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NERVES AND TISSUE REPAIR

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Studies have been conducted with regenerating amphibian sciatic nerves in organ culture to characterize further transport and release of transferrin in growing axons of peripheral nerves. The hypothesis under investigation is that transferrin, the iron-transport protein which is required as a permissive factor for cell growth, is delivered axonally to target cells and is involved in the nerve-dependence of cell proliferation during repair in avascular tissues lacking an adequate supply of transferrin from capillaries. Amphibian (axolotl) limb regeneration is a well-characterized model system in which the early period of growth shows complete dependence on nerves. Organ culture of sciatic nerves, combined with an assay for axolotl transferrin developed earlier, allows quantitative study of the release of this factor as well as its transport. As reported elsewhere for regenerating nerves *in vivo*, distal delivery of transferrin by fast axonal transport was found in cultured nerves. Moreover, transferrin was secreted and released to the medium in significant quantities at the axonal growth cones. These results have important implications for understanding the trophic effect of regenerating nerves on cell proliferation during tissue repair.

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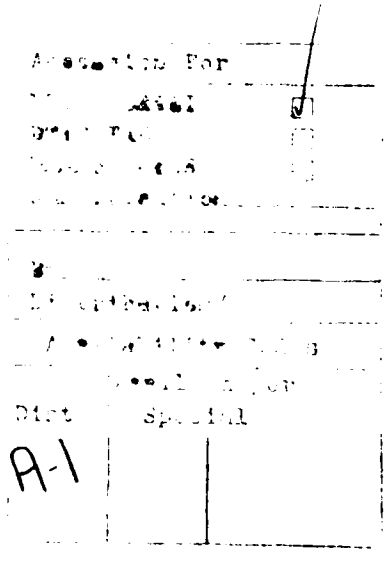
TABLE OF CONTENTS



INTRODUCTION

That peripheral nerves exert a "trophic" influence on certain processes of tissue repair or regeneration has long been recognized (Drachman, 1974). Reparative processes that depend on an adequate nerve supply include regeneration of injured or grafted skeletal muscle (Carlson, 1988), healing of corneal epithelial abrasions (Beuerman et al., 1988) replacement of taste buds (Zalewski, 1974), and regeneration of amputated limbs in urodele amphibians (Carlone and Mescher, 1985). Of these processes amphibian limb regeneration has been studied by far the most extensively in attempts to characterize the trophic activity. The molecular basis for the neural effect on tissue repair remains unknown but various factors have been suggested (Brockes, 1987; Carlone and Mescher, 1985). We are examining the role of the iron-transport factor transferrin (Tf), a protein abundant in nervous tissue which is required for maintenance and proliferation of cells (Mescher and Munaim, 1988).

The iron requirement for survival and growth of animal cells is based on the need for this atom in the prosthetic groups of many important enzymes, including enzymes necessary for aerobic respiration and for DNA synthesis (de Jong et al., 1990). Certain specialized cells also need iron as a cofactor in proteins related specifically to the cells' function, such as hemoglobin or enzymes involved in synthesis of collagen or production of various neurotransmitters. The principal mechanism by which cells of vertebrate tissues obtain iron under physiological conditions is receptor-mediated endocytosis of Tf (May and Cuatrecasas, 1985). Work with several types of cells has established the basic pattern of iron-Tf uptake: Tf complexed to its receptor is internalized via clathrin-coated vesicles, the iron atoms are transferred to other cytoplasmic proteins, and the receptor-apoTf complex is returned to the cell surface where Tf is released to bind iron again for another delivery cycle (Huebers and Finch, 1987).

Increased numbers of Tf receptors appear on the cell surface in response to mitogenic growth factors and such receptors are very abundant on all proliferating cells (Davis and Czech, 1986; Huebers and Finch, 1987). Studies with synchronized cells have shown that the increased expression of Tf receptors coincides with the onset of DNA synthesis (Seligman, 1983). The importance of Tf for DNA replication is apparently due to the iron requirement of ribonucleotide reductase, the rate-limiting enzyme for DNA synthesis (Eriksson et al., 1984; Reichard, 1988).

Additional evidence for the importance of Tf in cell growth comes from observations that proliferation *in vitro* can be blocked by addition to the medium of monoclonal antibodies to the Tf receptor or by chelation of iron from the medium (Seligman, 1983). Moreover, normal cells cultured in defined media will only grow if Tf is present (Barnes and Sato, 1980). *In vivo*, adverse effects on growth caused by severe iron deficiency have been shown both clinically and experimentally (Morgan, 1981; de Jong et al., 1990)

Tf is primarily synthesized and secreted in the liver and delivered to cells throughout the body in the plasma. However, local synthesis of Tf occurs in tissues where cells do not have direct access to plasma proteins. In the seminiferous tubules of the testis, for example, developing germ cells bind Tf released from Sertoli cells, which both synthesize this protein (Skinner and Griswold, 1980) and take it up selectively from the interstitial fluid (Morales and Clermont, 1986). In rodent brain Tf has been shown to be synthesized by both the choroid plexus (Dickson et al., 1985) and oligodendrocytes (Bloch et al., 1985), apparently to meet the iron requirements of neurons which also lack direct access to Tf from plasma. Transcytosis of Tf has also be demonstrated across epithelial cells of the blood-testis barrier (Morales and Clermont, 1986) and the blood-brain barrier (Fishman et al., 1987).

Peripheral nerves are particularly rich in Tf (Markelonis et al., 1982; Meek and Adamson, 1985), but the cells responsible for uptake and/or synthesis of the factor in this

tissue are not known. Recent work has shown increased uptake of Tf and iron by Schwann cells of rat sciatic nerve during post-traumatic nerve regeneration (Raivich et al., 1991). The importance of iron in neural tissue probably involves its role in enzymes for aerobic respiration and neurotransmitter synthesis (Dion et al., 1988) and neuronal growth seems to require increased levels of iron delivery. Studies on the synthesis and possible significance of Tf in peripheral and central nervous tissue have been extensively reviewed elsewhere (Mescher and Munaim, 1988).

In attempts to characterize the nerve's trophic agent required for growth of vertebrate limb regeneration blastemas, extracts of amphibian brain have been used with various *in vivo* and *in vitro* bioassays (Singer, 1978; Carlone and Mescher, 1985). Measuring cell proliferation in cultured regeneration blastemas, initial studies with heterologous Tf in this laboratory showed stimulation of DNA synthesis and mitotic activity, with a biphasic dose-response curve and an optimal response similar to those obtained with brain extract (Mescher and Munaim, 1984). The mean mitotic index of blastemas cultured with an optimal concentration of Tf was not significantly different from that of blastemas of the same age *in vivo* (with normal innervation). We subsequently showed that the growth-promoting effect of brain extract was dependent on its content of iron, which suggested that Tf may be involved in the stimulatory effects of neural extracts in this bioassay (Munaim and Mescher, 1986).

For *in vivo* investigations on the role of neural Tf during limb regeneration, we purified Tf from the salamander *Ambystoma mexicanum* and produced a variety of monoclonal and polyclonal antibodies against this factor. We have used these antibodies in two ways. First, we have developed a noncompetitive, solid-phase enzyme-linked immunosorbent assay (ELISA) for *Ambystoma* Tf that is capable of measuring as little as 1 ng of Tf per ml of tissue extract. This ELISA allows us to determine concentrations of Tf in

regenerating tissues and in nerves. Secondly, we are using the antibodies in immunostaining procedures to localize cells and tissues where Tf is concentrated.

We have found that interruption of the nerve supply to mid-bud stage forelimb regenerates (six days after amputation) of larval *Ambystoma* reduces the Tf content of distal blastema tissues by more than 50%. Similar reductions were found at various times after axotomy, ranging from six days to one day before distal tissues were harvested for assay. Care was taken not to disrupt the vascular supply to the limbs during the denervation procedure. Since denervation inhibits cell proliferation in limb stumps and Tf uptake is greater in growing than in nongrowing cells, reduction of the Tf content in distal limb tissues by denervation could be an indirect effect in which blocked growth causes decreased uptake of Tf by cells. As a control therefore, larval salamander forelimbs were X-irradiated (2000 rads) unilaterally and amputated bilaterally one day later. Six days after amputation the shielded limbs had mid-bud stage blastemas, but the X-irradiated limbs showed no sign of regeneration. However, Tf concentrations in distal tissues were similar in both X-irradiated and regenerating limbs, indicating that Tf content was not dependent on the state of growth in the tissue.

We used the same assay for Tf content in recent work demonstrating that this factor is transported in regenerating *Ambystoma* peripheral nerve (Kiffmeyer et al., 1991). This study will be discussed very briefly here; experimental details, statistical analysis and complete discussion of the data are included in the publication cited and in the Final Report of the previous USAMRDC contract. Using a standard procedure for the study of axonal transport *in vivo* (Bisby, 1982), sciatic nerves were exposed in regenerating limbs of adult salamanders and ligated at a distal level in the stylopodia. One day later the animals were killed and entire sciatic nerves, including associated dorsal root ganglia, were removed and cut into segments. Each segment was homogenized and its Tf content determined by

the ELISA. Consistently, a 2 to 3-fold accumulation of Tf was present proximal to the ligature, suggesting axonal transport of the factor distally. Control experiments, in which axonal transport is disrupted by local application of colchicine to the neurons, clearly indicated that Tf accumulation due to axonal transport rather than to edema or vascular constriction. Such controls are particularly important when testing for axonal transport of a protein abundant in plasma.

Immunocytochemical investigations were performed on both sections and teased preparations of *Ambystoma* brachial nerves. The results with individual myelinated fibers in teased nerves indicate that Tf is present in both axons and Schwann cells (Kiffmeyer et al., 1991). Longitudinal sections of nerve and dorsal root ganglia also show Tf localization in neurons and Schwann cells (Tomusk and Mescher, 1988). These studies showed further that the protein is particularly abundant in the perineurium and perineuronal glial cells of the ganglia, structures which constitute major features of the blood-tissue barrier in the peripheral nervous system. Electron microscopy reveals that the flattened processes of perineurial fibroblasts, which are present as multiple lamellae surrounding each fascicle of nerve fibers, have numerous vesicles and pits opening to both sides of the cell. Such ultrastructural characteristics are typical of flattened cells involved in "transcytosis", i.e., transcellular transport and exocytosis of endocytosized material.

Now the most important question regarding the possible role of Tf in the trophic effect of nerves is whether this factor is released distally from regenerating nerves. Released from axons at a significant concentration, Tf would be expected to exert a permissive influence locally on cells if their supply of iron-Tf from plasma or other sources was not adequate to support growth. As reviewed in detail elsewhere (Mescher and Munaim, 1988), studies of limb regeneration indicate that the early, nerve-dependent blastema is essentially avascular and is characterized by extensive extracellular proteolysis. Such

microenvironmental conditions would reduce the supply of Tf available to blastemal cells from capillaries. Therefore neural Tf may provide an explanation for the nerve-dependence of cell proliferation in this system: upon its release from axons, Tf would bind iron available locally and be taken up by cycling blastema cells.

The overall hypothesis being tested in this investigation is that neurons of peripheral nerves accumulate Tf from the surrounding glial cells, transport it distally along axons, and release it from growth cones. As described briefly above, we have obtained good evidence for axonal transport *in vivo*. We are now investigating further the physiological importance of Tf in peripheral nerves by studying axonal transport and release of the factor, which requires development of a suitable *in vitro* model, and by determining the mechanism by which Tf accumulates in peripheral nerves. The specific purpose of these experiments is to answer several questions, all of which are of fundamental importance to understanding the physiological and developmental significance of Tf in peripheral nerves. The major questions to be answered, as indicated in the proposal, include the following:

(1) Can axonal transport of Tf be demonstrated in regenerating peripheral nerves in organ culture?

(2) Is Tf released at regenerating nerve endings (growth cones)?

(3) Is Tf taken up by neuronal cell bodies and/or axons across the blood-nerve barrier?

(4) Do neurons synthesize Tf?

(5) Do glial cells or perineurial cells synthesize Tf?

(6) Is synthesis or uptake of Tf in peripheral nerve increased during nerve and/or limb regeneration?

As described more fully in the next section, several different methods of approach are being used to answer these questions. Axonal transport and distal release of Tf must be investigated in nerve organ culture in order to detect and quantify the secretion of Tf from

growth cones. To undertake this we have adapted methods of sciatic nerve organ using serum-free culture medium multicompartment chambers, a method others have used to show axonal release of various unknown proteins. Combining this approach with the ELISA for quantitative measurement of axolotl transferrin developed with the previous USAMRDC support has allowed us for the first time to demonstrate axonal transport and release of a specific protein from distal ends of regenerating axons. As discussed in a recent review (Mescher, 1992), this work has considerable significance not only for the role of nerves in promoting regenerative growth as studied here, but also for other systems in which growth and maintenance of a structure depends on peripheral nerves.

The remaining questions to be answered in this project involve synthesis of Tf by cells of peripheral nerves and uptake of Tf by neurons and other cells. These investigations have begun and will be reported on below, but results are not yet available. The method of approach for the binding study involves light microscope autoradiographic analysis following binding of radiolabelled Tf. Studies of Tf synthesis will employ cDNA probes for axolotl Tf mRNA generated by the polymerase chain reaction (PCR) used in both Northern analyses and *in situ* hybridization. As discussed in the next section, the latter approach represents a change from the method originally proposed.

BODY

We have completed the organ culture studies related to the two questions regarding the transport and release of Tf from regenerating peripheral nerves. Detailed results have been published (Kiffmeyer et al., 1991; Kiffmeyer, 1991; Mescher and Kiffmeyer, 1992) and will be reviewed here. This work confirmed the *in vivo* results described above and produced the first demonstration that Tf is released in physiologically significant quantities from distal regenerating ends of axons. These results are consistent with and extend reports from other investigators showing increased uptake of iron-Tf (Raivich et al., 1991) and other plasma

proteins (Boyles et al., 1990) in regenerating rat sciatic nerves. Moreover, *they demonstrate for the first time axonal transport and distal release of a "trophic" factor with well-understood and important significance as a requirement for cell growth*. Such a demonstration has never been accomplished *in vivo* in this or any other nerve-dependent developing system and the results have major significance for the field of regeneration.

To determine whether transported proteins are secreted distally at growth cones of regenerating axons an *in vitro* approach using organ culture of injured, regenerating peripheral nerve was necessary. Axonal growth into connective and mesenchymal tissue during regeneration of amputated amphibian limbs precludes direct study of distally released factors *in vivo*. However axonal regeneration following crush injury to a nerve is followed by axonal regeneration within the morphologically intact perineurium rather than in non-neural tissue. Dissection of such regenerating nerves with ganglia attached and perineurium intact can be followed by their transfer to organ culture. This approach allows isolation of material secreted distally from the growth cones of regenerating axons. This *in vitro* method of studying distally secreted proteins was developed initially by others using frog sciatic nerves (e.g., Tedeschi and Wilson, 1986, 1987) and was adapted in our laboratory for use with axolotl nerves since this urodele species rather than frogs is of particular interest with regard to the question of limb regeneration.

Axonal regeneration was stimulated bilaterally by standard crush injuries to adult axolotl sciatic nerves at the level of the knee. Four weeks later each injured nerve was dissected from the limb, including dorsal root ganglia, the site of the crush injury, and at least 1 cm of nerve distal to the injury. Each nerve was cleaned of extraneous connective tissue and blood vessels and placed in a multicompartment culture slide (Labtek) inside a 10-cm culture dish. All chambers and the dish were filled with serum-free Liebovitz L-15 medium (GIBCo), diluted to 80% for amphibian osmolarity and containing GIBCo

antibiotic-antimycotic. For each nerve, ganglia were placed in one chamber and the distal portion containing the regenerating growth cones was placed in a second chamber. Nerve tissue draped across the chamber walls was covered with petroleum jelly to prevent desiccation. The intermediate region of nerve was bathed in medium of the culture dish between the two chambers, thereby preventing passive diffusion of material between chambers along the nerve. The multicompartment culture system is shown schematically in Figure 1. After different periods of culture (typically 24 hr) in a humidified chamber at 22°, medium from the proximal and distal chambers was assayed for Tf in triplicate using the ELISA described above.

Results with six cultured nerves are shown in Figure 2. In every case there was greater release of Tf from the regenerating nerve distally than from the ganglia, so that the mean Tf concentration in the distal chamber was twice that in the chamber containing the ganglia. Cultures maintained for three days with media sampled every twelve hours revealed that release was linear through approximately the first day, then leveled off (Fig. 3). The decline in Tf release after 24 hours may be due to depletion of the protein from nerves or to a reduced rate of axonal transport in the cultured nerve.

Although *in vitro* preparations of injured, regenerating frog sciatic nerves have been used by others to investigate fast axonal transport and release of material during neuronal growth (Hines and Garwood, 1977; Tedeschi and Wilson, 1987; Synder, 1988), it is particularly important with a plasma protein to show that release is associated with axoplasmic transport and not simply passive diffusion from interstitial tissue space. In our preparations a greater volume of tissue was present in the proximal ganglionic chamber, suggesting that the two-fold higher concentration of Tf found in the distal compartment was not due to diffusion from the tissue. To test this point further, colchicine was added to the compartment containing the ganglia in order to block axoplasmic transport. In every case

this was found to prevent accumulation of Tf in the medium of the distal chamber surrounding the growth cones (Fig. 4), indicating that this accumulation is dependent on anterograde transport in the axons.

The average Tf concentration in the distal medium after 24 hrs of culture exceeded 200 ng/ml (Kiffmeyer et al., 1991). Released locally from regenerating axons within the microenvironment of a blastema or other site of nerve injury *in vivo*, this amount of Tf would represent a significant contribution to the micromolar concentrations required for optimal proliferation of blastema cells (Albert and Boilly, 1988) and mammalian cells (Barnes and Sato, 1980).

Our finding that Tf is fast-transported and released in regenerating axons is consistent with data from other groups studying axonal transport of secretory proteins. Using two-dimensional polyacrylamide gel electrophoresis to analyze material released *in vitro* from regenerating sciatic nerves of frogs, Tedeschi and Wilson (1987) found that approximately five newly synthesized, fast-transported polypeptides were secreted in significantly greater amounts. The spots were not identified, but prominent among them was one for a glycoprotein with a size and isoelectric point similar to that of Tf. In a study using similar techniques, Stone and Hammerschlag (1987) identified Tf among the fast-transported proteins in frog sciatic nerve by testing various antisera on Western blots of the two dimensional polyacrylamide gels.

To determine whether Tf is taken up by nerves of injured and uninjured peripheral nerve, we are primarily using methods of light microscopy and autoradiography. With control limbs or limbs that were amputated at various times earlier, we have removed nervous tissues and regeneration blastemas for cryostat sectioning. Nervous tissues embedded include dorsal root ganglia and attached lengths of brachial or sciatic nerve as well as segments of spinal cord at the level of the regenerating motor nerves. Sectioning of

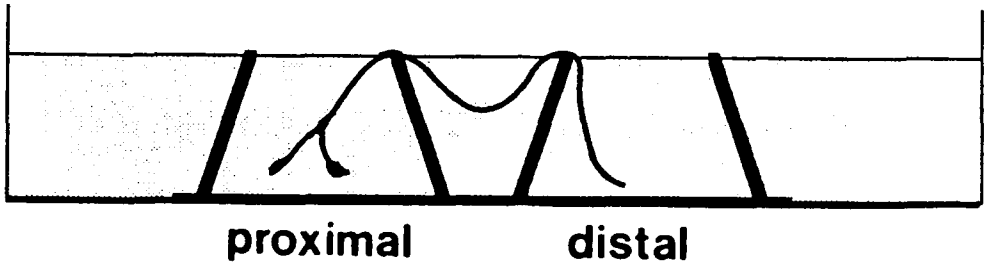


Figure 1. Multichamber system for investigating axonal transport and release of proteins in sciatic nerves *in vitro*. Shown are two chambers on a Lab-Tek slide inside a culture dish. Both chambers and the dish are filled with serum-free medium. Ganglia and the proximal part of the nerve are placed in one chamber and distal portions of the nerve, including the site of axonal regeneration, are placed in the other chamber. The intervening part of the nerve is covered by medium in the dish. At various times of culture a sample of medium is removed from each chamber are tested for its Tf content by ELISA.

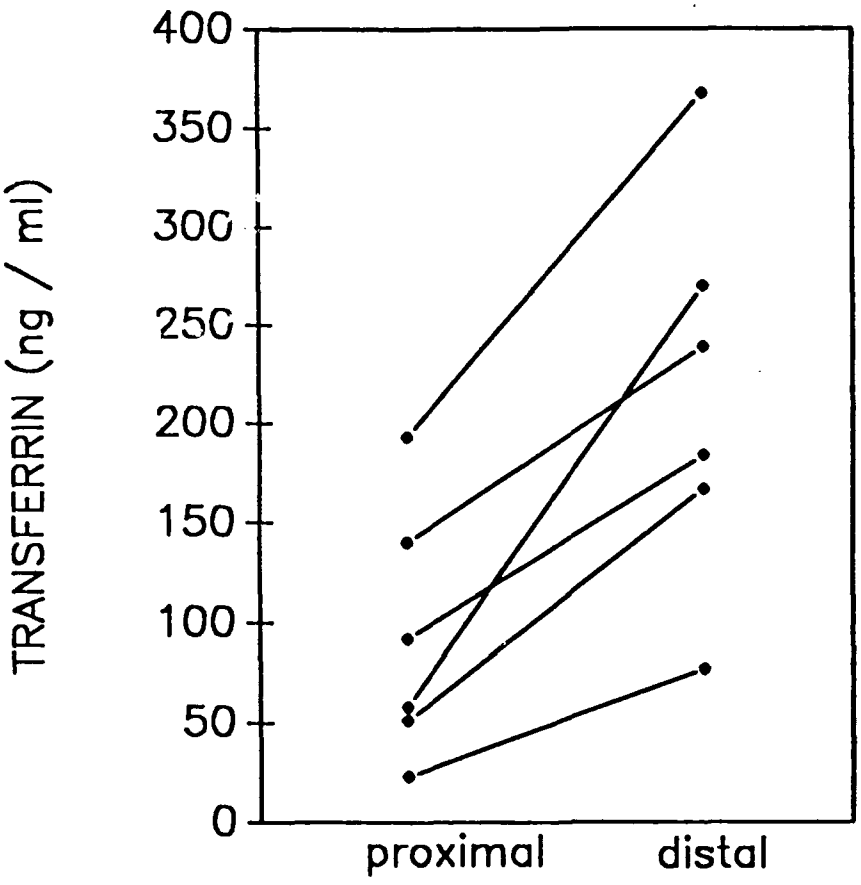


Figure 2. Tf concentrations in medium of culture chambers containing proximal and distal regions of regenerating adult axolotl sciatic nerves maintained in organ culture for 24 hours as described in Methods and Materials. Each line represents the results with one nerve. The average concentration in the distal chambers, which contained the regenerating ends of axons injured by crush 4 weeks earlier, was approximately twice that in the proximal chambers containing the dorsal root ganglia.

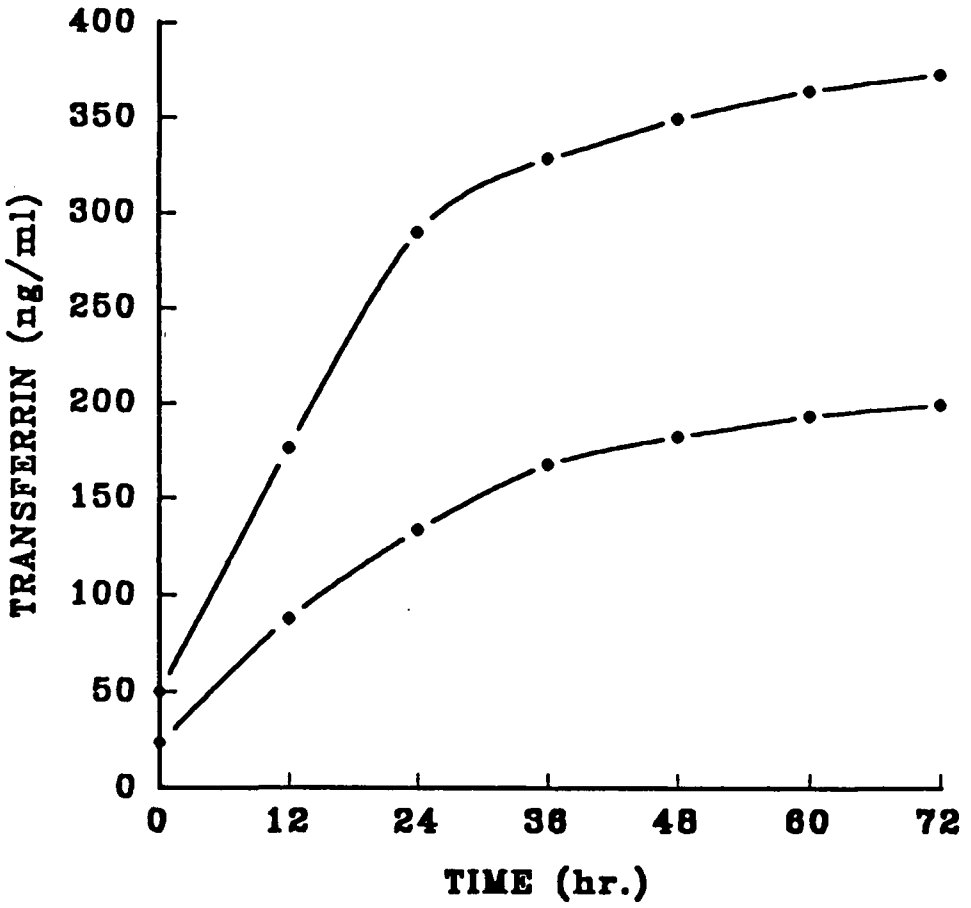


Figure 3. Tf concentrations in distal chambers of two representative regenerating adult axolotl sciatic nerves after various periods of culture. Tf is released maximally during the first 24-36 hours followed by a gradual decline in the rate of release.

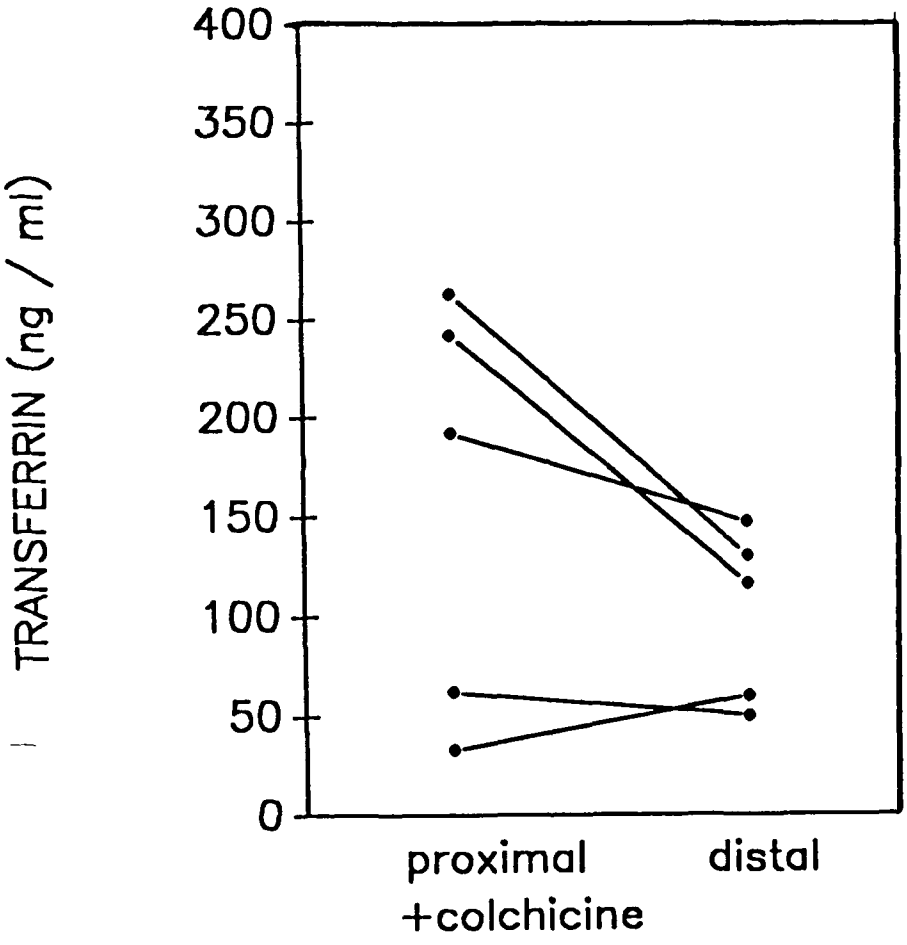


Figure 4. Tf concentrations in medium of culture chambers containing proximal and distal regions of regenerating adult axolotl sciatic nerves like those of Fig. 2, but with the addition of colchicine to medium of the proximal chamber. Colchicine in the medium surrounding the ganglia prevented release and accumulation of Tf in the distal chamber, indicating the importance of axonal transport for this accumulation.

these tissues is currently underway. All slides will be treated with ^{125}I-Tf, washed, and exposed to photographic emulsion in standard procedures for light microscope autoradiography to demonstrate cells binding Tf. The percentage of labelled cells and/or the density of label will be used to indicate quantitatively the effect of injury on uptake of Tf by sensory and motor neurons, Schwann cells, and other cells. Analysis of Tf binding in the blastema will indicate the nature and number of limb cells taking up Tf during this period. All cycling cells are expected to bind Tf and be labelled in this study; of major interest is exactly when Tf receptors appear during the process of cellular dedifferentiation that leads up to active cell proliferation.

The other area currently under investigation involves the question of Tf synthesis by cells in peripheral nerve. The molecular approach we are using here will employ labelled cDNA probes for axolotl Tf mRNA. As described in the proposal, the mRNA will be quantified by Northern analysis and localized among the different cells in peripheral nerve by *in situ* hybridization and light microscope analysis. Unlike what was proposed, we are generating the probes by reverse transcriptase-PCR rather than by screening of axolotl cDNA libraries. PCR primers for axolotl Tf have been designed based on published sequences for the Tf gene in mouse, rat, and the frog *Xenopus laevis*. Preliminary work has shown that the method produces an electrophoretic band of the expected size using RNA from axolotls or *Xenopus* and its identity as part of the Tf sequence was confirmed by the use of nested primers. Before labelled probes are generated for Northerns or *in situ* hybridizations, this PCR product will be sequenced to characterize it fully as a probe for Tf mRNA.

The PCR approach is much less labor intensive than the method originally proposed which involves construction and screening of cDNA libraries, but the results obtainable will be the same. This molecular analysis should show whether motor neurons, sensory neurons,

nerve upregulates such expression. To our knowledge no other laboratory is investigating synthesis of Tf in the peripheral nervous system, but results of similar work by others in the central nervous system indicate that certain neurons as well as oligodendrocytes contain Tf mRNA. Our study with peripheral nerves represents an important extension of that work which again will be directly relevant to our work on axonal transport and release of this factor in regenerating nerves.

CONCLUSIONS

Using reagents generated and assays developed with support of the earlier USAMRDC contract, we have demonstrated several points highly significant to the issue of how nerves support growth of the regenerating amphibian limb:

(1) The concentration of Tf in injured sciatic nerves of adult axolotls increases up to twenty-fold during the process of regeneration (Kiffmeyer et al., 1991).

(2) This Tf is contained in both axons and Schwann cells of the sciatic nerve (Kiffmeyer et al., 1991).

(3) Tf appears to be taken up across the perineurium (Tomusk and Mescher, 1988).

(4) Tf is transported by fast axonal transport in sciatic nerves (Kiffmeyer et al., 1991).

(5) Post-traumatic axonal regeneration affects the quantity of Tf transported, but not the rate of transport (Kiffmeyer et al., 1991).

(6) Tf is released at the distal ends of regenerating axons in a physiologically significant quantity (Kiffmeyer et al., 1991).

(7) In organ culture, distal release of Tf sciatic nerves continues for at least 24 hours, then levels off (Mescher and Kiffmeyer, 1992).

(8) Both *in vivo* and *in vitro*, Tf transport and release depends on microtubule-based axonal mechanisms, consistent with the view that normal fast axonal transport of Tf-containing vesicles is involved.

(8) Both *in vivo* and *in vitro*, Tf transport and release depends on microtubule-based axonal mechanisms, consistent with the view that normal fast axonal transport of Tf-containing vesicles is involved.

These results are all consistent with the hypothesis that Tf is a trophic factor by which nerves support limb regeneration. Without reviewing the literature on amphibian limb regeneration here, it should be noted that these results are entirely consistent with all previous work leading to characterization of the putative trophic factor(s) of nerves. Because of this work Tf is currently accepted as the most likely candidate for this long-sought factor, more than one of which may actually be involved in nerve's trophic effect.

The work on Tf uptake and synthesis by cells of peripheral nerves will greatly extend the results already obtained and lead to increased understanding of the neural role in tissue repair. Moreover, these studies will allow comparison in peripheral nerves with data obtained in similar investigations done by others on CNS tissue. Five laboratories in the U.S. and France are engaged in studies of Tf-iron in the CNS. To our knowledge this laboratory and one in Germany are the only ones working on Tf in peripheral nerves.

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