is not known, but some have been termed "growth associated proteins" (GAPs), as they are also expressed in the developing peripheral nervous system (104). Results from conditioned lesion experiments, where a nerve trunk is crushed and then two weeks later transected, suggest that GAPs are responsible for the reduced delay of the regenerative response in conditioned nerves (104). After injury to rabbit hypoglossal nerve, the induction of GAP synthesis and the transport of those proteins, along with the finding that central nervous system neurons do not synthesize GAPs, led to the suggestion that the ability of a nerve to regenerate is determined by its genes' capacity to synthesize GAPs (121). In one study, GAP synthesis stopped only when reconnection with a target was achieved by a regenerating nerve (8). It has been speculated that a 43 kD GAP protein, "GAP-43", inhibits proteases present in the growth cone which break down the cytoskeletal elements arriving via axonal transport, which are necessary for elongation (8).

Cells associated with peripheral nerve but are non-neuronal, are believed to be responsible for some of the

responses observed after transection. For example, polyamine synthesis increased at a rapid rate after nerve injury and was localized to non-neuronal cells (135). The enzyme transglutaminase catalyzes the binding of polyamines to glutamine residues on polypeptide chains, leading to cross-linking between adjacent chains. This modification of protein structure has been associated with the cellular response to nerve injury (135). Posttranslational protein modification was found to occur close to the site of crush injury of sciatic but not optic nerves (118). The authors suggested that this protein modification by non-neuronal cells may reflect the capabilities of peripheral nerve to regenerate as compared to central nerves (118).

Transection of a peripheral nerve triggers a series of events which prepare the nerve cell body for regeneration. The nerve cell body is transformed from one that produces proteins and materials necessary for transmission, to a regenerating one with a much higher metabolic demand (7,29,43,121). Larger amounts of actin and tubulin are produced and GAPs are synthesized and transported to the regenerating nerve tip. There also appears to be synthesis and modification of proteins in

the immediate vicinity of the nerve lesion. Wallerian degeneration results in degeneration of all axons and digestion of myelin all the way to the target tissue, but Schwann cell basal lamina is spared, perhaps providing a trail for regenerating axons. By the time that axons begin to elongate, much of the groundwork for the success or failure of regeneration has been laid.

b. Surgical Repair after Peripheral Nerve Injury.

Functional improvement of denervated target tissue is the aim of surgical repair of transected peripheral nerves. However, successful reinnervation of target tissue depends on many surgical factors. Surgical nerve repair can create obstacles for the regenerating nerve including: the sutured reapposition site, intraoperative trauma, tension at the sutured reapposition site and grafting material. Surgical repair often involves suturing nerve stumps in apposition. When the continuity of the perineurium is broken by suture material, a tissue response is elicited by the introduction of the foreign object and entry of infective agents into the nerve funiculus is facilitated (132). A sutured reapposition site represents an interruption of continuity of nerve tissue and a barrier to be crossed by regenerating axons. The trauma of injury and repair can cause an inflammatory tissue reaction, including the release of chemicals and substances which may impede axonal elongation. Intraoperative trauma can lead to excessive collagen deposition in the form of a scar, preventing axonal outgrowth and diminishing perfusion. Nerve stump

reapposition under tension decreases blood flow and stretches axons (25,70,79). Signs of reduced blood flow are evident when the nerve is stretched by 8% of its length. If a peripheral nerve is stretched to 15% of its length, complete stasis occurs in all vessels in the nerve (70). It has been suggested that a regenerating nerve might have a better chance of success when crossing over two sutured reapposition sites than one under tension (10). To eliminate tension, a nerve graft can be inserted to bridge an interstump gap, but there are drawbacks to this technique: grafting introduces two sutured reapposition sites and the site of failure is often the second coaptation site (10); there is difficulty matching nerve diameters, as degeneration causes nerve atrophy; a nerve must be sacrificed when using an autograft.

Depending upon the type and extent of peripheral nerve injury, a different surgical repair technique is followed. After a clean nerve transection, epineurial repair is often advocated since the nerve has not undergone undue damage and the funiculi can be visualized and re-aligned (132). The advantage of this approach is that it results in less injury to nerve tissue and blood vessels due to suturing and handling (132). However,

epineurial reapposition has been criticized because there is no attempt to insure that the regenerating axons will extend toward the appropriate target.

If peripheral nerve damage is sufficient to require removal of a small amount of nerve tissue, funicular repair is the surgical approach often chosen (91,132). Funicular repair involves dissecting out the proximal and distal funiculi to allow each proximal funiculus to be sutured to a corresponding distal funiculus by either perineurial or intrafunicular sutures. The funicular arrangement of peripheral nerve is a constantly changing plexus. When attempting funicular repair in the presence of a tissue defect, correct funicular alignment is difficult. This repair is said to improve reinnervation by properly aligning funiculi. However, during regeneration after retrograde degeneration in the proximal stump, extensive axonal sprouting occurs and "regenerating units" form in individual perineurial sheaths (83). This reorganization, likely changes the funicular pattern sufficiently to prevent a correct match of funiculi. Also, this procedure involves a greater amount of handling and suture material, which could cause further nerve and

blood vessel damage. There does not appear to be clear evidence that funicular repair is more successful than epineurial. When funicular and epineurial repair were compared in an ultrastructural study, no statistical difference was found between the number of regenerated fibres in each group (91).

After nerve crush injury or infection with epineurial damage, necrotic tissue must be removed often leaving a significant interstump gap. In the past, most nerve grafting efforts to bridge interstump gaps ended in failure (10,124,125). To avoid nerve grafting, surgeons stretched nerves across distances and performed anastomosis under tension, often with a joint in a flexed position. This caused damage to the nerve, offered little opportunity for regeneration and caused joint contractures. The introduction of the surgical microscope and a better knowledge of peripheral nerve response to injury, resulted in an improved success rate of nerve grafting and it became the treatment of choice over anastomosis under tension (10).

Nerve grafts are sutured in a similar fashion to end-to-end repair, that is, funicular or epineurial. A cable graft is a group of nerves grafted together and a full

thickness graft measures the same diameter as the nerve being repaired. Funicular grafting will only achieve its aim if the funicular pattern of the graft at the reapposition site is identically matched in the proximal and distal stumps (44). An autologous nerve graft is most often used, because homografts and heterografts have lower rates of success (132).

In a comparative study of an "empty perineurial tube" graft to an autologous nerve graft, all the fascicles from a 25 mm piece of sciatic nerve were removed, leaving an empty perineurial tube. This was grafted to the proximal and distal stumps of a transected nerve with 10-0 nylon sutures. Using EMG recording, angiograms, light and electron microscopy endpoints, the authors reported the perineurial tube technique was superior, yet they showed no statistical difference between the two (106).

There are other nerve-grafting techniques which are used clinically and appear useful under particular circumstances. Two examples are: pedicle grafting, which uses part of the divided nerve as the grafting material, maintaining some blood flow, and nerve crossing, which uses an adjacent uninjured nerve to partially or completely reinnervate the distal stump of the injured

nerve (132).

One of the problems with nerve grafting is having to lay the graft on a scarred bed. Scar tissue restricts blood flow, reducing the vascularization of the nerve graft and nerve stumps (10). Nerve grafts frequently become necrotic in the centre because they are avascular, while the peripheral cells remain viable due to diffusion from surrounding fluid (78). Free vascularized nerve grafting, by providing a rapid revascularization of the graft and injured nerve stumps, offers advantage over some of the problems with conventional grafting. Despite its advantages, free vascularized nerve grafting is limited by the availability of a donor and the fact that both a nerve and an artery must be sacrificed with each procedure (10).

Other biological non-neuronal tissues have been tested as bridging materials including: formalinized calves' arteries, fascial tubes, catgut and decalcified bone tubules. When used as nerve grafts, these materials most often resulted in an inflammatory tissue response with little or no axonal elongation (58). A bridging material which has been successfully used is collagen. A cell-free matrix of collagen derived from bovine skin was

produced in 1977 (56). Collagen matrix is a liquid at 4° C. and gels at body temperature within an hour. It has been used in several studies as a substrate for peripheral and central axonal growth across a gap (22,24,35,47).

Nerve guide tube repair is a technique used to bridge interstump gaps being tested in animal models (57,67,68,71-76,88,115,149-155). Nerve guide tubes made out of a variety of materials, are useful because they minimize the amount of suture material needed, support the nerve and regenerate, allow for observation, provide an opportunity for sampling contents or administering substances to modify the regenerative response of the nerve and will reduce tension at the reapposition site.

Lundborg (71) entubulated the sciatic nerve with a mesothelial chamber which the rat produced itself. The subcutaneous implantation of a silastic rod surrounded by a coiled wire, produced a tubular mesothelial structure upon the removal of the rod a few weeks later. The tube, kept patent by the coil, was sutured to the proximal and distal stumps of a transected sciatic nerve in the same animal. Using this technique, axons regenerated across a 12 mm. gap in six weeks (72). In a silicone nerve regeneration chamber developed by the same group later, a

gap greater than 10 mm. could not be bridged by axons (73).

Nerve guide tube studies have addressed many questions regarding peripheral nerve regeneration. The subject of one study was the progress of reconnection and axonal regeneration across a 10 mm gap in a silicone chamber (150). The chamber initially filled with fluid which solidified to a non-cellular bridge consisting mostly of fibrin and which connected the proximal and distal stumps. This was followed by the migration of epineurial and Schwann cells and fibroblasts (from both proximal and distal stumps) making it a cellular bridge. Finally, axons elongated across the cellular bridge to the distal stump (150).

Fluids, gels and mixtures of putative growth-promoting substances placed in the interstump gap of nerve guide tubes have been tested in an attempt to increase the rate and/or density of regenerating axons. If an empty silicone chamber was sutured to the proximal and distal stumps, axons could only regenerate a distance of 10 mm. When the gap was filled with phosphate buffered saline, axons grew across a gap of 15 mm (155). A mixture of laminin, testosterone, ganglioside and catalase was

injected into silicone regeneration chambers which were sutured to a transected peripheral nerve. After one month, a greater density of axons, Schwann cells and blood vessels was observed in the chamber filled with the mixture of growth promoting factors than an empty one (86). A laminin-enriched gel was inserted into polyethylene regeneration chambers and then implanted. A greater rate of nerve fibre regeneration was reported in the chambers with laminin-enriched gel, as compared to empty chambers (74). On the other hand, when an 8 mm interstump gap in a nerve guide tube was filled with oligodendrocytes suspended in collagen, axonal growth was impeded. At eight weeks, 1 out of 12 of the nerves regenerated through the medium, while 6 out of 9 tubes implanted empty resulted in nerve regeneration. After testing regeneration through the collagen alone inside the tube, it was concluded that central nervous system cells were responsible for this interference of axonal growth (47).

Porous nerve guide tubes allow substances from outside the tube to enter, where they may affect axonal regeneration. Depending upon the pore size and materials the tube is made of, particular substances could be

selected to enter or excluded from entering the chamber. When a perforated silicone tube was used to bridge an interstump gap, it was found to contain a greater number of regenerated axons as compared to an impermeable silicone tube (48). A nerve guide tube made of "expanded" Gore-Tex, a porous carbon and fluorine polymer (30 um pores), was compared to autogenous nerve grafting of transected rat sciatic nerve. After four months, the results were assessed using gastrocnemius muscle weight, light microscopy and electrophysiology, and no difference was found between the two groups (36). A polyglactin tube (with pores 0.4 X 0.4 mm) was compared to autologous nerve grafting using EMG and morphometry. The results showed no differences between the groups with respect to nerve fibre caliber or degree of axonal myelination (80). Madison (75) found that regeneration was most dense in "large pored" collagen-based tubes implanted in mouse sciatic nerves as compared to: small-pored tubes, the addition of a laminin enriched gel to large-pored tubes, or laminin enriched gel in small-pored tubes. In contrast, when implanted semi-permeable acrylic Amicon tubes were compared to impermeable PVC or silicone tubes, the silicone tubes were found to contain the highest density

of myelinated axons (57). Although not conclusive, it appears that porous nerve guide tubes may improve the density and rate of regenerating axons by allowing factors from outside the nerve guide tube to enter and bathe the nerve tips.

Biodegradable nerve guide tubes have been proposed for use in human surgery (88). In one study, rat sciatic nerve regenerated across a 10 mm gap in a biodegradable tube (115). However, it was reported that biodegradable tubes might induce a compressive neuropathy secondary to shrinking during the degradation process (76). Another modification of the nerve guide tube technique is the introduction of dividers. A nitrocellulose strip was used to divide a silicone chamber into two parts. Two nerve regenerates grew, one on each side of the divider. It was suggested that this approach may aid in the formation of fascicular patterns and also allow the introduction of growth-promoting substances, which could be bound to the nitrocellulose divider (20).

Depending upon the nature and extent of nerve injury, there are many surgical approaches which can be taken. Epineurial and funicular repair reappose injured nerves, but there are drawbacks to each type of surgery. Nerve

grafts are currently used to bridge interstump gaps. One direction of research is towards the improvement of the materials used and the structure of nerve guide tubes as well as the modification of the internal environment. With improved surgical technique and materials, entubulation may provide an approach which will affect the success of peripheral nerve repair.

c. Tropic and Trophic Influences on Peripheral Nerve Regeneration.

"Tropism" as it relates to the peripheral nervous system, is an effect generated by some object or substance which will influence the direction of axonal growth (27). A "trophic" influence on a peripheral nerve, refers to a substance which will nutritionally support axonal growth when present (27). But, these two terms may lose their unique definitions, when used to describe an event such as the development of the peripheral nervous system or its regeneration. If there are "trophic" factors supporting the outgrowth of fibres, these same factors could be considered "tropic" if they also guide the direction of that outgrowth. The combined effect of the two may be considered growth- or regeneration-promoting properties.

In 1944, Weiss and Taylor (149) used a Y-shaped arterial cuff (made from the bifurcation of the abdominal aorta of a donor rat) to test tropic influences on regenerating axons. The proximal stump of a transected sciatic nerve was sutured into the single trunk inlet of the inverted "Y", and a nerve graft "target" was sutured into one of the two distal trunk outlets. In the other

distal trunk outlet, they tested the tropic influence on regenerating axons of tendon, empty space or another nerve graft target. They found that the same number of regenerating axons reached every target and reported that tropism did not exist in peripheral nerve regeneration. Their conclusions held for several years, but were challenged by Politis et al (95) who repeated the experiment in 1982 with Y-shaped polyethylene tubes. They found a greater number of axons extended into nerve grafts than into other distal implants. These results and others suggested that axonal regeneration after peripheral nerve transection could be guided by substances in the distal stump (95,96). Williams (154) also repeated Weiss and Taylor's early study, using Y-shaped silicone tubes. The targets presented to regenerating axons were aorta, nerve tissue, tendon or an empty space. As many axons extended to aortic tissue as the nerve implant, but the other targets attracted none. It was concluded that the distal nerve implant and aorta possessed regeneration-promoting properties (154).

Lundborg et al (72) studied the effect of the distal nerve stump upon axonal regeneration using a rectangular mesothelial chamber. The proximal stump was sutured into

one corner of the chamber and the distal stump was inserted in the diagonally opposite corner, or left out. Regenerating nerves made a diagonal path to the distal stump, but when it was absent, only undirected axonal growth occurred. In another experiment (48), the proximal nerve stump was sutured into one end of an impermeable nerve guide tube while the other end was closed with either a solid plug, a 5 um Millipore filter or a 1.2 um Millipore filter. With the solid plug preparation there was abortive nerve growth. A nerve cable reached the 5 um filter, while the 1.2 um filter preparation had minimal growth (48). These results support the view that the distal nerve stump exerts a tropic influence on regenerating peripheral axons (72).

Trophism as applied to the study of nervous system development and connection first took form in 1909 with an experiment by M.L. Shorey, who showed that removal of neural targets during development resulted in a decrease in nerve cells in corresponding areas of the chick nervous system (101). Since that time, similarities have been found between the trophic interactions during development and regeneration. Nerve growth factor, for example, is essential for the development of sympathetic nerves, but

also may be involved in peripheral nerve regeneration (59,66,107). Myelin-associated glycoproteins are synthesized in the developing cat peripheral nervous system (156). Protein analysis during remyelination after transection revealed higher levels of myelin-associated glycoproteins and supported the theory that these proteins play an essential role in the Schwann cell/regenerating axon interaction (156).

Lubinska (69) proposed that a trophic factor was distributed throughout the axon, where it inactivated Schwann cells, preventing nerve fibre degeneration. This theory was supported by a multiple transection study measuring the time of onset of Wallerian degeneration in various parts of the nerve. The conclusion was that the isolation of nerve tissue from the trophic factor by transection was the critical trigger for degenerative changes in the nerve (69).

Some of the trophic factors occurring endogenously in the peripheral nervous system which have been studied are: nerve growth factor (NGF), gangliosides, fibronectin and laminin.

NGF has been purified, characterized and shown to be essential for the <u>in vivo</u> survival of embryonic sensory

and sympathetic neurons (136). If animals are injected with NGF antiserum for eight days after birth, the result is the disappearance of 99% of the sympathetic nerve cells (66). Results of other studies suggest that NGF might be synthesized by target tissues and at that site, regulate the survival and differentiation of sympathetic and sensory neurons (117).

Schwann cells are probably responsible for the synthesis of NGF after axotomy (59,107). NGF receptors have been found to be expressed by Schwann cells after neurotomy (134). This receptor expression was correlated in time with transection, and disappeared at the same time as regenerating axons arrived and made contact with the Schwann cells (134). It appears that NGF and Schwann cells have an important relationship which may be influenced by traumatized or regenerating axons, but the implications of this relationship are not clear.

A small protein has been isolated from mammalian brain which is similar to NGF (4). This brain-derived protein exhibits growth-promoting qualities. It is reported to be necessary for in vitro survival of some NGF-insensitive sensory neurons and some neural crest-derived NGF-sensitive neurons (4). NGF is necessary for

the survival of explants of sensory and sympathetic neurons, stimulates neurite outgrowth and triggers neurotransmitter expression (62,65). Barde and Thoenen (4) suggest that some neurons may require both NGF and the brain-derived protein during development.

Gangliosides are glycolipids found in neuronal membranes. They have been implicated in neuronal differentiation, growth and synapse formation in the peripheral and central nervous systems (33). Intraperitoneal injection of gangliosides, increases the sprouting of nerves in rats after muscle denervation and augments the number of regenerating axons after crush injury of rat sciatic nerve (32,128). Monosialoganglioside (GM1) was added to the medium of explanted day 8 chick embryo dorsal root ganglion cells which had been induced to sprout by NGF and this resulted in enhanced growth (63). Without NGF, GM1 had no effect (63). The mechanism by which gangliosides can enhance growth is thought to be via cell membranes. They may induce receptor expression or alter membrane-associated enzyme activity, increasing the cell's tolerance to ionic imbalance often present in traumatized nerve (32,33).

Letourneau (65) showed the ability of elongating

neurites *in vitro* to recognize surfaces containing the membrane glycoproteins fibronectin or laminin and preferentially extend along their path. Davis *et al* (21), described an intimate relationship of laminin and substrate-binding neurite-promoting factors in regenerating peripheral nerves. Other putative growth-promoting substances such as thyroxine, spermine and melanocortins have been used to treat animals in an attempt to improve axonal regeneration. Peripheral nerve regeneration is slowed in hypothyroidism, if the thyroid gland is removed, the normal rate returns if the animal is treated with thyroxine and hyperthyroidism results in accelerated nerve regeneration (17,77). A comparison was made between treating a rat with subcutaneous injections of alpha-melanocyte stimulating hormone, thyrotropin-releasing hormone, testosterone and an intraperitoneal injection of spermine after crush-injury of the sciatic nerve. The results revealed that only spermine injected animals had a faster axonal regeneration time. It was suggested that polyamines may accelerate axonal regeneration in the peripheral nervous system (52). In another study, melanocortins (ACTH and MSH-like peptides) were administered subcutaneously to animals with a

peripheral nerve transection. The results implied these peptides stimulated axonal regeneration (140). Finally, subcutaneous injections of $ACTH_{[4-10]}$ were given every other day to rats with a crush-injury to the sciatic nerve. The authors reported an acceleration of muscle reinnervation and an increased density of nerve fibres in treated animals (31). It is interesting that many endogenously occurring substances exhibit growth-promoting properties, but perhaps their concentrations need to be increased under conditions of trauma to be effective. In order to better use their influence, further investigation is required regarding their mechanisms of action.

Exogenous trophic factors have also been the subject of investigation. Pomeranz (97) has shown that with DC stimulation, sprouting was enhanced in chronically denervated rat sciatic nerves. In vitro studies showed neurites of frog (Xenopus) neurons accelerated their growth and curved their path in response to weak extracellular electrical fields (93). The dye pyronin was administered to mice through their drinking water and this was reported to accelerate axonal sprouting of crushed soleus nerve (53).

Tropic and trophic factors may be important in the

nerve's response to injury and could possibly be manipulated to improve peripheral nerve regeneration after nerve transection. The distal stump is clearly important for successful regeneration. Growth promoting factors which are found in the nervous system, such as NGF, gangliosides and fibronectin, may have important roles in regeneration. Appropriate treatment using influences not normally occurring in the nervous system, such as electrical fields, may also enhance regeneration. The knowledge of the effect of these substances upon regeneration can be incorporated during surgical repair after peripheral nerve injury.

The surgical approach used will influence the outcome of peripheral nerve trauma. Excessive stretching, handling, or suture material, can induce further tissue reaction and hamper the regenerative process. The use of autologous nerves grafts to bridge nerve gaps, has gained popularity and success (10,132) However, further research into entubulation techniques may provide an better environment to protect and enhance axonal elongation.

B. INTRODUCTION OF THE EXPERIMENTAL PROTOCOL

Although much has been learned about mammalian peripheral nerve regeneration in the past 25 years, the process which reconnects a transected nerve prior to axonal regeneration and the possible influences over this phenomenon have not been well explored. One difficulty during surgical repair of transected peripheral nerve is the bridging of an interstump gap. A gap is often formed by the removal of nerve tissue due to extensive trauma or necrosis. Nerve guide tube repair after neurotomy, provides an experimental model to study the formation of the tissue bridge which reconnects the proximal and distal stumps prior to axonal regeneration (138,150). The nerve guide tube aids nerve repair by supporting the nerve tips and tissue bridge, helping to align the nerve stumps and allowing for manipulation of the environment surrounding the nerve tips (22,24,25,73,155). Using a polyethylene (PE) nerve guide technique developed in our laboratory, regenerating axons of transected rat sciatic nerve are able to cross a 3 mm gap within 4-6 weeks (24,35). This technique is also useful to study the formation of the tissue bridge which reconnects the proximal and distal

nerve stumps prior to axonal regeneration (138,150).

Initially, we were interested in studying possible tropic or trophic influences on axonal regeneration after transection. However, in our pilot study, when examining the nerve preparations nine days after transection, we found that although axons were not crossing the 3 mm gap, the nerve stumps had reconnected. Upon reviewing the literature, we found that little work had been done on this subject. Thess observations led us to become interested in studying the formation of the tissue bridge which reconnected the proximal-distal nerve stumps prior to axonal elongation. It is known that this tissue bridge is composed of collagen, fibrin, fibroblasts and blood elements but contains no axons (138,150). However, neither the process which results in reconnection of the proximal-distal nerve stumps prior to axonal regeneration nor the factors which might influence tissue bridge formation are well understood (126).

Pilot studies involving fourty-nine Long Evans Hooded male rats, used a slightly modified polyethylene (PE) model to examine the sciatic nerve nine days after transection. As nerve cell bodies and target tissue are known to have essential roles in the success of axonal

regeneration, these factors were considered in our study of tissue bridge formation. After observing nerve reconnection under many conditions, we found that the tissue bridge would reconnect the proximal-distal nerve stumps despite removal of the influence from either nerve cell bodies or target tissue and suspected that tissue bridge formation was a local event. The results of the pilot study led us to design the current experiment.

As mentioned, we were interested in examining the possible influences on the development of the tissue bridge reconnecting the proximal-distal nerve stumps nine days after nerve transection. In this experiment, we examined three of the possible influences on tissue bridge formation preceding axonal regeneration, they were: i) nerve cell bodies and target tissue, ii) factors outside of the nerve guide tube and iii) factors within the nerve.

In this study, we used the same PE nerve guide technique as in the pilot projects and designed three groups of nerve preparations, TRIPLE TRANSECTION, LIGATION and FILTER to examine the above mentioned influences on tissue bridge formation (Fig. 2,3,4).

In Group TRIPLE TRANSECTION, we investigated whether a tissue bridge could form if: 1) the proximal and distal

nerve stumps were isolated from their respective nerve cell bodies and target tissue, 2) factors outside the PE catheter were prevented from entering the interstump gap. To do this, the sciatic nerve trunk was cut three times, in the middle, proximally and distally, thereby removing the nerve cell bodies' and target tissue's influence on tissue bridge formation. The PE catheter was then plugged with petroleum jelly or heat-sealed with plastic discs, to prevent the entry of outside factors into the interstump gap.

In Group LIGATION, we examined the relative influence of intrinsic neural or vascular flow from the proximal or distal nerve stumps on the rate of tissue bridge formation. The sciatic nerve was ligated either proximally or distally to the transection site and then transected in the middle.

Finally, we designed Group FILTER to exclude the possibility that the heat-sealed TRIPLE TRANSECTION nerve preparations did not reconnect due to a lack of oxygen or other nutrients. We modified the heat-sealed TRIPLE TRANSECTION nerve preparation by cutting a window in the PE catheter and covering it with a MicronSep 0.45 um pore

nylon filter*. The filter would allow diffusion of oxygen and other fluids into the nerve preparation while excluding the physical penetration of microvessels.

Nerve guide tube repair is used to aid bridging interstump gaps after peripheral nerve transection. Regenerating peripheral nerve fibres require particular conditions for success, including a favorable surface to grow upon (33,65,102,112,132). The tissue bridge observed at nine days provides a surface for axonal elongation, but the progress and regulation of its formation has not been well studied. A better understanding of this process is critical because an optimal tissue bridge reconnecting transected nerve stumps, may improve the density and rate of axonal regeneration which follows.

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* 11769 MicronSep, Honeoye Falls, NY, 14472.

C. METHODS

In the current study, twenty-two Long Evans Hooded male rats (350-445 g) were used (forty-nine additional rats were used in the pilot experiments). With each rat, both sciatic nerves were exposed but each underwent a different procedure. A systematic approach was taken to isolate the interstump region from potential sources of influence and observe tissue bridge formation.

a. Surgical Method

All animals were anaesthetized with ketamine 60 mg/kg intramuscularly, pentobarbital 21 mg/kg intraperitoneally and atropine 0.1 cc. intramuscularly, and prepared for surgery.

The rat was placed in a prone position and restrained in a head holder with its hindlegs taped to the table. Body temperature was monitored with a rectal thermometer and maintained at 37°C with a heating blanket.

Using a clean surgical technique, a 4 cm. long skin incision was made over each thigh. The gluteus maximus and biceps femoris muscles were divided and retracted with

sutures. The sciatic nerve was dissected free of loose connective tissue and surrounding muscle from the sciatic notch to the nerve's trifurcation. This provided a field of approximately 2.5 cm. The nerve to biceps femoris muscle, which branches from the sciatic nerve was cut close to its origin and this site was designated the middle of the nerve exposure. A 10-0 nylon marker stitch was made there, to be used later as a reference point.

A 1.5 cm long piece of PE 280 tubing (inner diameter = 2.15 mm. and outer diameter = 3.25 mm.) was sliced with a scalpel longitudinally and pierced once at each end with a cutting needle, to ease suturing. The catheter was then disinfected with alcohol and rinsed with sterile saline.

The prepared PE catheter was slipped around and sutured to the nerve with one 10-0 nylon epineurial stitch at each end of the catheter. The longitudinal slice and holes were sealed with fast-acting glue (Fig. 1a). The PE catheter and nerve were then transected with one cut, using a sharp razor blade held in a blade breaker. Since the catheter offered firm support for the nerve during transection, nerve torsion and compression were minimized.

At this point different steps were taken to pose particular questions, these nerve preparations are

SURGICAL METHOD

FIGURE 1.

a. A 10-0 nylon epineurial marker stitch was made at the middle of the nerve exposure. A polyethylene catheter (PE cath) was slit longitudinally and needle holes were made at each end of the catheter to ease suturing. The PE catheter was placed around the sciatic nerve and anchored to it with one 10-0 epineurial stitch at each end. The slit and needle holes were sealed with fast-acting glue.

b. The PE catheter and nerve were transected with a sharp blade at the marker stitch (m). This left two pieces of catheter, a proximal and distal stump (-------- = transection site).

c. The PE catheters were reapposed, as before the transection, leaving an interstump gap of 3 mm. A flexible, plastic external catheter (ext. cath.), also slit longitudinally, was placed around the PE catheters to maintain their position.

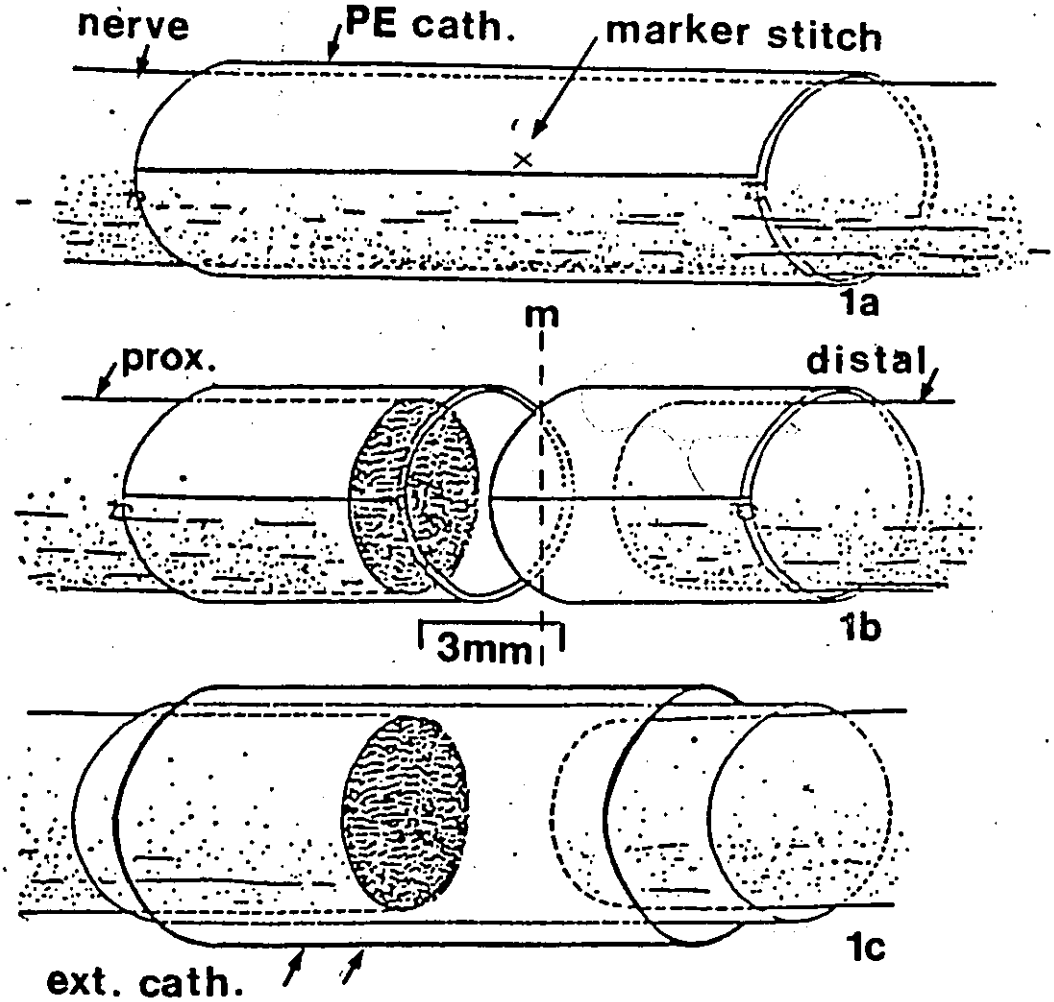

nerve
PE cath.
marker stitch
1a
m
prox.
distal
3mm
1b
1c
ext. cath.

FIGURE 6.

Longitudinal section of Group TRIPLE TRANSECTION with petroleum jelly nine days after transection (Lendrum-Fraser stain).
ps = proximal stump, ds = distal stump, fm = fibrin matrix, col = collagen fibres, bv = blood vessel-like formation.

a. proximal and distal stumps reconnected with a tissue bridge nine days after transection. Extravasated blood present close to nerve stumps, cells have migrated into the gap region.

b. higher power of a middle section of tissue bridge. Blood cells seen in a capillary-like formation. Collagen fibres formed along the nerve axis and fibrin matrix surrounding the collagen fibres in the middle of the interstump region.

described below. In every case, when the procedure was completed, the two catheters were reapposed, returned to the position they were in before transection, restoring the original alignment of the nerve (Fig. 1b). The gap between the proximal and distal stumps (consistently 3 mm ± 0.1 mm) and the distance between the marker stitch and the proximal stump were measured. This point did not move relative to the lesion site during the recovery period and therefore offered a reliable measurement of tissue outgrowth. An external catheter (Tygon flexible plastic tubing, i.d. = 3.75 mm. o.d. = 4.80 mm.), which was also sliced longitudinally and disinfected, was wrapped around the PE catheters to maintain their position (Fig. 1c). The muscles were closed with three 3-0 nylon sutures and the skin closed with skin clips. Betadine was immediately applied to the skin.

After surgery, each animal received an intramuscular injection of penicillin. The animal was allowed to recover in the laboratory, with a heating blanket under half of the cage. After 24 hours, a neurological examination was performed to ensure total transection had occurred. Animals were housed in single cages in a temperature, humidity and light controlled environment.

Food and water were available ad libitum.

There were two animal deaths during the entire study (including the pilot studies) and no infections occurred. With the sciatic nerve transected, the animals were capable of grooming, eating and drinking.

The characteristic gait of an animal with its sciatic nerve transected was weight-bearing on hindlimb heels, with the hindlimb brought forward in a circumductory manner. No weight-bearing on the plantar aspect of the foot with splaying of toes was observed.

b. Nerve Preparations

i. Pilot Studies

TRANSECTION--The nerve and PE catheter were transected in the middle, as described in Surgical Method, to observe tissue bridge formation after nine days (n=8) (Figure P1).

CELL BODY/TARGET TISSUE--Two nerve preparations were designed to exclude the influence on the interstump gap of elements from either: a) proximal sources called "nerve cell bodies influence" or b) distal sources called "target tissue influence".

1) The nerve and catheter were transected in the middle and then the nerve trunk was transected a second time 5 mm proximally. This resulted in an isolated piece of nerve tissue within the catheter and the distal nerve stump which was still connected to the target tissue (Figure P2a). This nerve preparation tested the nerve cell bodies' influence on the formation of the tissue bridge (n=10).

NERVE PREPARATIONS PILOT STUDIES

FIGURE P1. TRANSECTION

The nerve and PE catheter were transected one (-------- = transection site), in the middle (m) of the nerve preparation.

FIGURE P2. CELL BODY / TARGET TISSUE

a) The nerve and PE catheter were transected at m and 5 mm proximally (p).

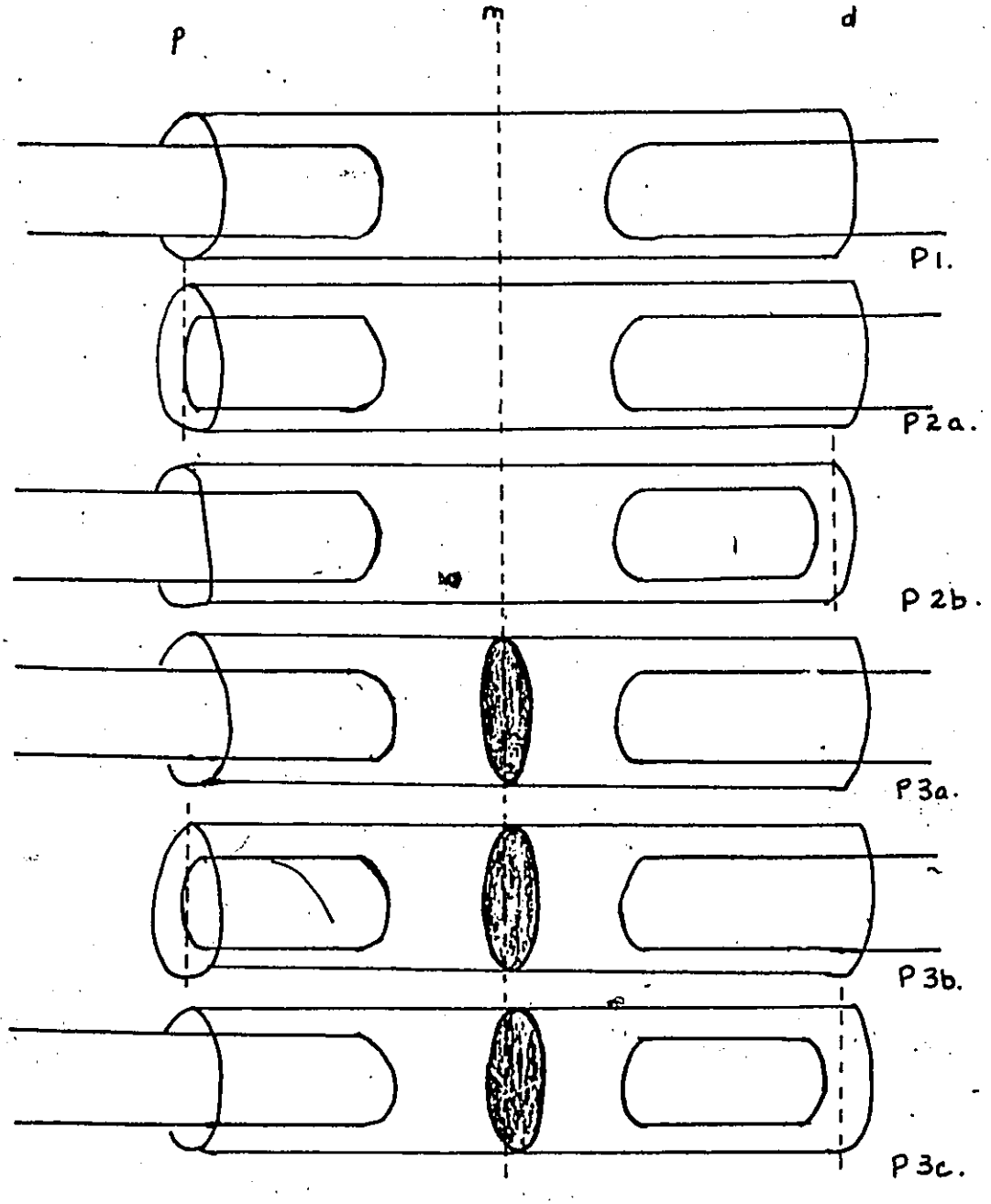
b) The nerve and PE catheter were transected at m and 5 mm distally (d).

FIGURE P3. NERVE STUMP BLOCK

a) The nerve and PE catheter were transected at m, then each open end of the cut catheter was heat-sealed with a 5 mm thick impermeable plastic disc.

b) The nerve and PE catheter were transected at m and p, then heat-sealed as described above.

c) The nerve and PE catheter were transected at m and d, then heat-sealed as described above.


p
m
d
P1.
P2a.
P2b.
P3a.
P3b.
P3c.

2) The nerve and catheter were transected in the middle and then the nerve trunk was transected a second time 5 mm distally (Figure P2b). This tested the target tissue's influence on the formation of the tissue bridge (n=7).

NERVE STUMP BLOCK--Three nerve preparations were designed to test the possible influence of one nerve stump upon the other with respect to tissue bridge formation.

1) The nerve and catheter were transected in the middle, then both inside open ends of the catheter were heat-sealed with 1 mm thick impermeable plastic discs (Figure P3a). Although this prevented nerve stump reconnection, the growth from each nerve stump could be measured (n=7).

The heat-sealing procedure did not appear to overly heat the nerve tissue. This was tested by inserting a probe thermometer into a piece of catheter while the cauterizer's hot tip (used to seal the discs) touched the catheter repeatedly. The thermometer was in the place where the cut end of nerve would be, and the temperature never exceeded 37°C.

2) The nerve and catheter were transected in the middle and the plastic discs sealed, as described above, then the proximal nerve trunk was transected a second time (Figure P3b). This was done to isolate the proximal nerve stump and test its influence on the formation of the tissue bridge (n=4).

3) The nerve and catheter were transected in the middle and the plastic discs sealed, as described above, then the distal nerve trunk was transected a second time (Figure P3c). The aim of this nerve preparation was to isolate the distal nerve stump and test its influence on the formation of the tissue bridge (n=6).

TRIPLE TRANSECTION (PERFORATION)--When the heat-sealed TRIPLE TRANSECTION nerve preparation did not reconnect, it suggested that entry into the catheter of an outside factor was necessary for the formation of the tissue bridge. The heat-sealed procedure was repeated, except that a 0.5 mm diameter cutting needle was used to punch eight holes in the middle of the catheter to allow the passage inside of external factors (n=9) (Figure P4).

DOUBLE TRANSECTION--With the PE catheter in place, instead of transecting the catheter and nerve in the middle, two transections were made, one at either end of the catheter, leaving 1 cm long isolated piece of nerve within the catheter (Figure P5). This preparation was designed to see if the tissue bridge would extend from the nerve tissue outward, in the absence of other nerve tissue (n=6).

NERVE PREPARATIONS PILOT STUDIES

FIGURE P4. TRIPLE TRANSECTION (PERFORATION)

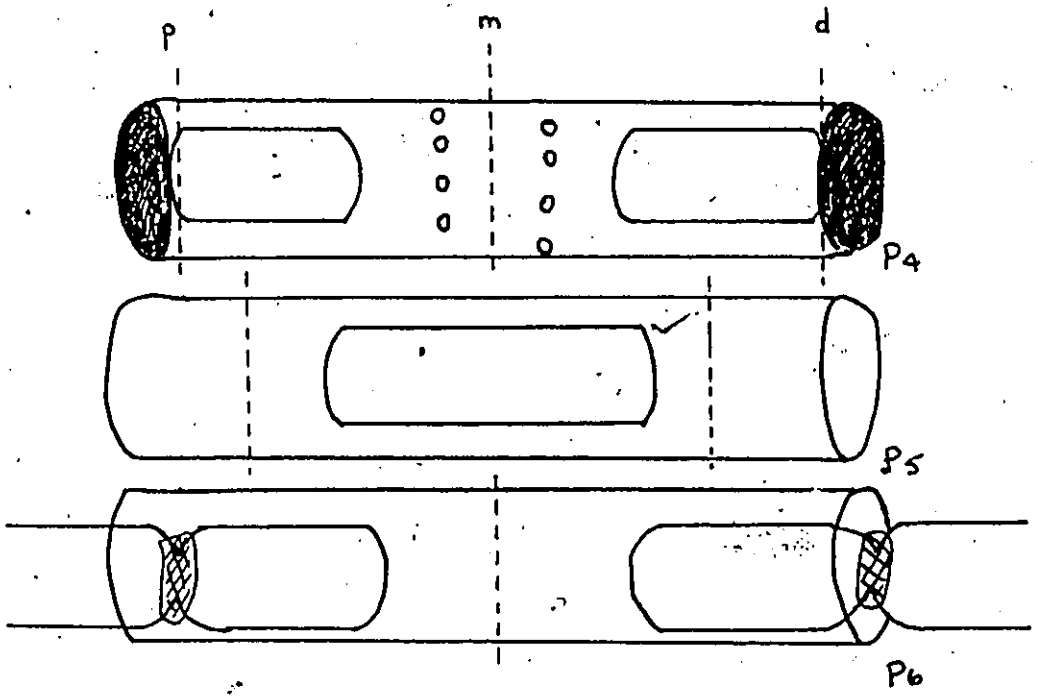
The nerve and PE catheter were transected once at the middle (m) and then 5 mm proximally (p) and 5 mm distally (d). The catheter was perforated eight times in the center with a 5 mm diameter cutting needle.

FIGURE P5. DOUBLE TRANSECTION

The nerve trunk was transected twice leaving a 1 mm long isolated piece of nerve within the catheter.

FIGURE P6. DOUBLE LICATION

The nerve trunk was ligated 5 mm proximal and 5 mm distal to the middle of the nerve preparation and then the nerve and PE catheter were transected at m.


p
m
d
P4
P5
P6

DOUBLE LIGATION--The nerve trunk was ligated 5 mm proximally and distally to the transection site (Figure P6). This study was done to see if a tissue bridge would reconnect the nerve stumps (n=4).

ii. Current Study

Group TRIPLE TRANSECTION.

Nine nerves were transected three times: at the marker stitch and 5 mm proximal and distal to it. This procedure removed the nerve cell bodies' and target tissue's influence on the interstump gap. The catheters were then reapposed and the open ends, facing the external fluid, were plugged with petroleum jelly (Fig. 2a).

In thirteen additional nerve preparations, the petroleum jelly was replaced by a 1 mm thick impermeable plastic disc which was heat-sealed to each open end of PE catheter (Fig. 2b). This nerve preparation maintained the nerve stumps in isolation from the influences described above while further preventing the entry of all factors from outside the catheter, into the nerve preparation. Because of the brief study period, we felt that cells in the nerve preparations would remain viable. Therefore, if

NERVE PREPARATIONS

FIGURE 2. Group TRIPLE TRANSECTION.

a. The nerve and PE catheter were transected three times (----- = transection site), 5 mm proximal to the middle of the nerve exposure (p), the middle (m), and 5 mm distal to the middle (d). The open ends of the PE catheter were plugged with petroleum jelly (not shown).

b. The nerve and PE catheter were transected three times at p, m and d. The open ends of the PE catheter were heat-sealed with impermeable plastic discs.

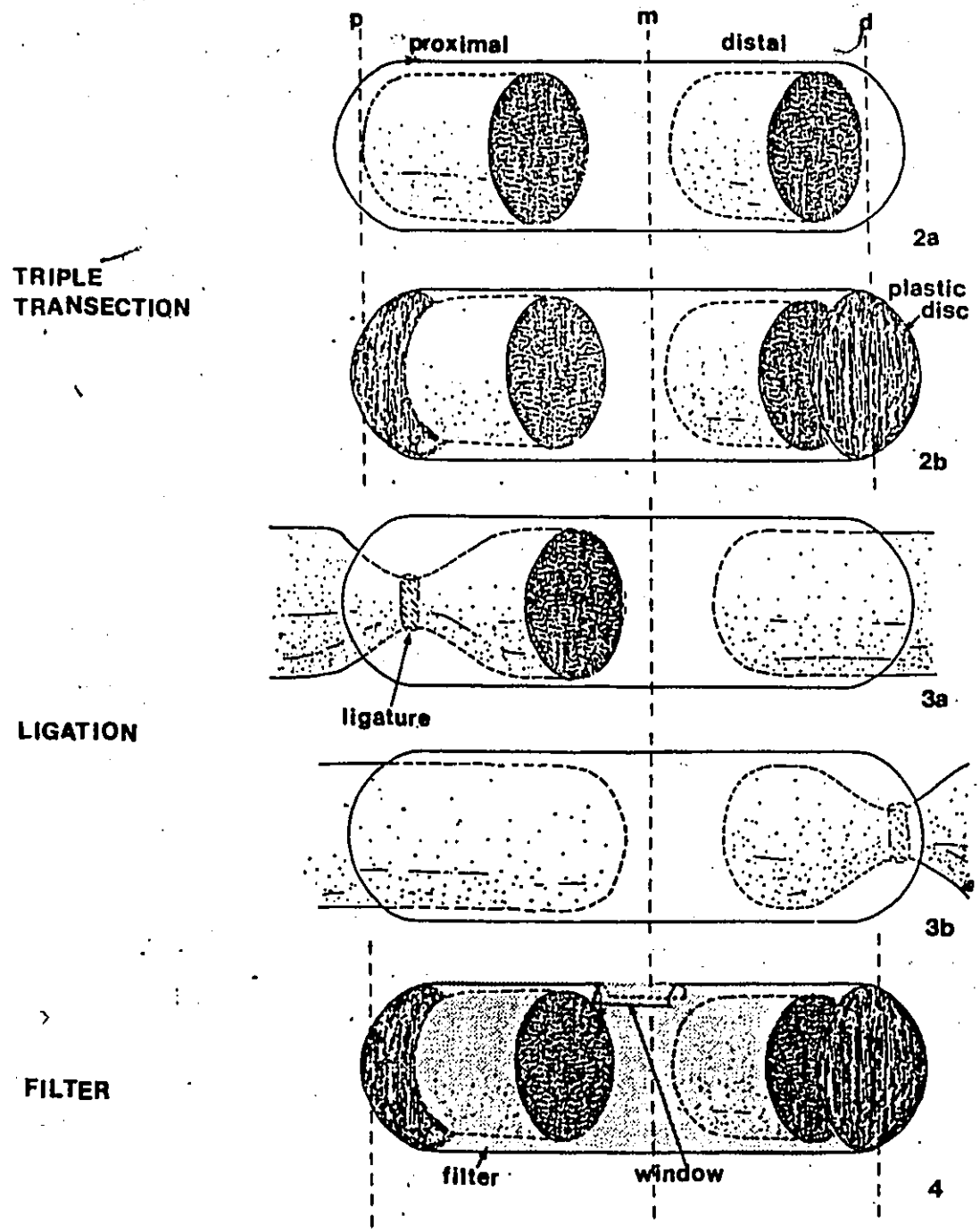
FIGURE 3. LIGATION

a. The nerve trunk was ligated 1.5 mm <u>proximal</u> to the marker stitch, ensheathed by the PE catheter and transected at m.

b. The nerve trunk was ligated 1.5 mm <u>distal</u> to the marker stitch, ensheathed by the PE catheter and transected at m.

FIGURE 4. FILTER.

A 2X2 mm window was cut in the PE catheter, the PE catheter and nerve were transected at p, m and d. The open ends of the PE catheter were heat-sealed with impermeable plastic discs. A 0.45 um pore nylon filter was wrapped around the catheter, covering the window. The filter and PE catheter were held in place with an external catheter (not shown).


p
m
d
proximal
distal
2a
TRIPLE
TRANSECTION
plastic
disc
2b
ligature
3a
LIGATION
3b
FILTER
filter
window
4

intrinsic fibroblasts and Schwann cells were responsible for tissue bridge formation, then nerve reconnection would be expected.

Group LIGATION.

In this Group, the entry into the interstump gap of intrinsic blood vessels, axoplasm and lymphatics was impeded from either the proximal or distal stumps by a ligature.

PROXIMAL LIGATION. Nine nerves were tightly ligated with a 3-0 suture 1.5 mm proximal to the marker stitch and then ensheathed with the PE catheter. The nerve and catheter were transected at the marker stitch and the surgery was completed as described in Surgical Method (Fig. 3a).

DISTAL LIGATION. Six nerves underwent the same technique as described in PROXIMAL LIGATION, except that the ligation was placed 1.5 mm distal to the marker stitch (Fig. 3b).

Group FILTER. Six nerves were transected and sealed as in the heat-sealed TRIPLE TRANSECTION nerve preparation. A 2X2 mm window was cut in the PE catheter and a MicronSep filter was placed around the PE catheter, covering the window (Fig. 4). The resulting increase in diameter was sufficient to prevent the external catheter from completely closing around the PE catheter and filter, leaving a gap where the filter was exposed to the surrounding fluid. This procedure was used to allow oxygen and other nutrients to diffuse into the nerve preparation, while preventing microvessels from entering the interstump gap.

D. MORPHOLOGICAL EXAMINATION

All animals were anaesthetized at the three or nine day time point. Two Group LIGATION animals were studied after three days to compare the tissue bridge extending from the ligated and non-ligated nerve tips before nerve reconnection and the rest were studied at nine days.

All nerves were removed from the catheters and immediately fixed in 10% buffered formalin except for the Group LIGATION rats which underwent left cardiac ventricular perfusion with heparinized saline followed by 10% buffered formalin. In three Group LIGATION animals, studied at nine days, a 70% india ink solution * followed fixation perfusion to fill blood vessels for vascular examination (144). Nerves were then removed and post-fixed in 10% buffered formalin.

Ink-perfused nerves were frozen with dry ice, sectioned 80 or 120 um on a sliding microtome and counterstained with cresyl violet. Other nerves were embedded in paraffin, blocked and sectioned 6 um on a rotating microtome. Sections were stained with hematoxylin and eosin for examination of tissue morphology, Lendrum-Fraser for fibrin and collagen (60), or Garvey's stain for mature fibrin (29).

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*CFC Liquid Inks, Aquablak, 115.

E. RESULTS

Gross examination revealed that all the external catheters were covered with thin connective tissue which was well vascularized at nine days. When the nerve preparation was clearly exposed, a blood vessel network was visible on the nerve stump and tissue bridge surfaces. This vascular invasion was evident except in the two nerve preparations where a tissue bridge did not form: heat-sealed TRIPLE TRANSECTION and FILTER.

Tissue outgrowth formed characteristic shapes in the different nerve preparation Groups, and also varied according to the time point at which they were examined (See Table 1.).

Histological examination revealed that the tissue bridge was composed of fibroblasts, red and white blood cells, collagen and fibrin. The blood cells in the middle of the tissue bridge were in blood vessel-like formation, while closer to the nerve tip, they were extravasated. In some cases, a fibrin matrix with a small number of scattered blood cells was observed extending from the nerve tip.

TABLE 1.

Shape of Tissue Bridge Reconnection

NERVE PREPARATION	PROXIMAL STUMP DISTAL
STUMP	
TRIPLE TRANSECTION	
Petroleum Jelly	
Heat-sealed	
LIGATION	
Proximal (three days)	
(nine days)	
Distal (three days)	
(nine days)	
FILTER	

a. Pilot Studies

Transection

The nerve and catheter were transected in the middle, and nine days later the proximo-distal stumps were reconnected with a tissue bridge which was either the same diameter as the nerve stumps or slightly tapered in the middle.

Cell Body / Target Tissue

In this Group, the nerve and catheter were transected in the middle and then again either 1) proximally or 2) distally. After nine days, a cone-shaped tissue bridge reconnected the proximo-distal stumps.

Nerve Stump Block

In all three nerve preparations, the proximal and distal nerve stumps were separated by an impermeable plastic disc. This prevented reconnection, but the distance of growth from the nerve tips was measured nine

days after transection.

1) After nerve and catheter transection in the middle, the tissue which extended from each nerve tip averaged 1 mm.

2) The proximal nerve stump was transected a second time after the middle transection. After nine days, tissue extended from the proximal nerve stump an average of 0.4 mm, and 1 mm from the distal stump.

3) The distal nerve stump was transected a second time after the middle transection. In this preparation an average of 0.4 mm of tissue extended from the distal stump and from the proximal stump, 1 mm of tissue was measured.

Triple Transection (Perforation)

This preparation was a triple transection with heat-sealed discs closing the open ends, also eight holes were made in the catheter. After nine days, two of the nine nerve preparations showed a thin, fragile tissue bridge reconnecting the proximo-distal stumps. In three nerve preparations, the proximal and distal nerve stumps each had tissue extending from it an average of 0.5 mm. There was no growth in the remaining four preparations.

Double Transection

This preparation produced no gap or reconnection, as there was only an isolated piece of nerve. However, tissue extension was measured from each end outwards. From the nerve tissue proximally, the tissue averaged 1.5 mm. and distally it measured 0.7 mm. The tissue was heavily stained for collagen and displayed very little matrix when examined.

Double Ligation

The sciatic nerve was ligated proximally and distally and transected. After nine days, three out of four nerve stumps reconnected, but the bridge was thin and fragile, unlike the ligation experiments in the current study.

b. Current Study

Group TRIPLE TRANSECTION.

All nine nerve preparations plugged with petroleum jelly reconnected with a tissue bridge. The shape of the tissue bridge was either the same diameter as the nerve stumps, or somewhat tapered in the middle of the bridge (Fig. 5). Red blood cells were seen throughout the length of the bridge (Fig. 6). Of the heat-sealed nerve preparations, 12 out of 13 did not reconnect, no cell migration or fibrin matrix was observed. In one nerve preparation, there was a thin strand of tissue connecting the nerve stumps.

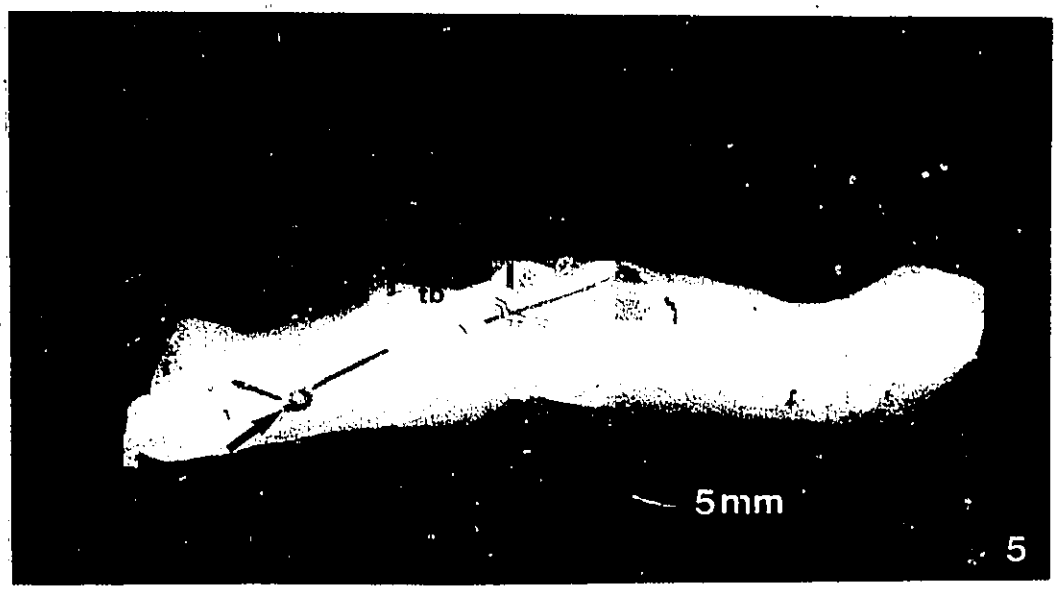
Group LIGATION.

At three days: The three nerves examined did not reconnect. A cylindrical fibrin matrix extended from the non-ligated side towards the middle of the catheter in a less dense pattern and containing fewer cells than seen at nine days (Fig. 7).

FIGURE 5.

Group TRIPLE TRANSECTION with petroleum jelly, nine days after transection.

The nerve preparation after the removal of all catheters. The proximal and distal nerve stumps have reconnected with an opaque, cylindrical tissue bridge (tb), of similar diameter to the nerve stumps. Marker stitch on the proximal stump (arrow).


5mm
5

6a

FIGURE 7.

Fibrin matrix present in the gap three days after transection. A section from a nerve preparation in Group LIGATION (Lendrum-Fraser stain). This is the beginning of the development of the tissue bridge, but has not yet spanned the interstump gap. The network is more open, with larger spaces and blood cells are less dense than seen at nine days.

FIGURE 8.

The fibrin matrix present in the interstump gap nine days after transection. A section from near the apex of the cone-shaped tissue bridge of a nerve preparation in Group PROXIMAL LIGATION with petroleum jelly (hematoxylin and eosin stain). Red and white blood cells are present (arrows).

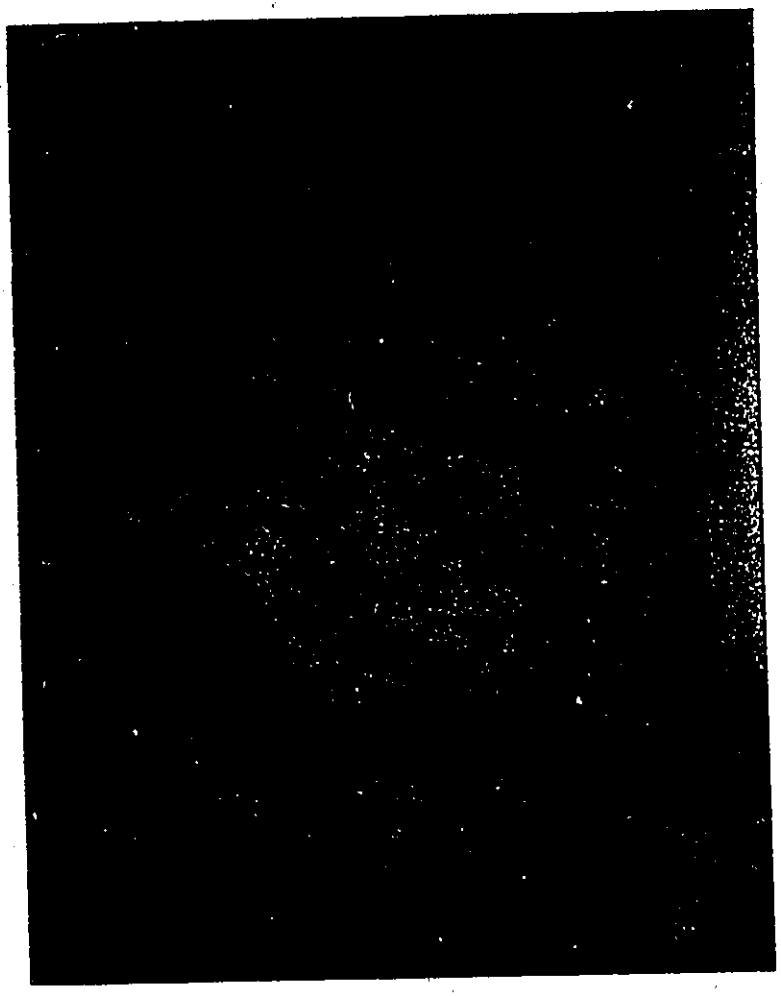
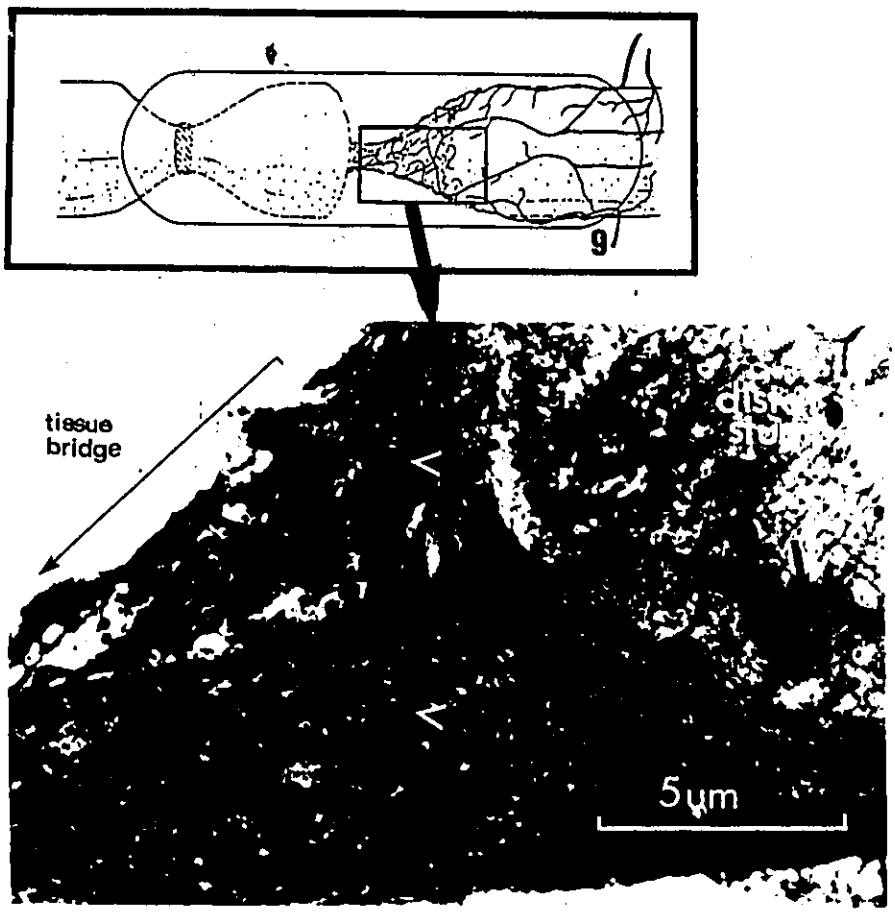


FIGURE 9.

Diagram of india ink perfusion of PROXIMAL LIGATION nerve preparation. INSET- photograph of distal stump and tissue bridge. Ink filled blood vessels are dense in the tissue bridge (white arrows), and somewhat more sparsely in the distal (non-ligated) stump (black arrow).


tissue
bridge
5µm

At nine days PROXIMAL LIGATION: Six out of seven nerves reconnected with a conical bridge, the base of the cone at the distal stump. A fibrin matrix was observed near the apex of the cone (Fig. 8). Ink-filled blood vessels appeared more dense in the non-ligated distal stump, forming a complex vascular network in the tissue bridge (Fig. 9).

At nine days DISTAL LIGATION: Four out of five nerves reconnected with a conical bridge, the base of the cone at the proximal stump. Morphology and perfusion results were similar to PROXIMAL LIGATION.

FILTER. None of the six nerves reconnected. Fibroblasts and neutrophils were observed, but no fibrin matrix was present.

F. DISCUSSION

In our pilot studies using the PE technique, we did not see regenerating axons in the tissue bridge nine days after transection, and at three days, the tissue bridge had not spanned the proximal and distal nerve stumps. Therefore, the nine day time point was used to study possible influences on tissue bridge formation prior to axonal regeneration. The three day time point was used to examine tissue formation prior to reconnection. The pilot studies revealed that neither the cell body or target tissue influence was necessary for the formation of the tissue bridge reconnecting the proximal and distal stumps.

Although it has been shown that a distal stump is required for successful axonal regeneration (73,151), this does not appear to be the case for tissue bridge formation. This was demonstrated by the NERVE STUMP BLOCK nerve preparation and further supported by DOUBLE TRANSECTION, where no nerve stump was present to influence the tissue growth which occurred.

Other studies provided clues as to what was occurring in the interstump gap. The TRIPLE TRANSECTION

(PERFORATION) nerve preparation supported our hypothesis that for tissue bridge formation to occur, elements from outside the catheter needed to enter into the interstump region. However, this approach was inconclusive because the hole size was large enough to allow penetration of blood vessels and other factors from the extracellular fluid into the catheter. In order to address this issue, the FILTER nerve preparation was designed, to exclude the possibility of blood vessel entry into the interstump region but to allow oxygen and nutrients inside. In the current study, when both proximal and distal ligation nerve preparations reconnected, DOUBLE LIGATION was designed. Reconnection after double ligation suggested that extrinsic blood vessels could enter the catheter, bypass the ligation and play a role in tissue bridge formation inside the interstump gap.

The pilot studies served to adjust the direction of the project, but it was the results of the current study which were the most interesting.

Nerve transection using this PE technique consistently results in a 3 mm interstump gap. A tissue bridge reconnects the proximal and distal nerve stumps in nine days and axons elongate across it within 4-6 weeks

(24,35). These findings agree with results from others using similar nerve guide models (73,138,150).

In Group TRIPLE TRANSECTION, petroleum jelly plugs blocked influences from the nerve cell bodies and target tissue and from other surrounding elements; nonetheless, the nerve stumps reconnected. The question of the permeability of the petroleum jelly was tested by incubating the nerve preparation (PE and external catheters plugged with petroleum jelly) at 37° C with Evans Blue used as a marker for diffusion. After nine days, there was no evidence of Evans Blue diffusion from inside out of the nerve preparation or the reverse. The results of the petroleum jelly TRIPLE TRANSECTION nerve preparations, suggested that the cell bodies and target tissue did not influence tissue bridge formation. However, it was still unclear whether intrinsic fibroblasts and Schwann cells formed the tissue bridge, or its formation was due to an outside influence which penetrated the petroleum jelly. This question led us to use the heat-sealed TRIPLE TRANSECTION nerve preparations, to more completely exclude the possibility of elements from the surrounding environment from entering the interstump gap. In the heat-sealed TRIPLE TRANSECTION nerve preparations,

after nine days, a tissue bridge did not form. At this point, we surmised that elements external to the nerve preparation were needed for the formation of the tissue bridge, and suspected that revascularization of the nerve stumps was involved, since we had observed blood vessel penetration of the petroleum jelly blocked nerve preparation.

To test the hypothesis that vascularization of the nerve stumps was critical for tissue bridge formation, the Group LIGATION nerve preparations were designed. Intrinsic vessel, axoplasmic and lymphatic flow were impeded from entering the interstump gap from either the ligated proximal or distal stump. After nine days, a conical tissue bridge extended from the non-ligated nerve tip, suggesting that factors carried or transported in the nerve, not necessarily originating from cell bodies or target tissue, could be involved in the tissue bridge formation. The results from the india ink perfusion showed that blood vessels had penetrated the tissue bridge and formed a rich vascular network. We also noted that at three days, the first elements observed in the interstump gap were fibrin and blood cells.

Finally, to exclude the possibility that the heat-

sealed TRIPLE TRANSECTION nerve preparations had not reconnected because the nerve tissue had reduced availability of oxygen and nutrients, we used the FILTER nerve preparation. The filter pore size would allow diffusion of oxygen and other nutrients into the nerve preparation while preventing microvessel penetration. Although intrinsic fibroblasts and Schwann cells had access to oxygen and other nutrients, a tissue bridge was not formed. Therefore, it appeared that these cells alone were not responsible for tissue bridge formation, or they lacked some factor(s) from outside the PE catheter which was excluded by the 0.45 um filter.

It became apparent that nerve cell bodies, target tissue, intrinsic cells and factors from outside the catheter, which were diffusible through a 0.45 um filter, were not essential for tissue bridge formation, although likely important regarding the ultimate regenerative response of the nerve. However, india ink perfusion demonstrated an elaborate vascular network which formed within and around the tissue bridge, the intrinsic flow from the unligated stump appeared to be important and we observed that the fibrin matrix is the first substance seen in the interstump gap. These observations were also

supported by the fact that when the animal's thigh was opened after the observation period, we were struck by the tremendous display of revascularization around the implanted catheter, and upon dissection, within it when blood vessel penetration was possible. This evidence strongly suggests that revascularization was fundamental to tissue bridge formation prior to axonal entry.

Neovascularization, and a prolonged increase of endoneurial permeability has been observed after peripheral nerve injury (90,145). Products of axonal breakdown and/or chemicals released during degeneration have been proposed to cause the change in permeability (145). The precursors of the fibrin matrix likely enter the interstump gap when transected nerve tips bleed and clot and via newly formed blood vessels with their increased permeability (70,90,127). The fibrin matrix appears to be the foundation of the tissue bridge. At three days, blood cells were observed within the fibrin matrix while fibroblasts were not yet seen (Fig. 8). When the filter prevented microvessels from entering the nerve preparation (Group FILTER), no fibrin matrix formed but other elements of the tissue bridge were also not seen in the interstump gap.

In response to vascular injury, the plasma glycoprotein, fibronectin is cross-linked to the fibrin clot during the generation of thrombin (84). The clot is composed of 95% fibrin, 4.4% fibronectin and about 0.7% alpha-antiplasmin (a plasmin inhibitor, also cross-linked to fibrin) (84). Fibronectin is widely distributed in mammals, in plasma, milk, urine, tears, synovial and cerebrospinal fluid, interstitial connective tissue, vessel walls and in association with most basement membranes (139). Several cell types secrete fibronectin, including fibroblasts.

As well as providing a substrate for migrating fibroblasts and other cells, the fibronectin-containing fibrin clot, may be a chemoattractant to them (139). In tissue culture, fibronectin supports cell adhesion and migration. In the developing organism, the extracellular matrix is implicated in cell attachment and motility as well as the induction and direction of cell migration (108). The fibrin matrix observed at three and nine days appears to have a similar role in regeneration as extracellular matrix does in development. By connecting the proximal-distal stumps, it provides a substrate for fibroblast migration.

Using this PE technique, we observe a two-step nerve regeneration process. In contrast to Williams' report (150), our findings indicate that revascularization is the first step of tissue bridge formation; with vessel penetration into the interstump gap and the formation of the fibrin matrix. The matrix appears to be the substrate upon which cells migrate and collagen is secreted, forming the tissue bridge observed at nine days. In the second step, axons may regenerate across the tissue bridge, under appropriate conditions, i.e. when the nerve cell bodies and target tissue are connected to the nerve stumps and when tropic and trophic influences are available to the regenerating fibres (48,95,102,151,154).

There is substantial evidence that peripheral axonal regeneration is dependent on adequate blood flow and that a vascular deficiency can result in nerve degeneration (10,13,19,55,78,100). Support for this view can be found in experimental models such as diabetes and microsphere- or pressure-induced ischemia and studies of surgical repair.

In galactose-fed rats, an animal model for diabetes, galactose enters the endoneurial compartment and is converted to galactitol which cannot escape the blood-

nerve barrier. This causes endoneurial edema resulting in a reduction of intrinsic blood flow and damage to myelinated fibres. To study regeneration, the sciatic nerves of galactose-fed rats were transected. It was found that cell migration, axonal elongation, myelination and vascularization were abnormal (100). In another experimental diabetic model, induced by streptozotocin, blood flow studies revealed resting nerve flow was 40% less than normal in diabetic animals (81). Others report a significant reduction of nerve blood flow in both types of experimentally diabetic rats (137). This supports the view that diabetic axonopathy is related to decreased blood flow, and that blood flow to peripheral nerve cannot be compromised without functional loss, including impairment of regenerative capability.

Axonal dependence on blood flow is further demonstrated by studies of ischemia. Occlusion of capillaries and pre-capillaries in sciatic nerve by microspheres results in wide-spread nerve degeneration, without distal gangrene (86). Pressure-cuff induced ischemia causes a nerve conduction loss within thirty minutes, whereas the distal stump of a transected nerve with an intact blood supply will retain its excitability

for up to 3 days (13,70,133). Peripheral nerves can sustain pressure-cuff ischemia for 4-6 hours with minimal ischemic damage when reperfused (70). The excitability of rabbit tibial nerve was shown to be directly dependent on blood flow *in vivo* and *in vitro* (13).

The effect of surgical repair on peripheral nerve blood flow has also been studied. Anastomosis under tension has been associated with both blood flow changes and impaired regeneration (10,25,70,80). Stretching a nerve as little as 8% of its length results in a decrease in intrinsic blood flow to the nerve tip (70). During surgical repair, nutrient vessels are often ligated or severed. Several authors have described minimal nerve damage after ligation of nutrient blood vessels (1,3,55). These studies were of the sciatic nerve and may reflect its adequate intrinsic supply due to an interfunicular arteriole running its length (131). The conclusion that the ligation or severance of nutrient vessels would not adversely affect a nerve's regenerative capacity should not be applied to other nerves without such perfusion. In contrast, sciatic nerve blood flow was measured after femoral artery ligation and found to decrease and result in a proportionately more ischemic core (123). These

conflicting results may relate to a difference in sensitivity of instruments, or perhaps, the resistance of peripheral nerve to ischemic damage.

There are some reports which do link the need for adequate blood flow to successful regeneration. Because intra-operative nerve mobilization disrupts nerve blood flow, Smith (123,124) advocated minimizing this procedure to avoid excessive ischemic nerve damage. An advance in surgical repair to bridge an interstump gap has been made with the free vascularized nerve graft (10). One aim of this technique is the rapid revascularization of the nerve graft which is "critical for its successful reinnervation by regenerating axons." (10). Porous materials shaped into nerve guide tubes are being tested with the intention to allow blood vessel penetration (19,75).

To put many of the preceding thoughts together, a synthetic graft might be designed to bridge interstump gaps. Each component must be tested independently, to determine their role in the model. A porous nerve guide tube would be used to provide support for nerve stumps and the regenerate, and allow as close an apposition as possible without tension or suture at the nerve tips. The pores (>8 um) would permit rapid microvessel penetration

and exposure of the regenerating axons to other circulating factors which may aid in stimulating growth. A mixture of fibrin, fibronectin and collagen would be used to fill the interstump gap. Different growth-promoting factors (gangliosides, MSH, fibronectin) could be mixed with the matrix. Finally, a stimulus for blood vessel proliferation, for example lipid angiogenic factor, derived from omentum, or the omentum itself (23) could be applied to the preparation. By maximizing all of the apparent factors necessary for optimal regeneration, one would expect improved results.

Central nervous system axons may have similar needs to peripheral nerve fibres. A recent study using an omental pedicle on transected cat spinal cord demonstrated the importance of blood flow for robust axonal regeneration (23). Kiernan (54) postulated that "axons of any type can regenerate only when their growing tips are bathed in extracellular fluid containing proteins derived from the plasma of the blood". This hypothesis has been supported by transplantation studies in the CNS (41).

It is well known that blood flow to living tissues is essential for survival and growth. Blood flow prior to axonal elongation appears to be fundamental for laying the

foundation for axonal growth. Axons require the diffusion or transport of substances from the blood to remain viable, and a decrease in blood flow affects conduction or regeneration, prolonged reduction or a complete arrest in blood flow causes degeneration (70,78,87,100,124,125,137). We believe that the provision of adequate blood flow to regenerating mammalian nerve is an essential component of repair and may be a key to improved regeneration in mammalian nervous systems.

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ABSTRACT

An interstump gap poses a difficult problem for the surgical repair of injured peripheral nerve. Nerve guide tubes are being tested to aid in bridging interstump gaps during nerve repair. Before transected rat sciatic nerve fibres grow across a 3 mm gap within a nerve guide tube, a tissue bridge reconnects the proximal and distal nerve stumps. Possible influences on this tissue bridge formation remain unclear. In this study, we chose to examine three of the possible influences on the formation of the tissue bridge which precedes axonal entry, they were: i) the nerve cell bodies and target tissue, ii) factors outside of the nerve guide tube and iii) factors within the nerve. To do this, we used a polyethylene (PE) nerve guide technique which was developed in our laboratory. At three and nine days, three groups of nerve preparations, TRIPLE TRANSECTION, LIGATION and FILTER, were examined for tissue bridge reconnection of the proximal and distal stumps, the morphology of the tissue bridge and the vascular pattern in the tissue bridge. Group TRIPLE TRANSECTION specifically tested the influence of the nerve cell bodies and target tissue as well as

other unknown factors from outside of the nerve preparation. The influences of the nerve cell bodies and target tissue were tested by cutting the sciatic nerve in three places, the middle, proximally and distally. The nerve tissue, which was inside the PE catheter, was isolated from factors from outside the nerve preparation by either petroleum jelly or plastic discs heat-sealed to the PE catheter. After nine days, a tissue bridge reconnection formed in the petroleum jelly nerve preparations but not in the heat-sealed ones, suggesting the need for some external factor(s) which could penetrate petroleum jelly. To test the possible influence of factors carried or transported in the nerve, nerve trunks in Group LIGATION were ligated either proximally or distally and then transected in the middle of the nerve exposure. Nerve preparations were examined three and nine days after transection. The nerve stumps reconnected in a manner suggesting an influence from the non-ligated side. India ink perfusion demonstrated an extensive network of blood vessels within and surrounding the tissue bridge. Group FILTER was designed to exclude the possibility that a reduced oxygen availability had prevented tissue bridge formation in the heat-sealed TRIPLE TRANSECTION nerve

preparations. This preparation also tested the role of cellular elements within the nerve preparation on tissue bridge formation. A modification of the heat-sealed TRIPLE TRANSECTION nerve preparation was made. A window was cut in the PE catheter, the nerve and PE catheter were transected, and the open ends were sealed as before. The window was then covered with a 0.45 um nylon filter. Despite their access to oxygen and other nutrients through the filter; the intrinsic fibroblasts and Schwann cells did not produce a tissue bridge nine days after transection. Nerve cell bodies, target tissue, factors inside the nerve and factors outside the nerve preparation which could diffuse through the 0.45 um filter, did not appear to influence tissue bridge formation. The results of this study indicate that revascularization of nerve stumps is essential to the development of the tissue bridge which precedes axonal elongation.