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Revascularization of free pheripheral nerve grafts

An Experimental Study in the Rabbit

by
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Gothenburg, March 1974

K.G.A.

INTRODUCTORY REVIEW

The restoring of nerve function after trauma is an important problem in reconstructive surgery. Today there is no ideal solution to this problem in spite of the fact that more than a century has elapsed, since Phillipeaux and Vulpian in 1870 reported a successful case of grafting of the lingual nerve in man.

The development of peripheral nerve surgery is characterized by the increased efforts that were made during the first and especially the second world war. In Great Britain a systematical work on peripheral nerve lesions was made by Seddon and his group, in Australia and in the U.S. other groups were working. In the following years many trials were made to improve the results - the Seddon group reporting only about 50 per cent satisfying results.

Concerning hand surgery Moberg (1958, 1962, 1964) has enforced the fact that full sensibility - meaning a good tactile gnosis - is of an uttermost importance for a good result, and that such a good quality very seldom is reached after peripheral nerve suture. Onne (1962) showed such good results chiefly among the young patient group.

The last decade has implied the use of even more refined techniques in different fields concerning nerve surgery. The anatomy has been revised by the use of electrone microscope, the physiology and histochemistry by the use of new techniques. The operation microscope has been an effective tool for the development of a safer dissecting technique, for a more exact apposition of fascicles and for the use of less foreign material.

The assembled effect of these innovations has been a more refined picture of the reaction to and treatment of trauma of the nervous system (Sjöstrand 1966, Ducker et al 1969, Morris et al 1972).

In peripheral nerve suture there often is a loss of substance either caused by the trauma direct or by secondary scar formation. As Millesi et al (1972) have shown, tension in the suture must be avoided in order to get a satisfactory result. Hence it follows that there is a need of the use of peripheral nerve grafts.

The problem of finding appropriate grafts has been discussed by many authors (for survey, see Sunderland, 1968). In animals the results often have been promising - clinical trials have given less good results.

A study of the literature reveals many different methods of grafting. There is, however, a lack of basic knowledge, i.e. concerning vascularization, immunological reaction, the factors determining scar tissue and the factors determining maturation and orientation of outgrowing axons.

The opinion is that a better knowledge of the factors responsible to vascular ingrowth, inflammatory reaction and scar formation may give a better ground for selecting the method of choice in future nerve grafting. Speaking of clinical results, the use of autologous grafts has given as best the same results as nerve suture (Sunderland 1968). Fresh homologous grafts in clinical work are reported to evoke an intense reaction, followed by an invasion of connective tissue. Many trials of pretreatment of homologous grafts are found in the literature. The aim of the pretreatment has been to diminish the inflammatory reaction. Marmor et al (1964) claim that pretreatment with freezing and irradiation of homologous grafts gives satisfactory results. Their human series are rather short and not easy to evaluate. Wilhelm (1972) also reports similar cases.

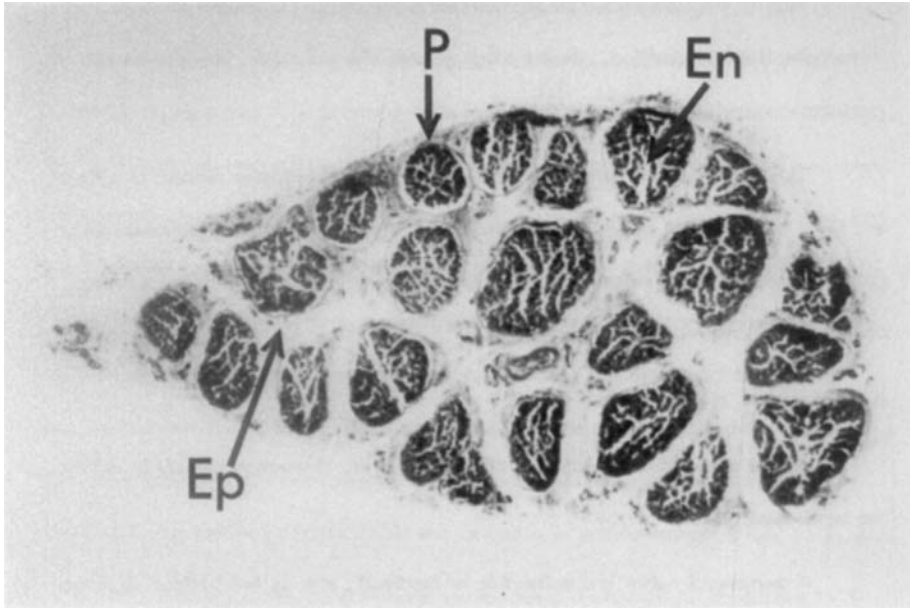

Fig. 1. Transverse section of a normal tibial nerve of the rabbit. Luxol fast blue. A = Axon. En = Endoneurium. Ep = Epineurium. Ev = Endoneural vessel. P = Perineurium.

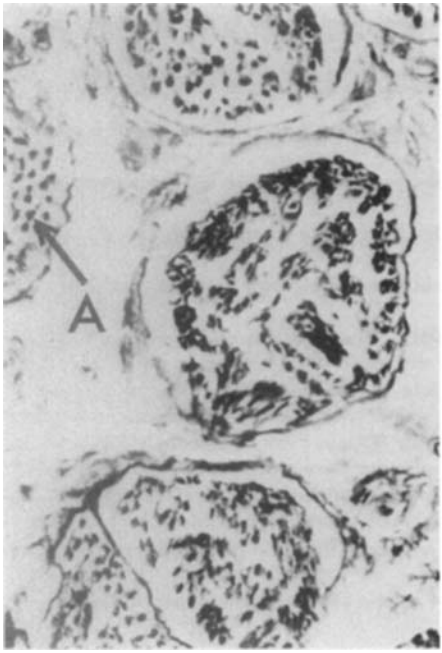

Fig. 2. Transverse section of normal tibial nerve. Palmgren.

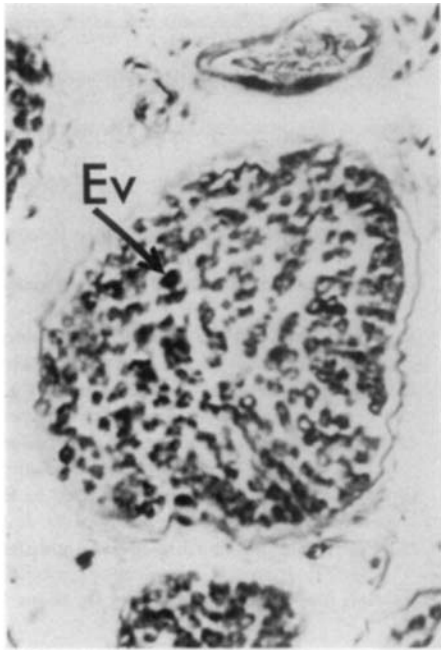

Fig. 3. Transverse section of normal tibial nerve. Luxol.

Thus it must once more be stressed that today there is no single ideal method of peripheral nerve grafting. Before starting more clinical trials, there is a need of more basic knowledge.

This study has the purpose to analyse the revascularization process in different types of peripheral nerve grafts, autologous, fresh homologous and homologous grafts pretreated in different ways. The specific questions, for which the answers are searched, are found on page No. 26.

Anatomical notes

As the nomenclature varies in different studies, it seems necessary to define the terms here used.

A peripheral nerve is a collection of fasciculi, also called funiculi (Sunderland). These consist of bundles of sheated nerve fibres. During their way the funiculi are uniting and dividing repeatedly, thus forming funicular plexa. These plexa are variable, and differences are noted even in contralateral nerves in the same individual (Sunderland). The funiculi are congregated in the epineurial tissue, a connective tissue richly vascularized. The most peripheral parts of a transverse section of a peripheral nerve often contain a more dense connective tissue, thus giving the impression of a distinct border. Each funicle is surrounded by a perineurium, consisting of several layers of flat cells and longitudinal, oblique and circular collagen fibrillae (Key and Retzius 1873, Röhlich and Knoop 1961). The thickness of the perineurium is linearily related to the diameter of the funicle (Sunderland 1957). The inner layer of the perineurium functions as a diffusion barrier (Olsson 1969, Lundborg 1973). Ontogenetically this layer is related to the pia-arachnoidea of the CNS (Shanta et al 1968). The nerve fibre or axon consists of a viscous fluid, in preparations often forming fibrillae. Surrounding it, there is the myelin sheath which is longitudinally

segmented - the different segments divided by the nodes of Ranvier. The myelin sheath is surrounded by a layer of Schwann cells. At the nodes these have direct contact with the axon. The Schwann cells form a syncytium without clear boundaries. Each Schwann cell nucleus corresponds to a myelin segment. The Schmidt-Landterman incisures are clefts in the myelin sheath. These are widened, if the nerve is stretched, thus hindering ruptures of the myelin sheath (Glees 1942). Peripherally to the myelin sheath there is the endoneurium, consisting of endoneural cells and connective tissue and forming a tube, often called the endoneural tube. The inner part of the endoneurium is sometimes looked upon as a separate membrane and is called the sheath of Plenk and Ladilaw. A more loose part of the endoneurium forms the intrafunicular septa, dividing the nerve fibres inside the funiculus in smaller groups (Figs. 1, 2, 3).

The vascular supply to peripheral nerves has a double origin (Lundborg 1970). There is the intrinsic system of longitudinal vessels, connected with numerous anastomosis, and there is the extrinsic system of vessels, giving blood supply segmentally. These vessels, approaching the nerve from the side, give proximal and distal branches. The branches make anastomosis to other branches of the extrinsic and intrinsic systems. The blood supply to peripheral nerves was primarily described by Isenflamm and Doerffler in 1786. Since that time many studies have been made (Adams 1941, Bacsich and Wyburn 1945, Sunderland 1945, Blunt 1956, Edshage 1964, Smith 1966, Nobel 1968, Lundborg 1970, Kline 1972).

In the peripheral nerve there are vessels in the epineurium, forming longitudinal stems with numerous anastomosis. In the perineurium also longitudinal stems - slightly thinner ones - make anastomosis and give obliquely running branches into the endoneurium. The endoneural vessels form a network of longitudinal stems with numerous anastomosis. Characteristic are the U-shaped loops, described by Edshage (1964).

Lundborg (1970), studying the vascular system with intravital microscopy, showed that the intrinsic system gave a satisfactory blood flow, even if the extrinsic blood flow was excluded in long segments of the peripheral nerve. In his studies on the vascular anatomy of human peripheral nerves, Sunderland already 1945 had shown the same thing. Other authors, e.g. Smith (1966) and Blunt and Stratton (1956), have stressed the importance of the extrinsic vascular system. The results of the studies thus are contradictionary. Lundborg's use of modern methods makes it probable that the intrinsic system - at least in rabbit peripheral nerves - is the more important one and alone is quite enough to secure an adequate blood flow. The extrinsic system acts more as a reinforcement during the course of the nerve.

Other ways of oxygenation of the peripheral nerve tissue have been discussed. Bentley and Schlapp (1943) and Blunt (1960) discuss the diffusion of oxygen from the capillaries in surrounding tissues. Sunderland (1968) discussed the lymphatic system as a possible oxygen carrier. These ways of oxygenation may be enough for a short period of time, but are certainly not enough for a long time use.

Lundborg (1970) showed the effect of section of the sympathetic nerve fibres to the vasa nervorum. There was hyperaemia and a greater sensibility to even small temperature changes.

GENERAL CONSIDERATIONS

Trauma to peripheral nerves is always followed by a degenerative reaction of the nerve distal to the lesion and a similar reaction of the nerve proximal to the lesion for a shorter or a longer distance. There also is a reaction of the nerve cell body and even in surrounding glial cells (Sjöstrand 1966, Ducker et al 1969, Engh and Schofield 1972). These extensive pathologic changes are demonstrable a few hours after injury. The degeneration after that proceeds in a reproducible

manner for two or three weeks. Regeneration starts before the first week is ended.

The degeneration in the distal nerve is named after Waller who first described it in 1852. The degenerative process in the proximal nerve end was described by Ramon y Cajal in 1912, who named it the proximal degenerative reaction. Several studies of the degeneration and regeneration of peripheral nerves have since then been made (Setterfield and Sutton 1935, Weddell and Glees 1941, Guth 1956, Lee Ching-Yuen 1963, Thomas 1966, Lehman and Hayes 1967, Mumenthaler 1968, Olsson and Sjöstrand 1969, Singer and Steinberg 1972). In the series of events that constitutes the Wallerian degeneration the earliest signs are noted in the axons. There is a fusiform swelling at irregular intervals or a general swelling of the axon. A more fluid portion of the axon appears to diffuse in the myelin sheath (Weddell and Glees 1941). The axons start to fragment, the particles scattering throughout the myelin sheath, or the axons break up in segments, which become twisted, but remain more or less in the center of the myelin sheath. In the myelin sheath there is a reticular network in the early stages and then the myelin breaks into droplets of various sizes. These form the so called digestive chambers or ellipsoids, in which the remnants of the axons disintegrate. 4 to 7 days after section the nuclei of the Schwann cells proliferate and come to surround the digestive chambers. These chambers are gradually resorbed. 14 days after nerve section all remnants of myelin and axon have disappeared from the fibres of peripheral nerves studied (Weddell and Glees 1941). When the resorption is complete, cell multiplication ceases. The hypertrophic Schwann cells decrease in volume and are arranged in cords, intimately covered by the endoneurium.

In the proximal stump there are similar changes. Morris et al (1972) followed these changes in rat sciatic nerve by electrone microscope. They found that the earlier stages of reaction (24 - 36 hours) appeared to be a wrinkling and distortion of the myelin lamellae, followed by a fragmentation of the myelin systems.

Retraction of the myelin from the region of the node of Ranvier was observed. Myelin debris in the cytoplasm of Schwann cells occurred as vacuoles, containing membranous material and pale staining globolous inclusions, which may develop from the membranes containing vacuoles. Cells, containing similar inclusions, were "free" in the endoneurium. The presence of similar cells, associated with axons and only partly covered by basement membrane, suggested that the cells "free" in the endoneurium were "transformed" Schwann cells, digesting their myelin. These cells were most frequent between 3 and 6 days after nerve section and gradually disappeared thereafter. There was no indication that cells, other than Schwann cells, were extensively involved in the traumatic degeneration of myelin.

The pathoanatomical reactions here described in the proximal and distal stump are accompanied by changed function in the peripheral nerve. 2 - 4 days after lesion the nerve distally is not apt to propagate impulses (Guth 1956). The course of events in the degeneration reaction is the same in different animals and man, but the time consumed varies. Axon remnants may be found up to 28 days after the lesion, and myelin remnants even 3 months after the lesion (Sunderland 1968). Blackwood and Holmes (1954) report myelin remnants in the distal stump after several months, even up to two years after lesion. The proliferation of Schwann cells shows a maximum 5 - 10 days after the lesion (Weddell and Glees 1941, Lee 1963, Sunderland 1968). The Schwann cells are transformed to macrophages (Morris 1972), but macrophages from the blood are also engaged (Olsson and Sjöstrand 1969) and macrophages from the mesodermal parts of the nerve (Holmes and Young 1942). When the proliferation of Schwann cells is high, the removal of axon and myelin is fast. After the removal of these remnants the Schwann cells are arranged in cords, called "Bungner bands" (von Büngner's Bänder, Blümcke et al 1962, 1966). These bands have been looked upon as a leading structure for the outgrowing axons (Holmes and Young 1942, Weddell 1942, Blümcke et al 1962). The recent work of Morris et al (1972) makes it probable that it is the axon itself that plays the leading rôle.

The regenerative process in the peripheral nerve has been studied by several authors (Speidel 1935, Setterfield and Sutton 1935, Sanders and Young 1941, Weddell 1942, Holmes and Young 1942, Gutman et al 1942, Gutman and Young 1944, Sunderland 1947, Bowden et al 1954, Guth 1956, Jacobson and Guth 1956, Blümcke 1962, Marmor 1964, Lehman and Hayes 1967, Millesi et al 1967, Mumenthaler 1968, Morris et al 1972). Signs of regeneration are early observed in the proximal stump. Already before one week has elapsed, there is elongation of the central part of the axons and "sprouting" from the axons also is observed. The result is numerous tiny fibers which soon are collected in different groups (Morris et al 1972). The regenerative activity was studied by Holmes and Young (1942) in sectioned rabbit nerves. They were able to show ingrowing axons in the graft after 7 days, the growth proceeded 3 - 5 mm a day. Initially a large number of axon sprouts were observed, finding their way into the graft. The greater part of the axons was to be found inside the endoneural tubes surrounded by Schwann cells. Myelinization was observed distal to the proximal suture after 15 days. Of the axon sprouts one or two in each endoneural tube showed a rapid enlargement of diameter. Estimated 10 mm distal to the suture, after 15 days the diameter was 1.2 microns, after 25 days 2.3 microns and after 100 days 7 microns. After that time the enlargement rate was slower. 100 days after grafting there were less fibres than initially. In most endoneural tubes there was only one fibre, in others two or three. Some thin axons were located outside the tubes or between the myelin of a central axon and the endoneurium.

Holmes and Young also showed a greater activity in nerves, in which a new section was made after different time periods. A maximum of proliferation of Schwann cells was shown, if a nerve section was made 2 - 3 weeks after the first one. The effect then declined and after 100 days it was very indistinct.

Regeneration never means restitution to the normal. There always remains a diminished axon diameter and myelin thickness. The endoneurium is unchanged or slightly increased (Sunderland 1956). The total diminishing of a fascicle therefore is depending on the number of axons it primarily held (Sunderland 1950, 1968).

Myelinization is said to begin as soon as the axon is surrounded by the Schwann cells. Myelinization proceeds distally, but always lags behind the leading axon tips (Gutmann et al 1942).

Several other factors have influence on the regeneration process. One is the initial delay, i.e. the time required for the neuron to recover, for axon growth to commence and for the axon to reach the place of injury. This delay depends on among other things - the age of the individual, the type of lesion and of the general condition of the individual (Young 1942).

When the axon tips have reached the injured zone, there is the problem of scar tissue and the exactness of apposition of the fascicles. In a graft there are next the problems of the distal suture. For the ultimate result there is the problem of the right axon reaching the right end organ and finally the function must return (Sunderland 1968).

Looking at this complex chain of events which must coincide for a good result, it is easier to understand the fact that only about half the cases show a satisfactory reinnervation. Furthermore, there are difficulties in the evaluation of the results. Studying only the histologic picture gives a meagre information on the functional status. Several methods have been tried in order to get a more complete picture of the final result.

Electrophysiological methods may be used both experimentally and clinically. Conduction velocity studies and electromyography studies both

give information on the restoration of the motor nerve fibres (Jacoby et al 1971).

The pinch method was used by Young and Medawar 1940 and is based on the increased excitability of new axons to mechanical stimulation. In light anesthesia the nerve is pinched by a fine forceps at intervals from the distal nerve. At the level of outgrowing axon tips a reflex response is elicited. The Hoffman Tinel Sign: a tingling sensation felt in the cutaneous distribution of an injured nerve, when the nerve stem is lightly percussed. This method makes it possible to follow the growing axons in clinical work.

The toe-spreading reflex was used by Lundborg (1970) in experimental work on the sciatic nerves. This reflex is elicited by holding the animal and then suddenly lowering it. The response is a spreading of the toes. The reflex requires a high degree of restoration of the nerve function, the neuromuscular union and the affected muscles.

Observations on muscle atrophy by measuring or by biopsy may be carried out. The estimation of sensory functions with pain, cold and touch is chiefly of clinical interest. In animals it is hard to make reliable estimations of such stimuli.

The two-point discrimination method is exclusively of clinical interest, giving an estimation of the higher qualities of sensory function.

The multiple factors influencing the reinnervation and restoration of nerve function and the different method used for estimation makes it impossible to specify fixed figures of the regeneration rate. In animal studies, however, using pure experimental models it is possible to obtain fairly accurate and reproducible estimations. Sanders and Young (1942) have shown this in their study on grafts in rabbit peripheral nerves. In his monograph 1968 Sunderland shows figures obtained from man and animal and they clearly demonstrate the divergences in the results noted.

Gutman et al (1942) found during optimal conditions in the rabbit the rate of 3.5 - 4.4 mm a day. The initial delay in the scar was 5 - 7 days and the final delay, prior to onset of functional recovery, was 20 - 34 days. These values applied both to motor and sensory fibres, the lower figures referring to crush injuries, the higher ones to suture.

Guth (1956) in his review on the regeneration in the mammalian nervous system says that in man where less favourable conditions usually are present the initial rate may be very high - up to 8 mm a day - with a gradual deceleration to low values of 0.5 mm a day distally. The initial and final delays are considerably greater than in lower animals, the combined delays occupying an average of 13 to 16 weeks. Further figures on human regeneration time can be found in Bowden and Scholl's report (1954).

SPECIAL CONSIDERATIONS TO PERIPHERAL NERVE GRAFTING

Sanders and Young in a study concerning degeneration and reinnervation of grafted nerves (1941) made observations on free grafts in rabbit peripheral nerves. Sunderland in his monograph 1968 made a survey over the work in peripheral nerve grafting up to that year. For more detailed information the reader is advised to look there. Some facts of interest for the present study will be presented here.

The study of Sanders and Young (1941)

The authors initially state: "The great difficulty in assessing the results of all this experimental and clinical work on the subject (i.e. nerve grafting) is that no measure of the efficacy of the various types of grafts has been devised. Each author has claimed that the same particular method of grafting gave success, but there has been very little comparison between methods on the basis of standard criteria. Moreover, there is very little information as to the nature of the histological changes, which proceed in the various grafts especially during the early stages." This statement was true in 1941 and it is true also today - more than 30 years later. Sanders and Young, using the peroneal nerve of rabbits, inserted different types of grafts with plasma clot technique. After 15 or 25 days the operation field was again exposed (using slight anesthesia) and with the so called pinch method the length growth of the sensory neurons were measured. The grafts were then removed, sectioned and stained in accordance to different histological methods. In a series of 63 autologous grafts (from the tibial nerve) the authors found a very feeble macroscopic reaction, an insignificant swelling at the suture sites and few adherences. After 15 days there was a good vascularization, mainly from the surrounding vessel

bed. After 25, 63 and 102 days the picture was the same. No tendency of ingrowth of connective tissue was found. 15 days post-operative all axons still remained in the graft, and after 25 days axons had passed the distal suture, their average growth being 41.7 mm, measured from the proximal suture (and as the grafts were 20 mm, they reached 21.7 mm into the distal stump). Related to regeneration after nerve suture (Young and Medawar (1940) give the mean figure of 3.9 mm/day after a latency of 8 days, before the suture line is reached) these figures are good ones, the latency 9.2 days and the growth rate 2.0 mm/day.

In their study Sanders and Young further found a degeneration process, which was close to normal. The vascularization was good, but the lesser vessels were more dilated than normally. The proximal and distal connections between nerve and graft look normal, i.e. resembling an ordinary nerve suture and the distal connection is established by Schwann cells already after 15 days, that is before the axons have arrived. The distal connection therefore does not give a further retardation of the axon growth. 25 days post-operative the myelinization process had started in the whole graft. That means, according to the authors, that viable Schwann cells were present.

Using pre-degenerated autogenous grafts the authors could not note any certain differences. There was no record of increased growth rate. The only certain difference was an increased consistency of the graft, which made the grafting more simple.

In another series of 35 homologous grafts (20 mm from the tibial nerve), inserted as in the previous series, a greater variability of findings was observed after 15 days. The grafts showed a yellow or reddish colour. They were surrounded by superboundant adherences and were swollen. The regeneration rate in this series

was slower - 15 mm after 15 days and 25.2 mm after 25 days. The authors could distinguish two different groups in this series. One group had characteristics like the autologous grafts, the other showed a very low regeneration rate. The authors concluded that factors beyond the conditions precedent for the experiment influenced the result. It must be kept in mind that immunological mechanism at that time had not yet been studied to any extent. Medawar published his first observations on immunology in 1944.

Histologically the authors showed a more prolonged degeneration process in the homologous grafts compared to the autologous. Even 25 days post-operative there were remnants of axons centrally in the grafts - axons which had not fragmented or had fragmented in a pathological way. There was an invasion of macrophages, in some cases a considerable invasion, especially around the vessels and at the suture lines. Vascularization was less pronounced, the vessels derived from the surrounding vessel bed. The Schwann cells formed syncytial bands and myelinization was observed at least in the proximal parts of the grafts. The authors concluded:

"There are therefore good reasons for supposing that the tissues of a fresh homograft in the rabbit can survive and become incorporated with those of the host nerve. In some cases this is accomplished with a small reaction, producing a condition approaching that of an autograft, though in other cases the state of affairs my be less favourable."

In another series the authors tested homologous grafts, which had been kept in Ringer solution at 2° C for different periods of time up to 21 days. The results - as observed up to 73 days - were as good or slightly better than the results obtained with fresh homologous grafts. There was a less pronounced macroscopic reaction.

In further series fresh heterologous grafts, homologous grafts pre-treated in

alcohol, and spinal cord grafts pre-treated in alcohol, were observed. These series had an intense macrophage reaction and sparse reinnervation in common.

In conclusion Sanders and Young stressed the importance of not making definite parallels between animal experimental results and clinical applications. They pointed out the difference in diameter of the grafts, and the greater difficulties in clinical work to find grafts with a fascicular pattern, resembling the damaged nerve stem.

Other studies of interest

Concerning autologous grafts there are several reports regarding both man and animal. The results obtained at best are as good as after nerve suture. Such results are observed in animals and in short grafts in man (Seddon 1963). In long human grafts the results are not so good (Seddon 1954, 1963). The degeneration in the autologous graft is of the same type as the degeneration distal to a suture, i.e. of Wallerian type. The degeneration may proceed more slowly - there is only a sparse invasion of inflammatory cells and an insignificant increase in connective tissue (Young et al 1940, Gutman et al 1942, Sanders and Young 1943, Davis et al 1945). Seddon (1947) and Björkesten (1948) reported promising results in man. In different series attempts have been made to analyse the clinical results. Sanders (1942), Seddon (1954) and Brooks (1955) all conclude that about half the cases showed satisfactory results, and that the results are better concerning motor activity than sensory activity.

Homologous grafts have been studied by several authors (Sanders and Young 1941, Seddon et al 1944, Spurling et al 1945, Tarlov et al 1945, Davis et al 1950, Campbell et al 1963, Lewis et al 1966, Metz et al 1969, Millesi 1969, Marmor 1970, Schröder and Seiffert 1970, Afanassieff et al 1971, Pollard et al 1971, Verhoog et al 1971, Meier et al 1972, Wilhelm 1972). Experimentally considerable differences

in results have been reported. Some authors have found relatively intact grafts (Sanders and Young 1941, Young 1942, Davis and Ruge 1959). In other studies a massive invasion of macrophages, followed by destruction of the grafts and ingrowth of connective tissue, is reported. This type of reaction is interpreted as an immunoreaction, started by the introduction of foreign protein (Seddon and Holmes 1944, Spurling et al 1945, Sanders 1954, Seddon 1963, Das Gupta 1967, Marmor 1967). Sanders (1954) showed that the strength of this reaction was proportional to the volume of the graft - short, thin grafts gave less reaction than long, thick ones. The walls of the endoneural tubes were thicker than in normal nerve stems, the amount of connective tissue in the nerve was greater, but the structures kept their long-striated pattern, thus acting as a guiding structure for the axon sprouts. Ingrowth of connective tissue in avascular parts of the grafts occurred and constituted an obstacle for the outgrowing axons.

The clinical trials using fresh homologous grafts have not been encouraging. An intense inflammatory reaction, followed by ingrowth of connective tissue, has been a hindrance of axon growth (Seddon 1963, Marmor 1967, Sunderland 1968).

In order to diminish this reaction different attempts to pre-treat the grafts have been made. Grafts have been kept in saline, serum or different buffer solutions for varying periods of time (Sanders 1942, Tarlov and Epstein 1945, Afanasieff and Recht 1971). Attempts have been made to leave nerve segments to degenerate in situ, in a second seance using them as grafts (Das Gupta 1967). Several trials to denaturize protein by the use of different chemicals, for instance alcohol and formaline, have been reported (Jianu 1909, Bielschowsky and Unger 1917, Sanders and Young 1941). Cooling, freezing and freeze-drying also have been tried to diminish the inflammatory reaction (Weiss and Taylor 1943, Tarlov and Epstein 1945, Davis and Ruge 1950, Verhoog and van Bekkum 1971). The use of proteolytic enzymes has been

tried (Seiffert 1967, Meier and Sollman 1972, Wilhelm and Ross 1972). Different types of irradiation have been used (Campbell et al 1963, Marmor et al 1964, Perl 1966, Roberts 1967, Cavanagh 1968). Especially Marmor and his co-workers have advocated this type of pre-treatment and they have reported both experimental results and clinical trials (1967). In order to obtain further immuno-suppression they have tried 6-/1-methyl (-4-nitro-5 imidazolyl)/, Imuran^{R} in animal experiments (Marmor et al 1967, Pollard and McLeod 1971). The use of freezed and irradiated homologous grafts in animals has given satisfactory results (Lewis and MacLaurin 1966, Marmor 1967).

In the search for methods, suitable for bridging peripheral nerve defects, the use of heterologous grafts has also been investigated. Neither the use of fresh heterologous grafts, nor the use of in different ways pre-treated grafts has given any good results. There are, however, occasional findings which give some promising results (Marmor et al 1966, Hirasawa and Marmor 1967, Marmor and Hirasawa 1968).

Tissues, other than nerve tissue, have also been tried without success (Sunderland 1968).

All these trials give a picture of the intense work which is carried out in different parts of the world in order to find a reliable method of peripheral nerve grafting. There is no critical survey to be found after that of Sunderland (1968). The criteria for good results are not standardized. Clinical trials, using pre-treated grafts, have not been reported to give enough good results for a recommendation of a general use of one type or another.

In clinical work today the grafting method of choice is the use of autologous grafts. The use of fresh or pre-treated homologous grafts is hazardous.

Irrespective of which type of graft being chosen there are additional facts to

consider. The length and thickness of the grafts is of significance (Sanders 1954). According to several authors the thick grafts run the risk of necrosis in the central parts (Maccabruni 1911, Bielschowsky and Unger 1917, Bunell and Boyes 1939, Tarlov and Epstein 1945, Brooks 1955). These observations were the ground for the trial of cable grafts, i.e. the use of a bundle of thin grafts instead of one thick graft (Bielschowsky and Unger 1917, Bunell 1927, Bunell and Boyes 1939, Seddon 1947). The source of these thin grafts were cutaneous nerves, each giving an insignificant sensory loss. The method, however, gave a more intense connective tissue reaction and scar formation, and necrotic areas could not be avoided.

The resemblance between the fascicular anatomy of the peripheral nerve and the graft has been discussed (Sunderland 1968). Theoretically a graft with the same cross-sectional fascicular pattern as that of the nerve should give the best chance for the outgrowing axons to reach their end organs. This ideal situation is not even obtained experimentally, as the anatomy of peripheral nerves is varied also concerning contralateral nerves in one individual (Sunderland 1968). The detailed anatomical studies of the peripheral nerves of man (Sunderland 1968) is of a great value in finding the most suitable graft in each case.

With the presumption that the ingrowth of connective tissue in the suture zones is an important hindrance for axonal growth, several attempts have been made to avoid this ingrowth by the use of protective wrappings of different kinds. A survey of the earlier literature is found in Weiss' review (1944). After that time arterial or venous sections, collagen film, millipore or silastic tubes and cyanoacrylat glues have been tried with more or less success (Campbell et al 1963, Lehman and Hayes 1967, Hirasawa and Marmor 1967, Dukker and Hayes 1968, Metz and Seeger 1969, Campbell 1970, Berger et al 1970, Sundin 1972, Carlsson 1973).

In the last decade the use of the operation microscope has made it possible to apply the microsurgical technique developed by Millesi et al (1967, 1969, 1971, 1972, 1973). This method has made it possible to dissect singular fascicles or at least groups of fascicles after resection of the epineurium. Intrafascicular grafting is then made, using cutaneous nerves as grafts. A minimum of foreign material is used, the grafts have to be made a little longer than the defect to avoid tension. The technique today is used in several clinics, but it is still too early to evaluate the results (Buck-Gramcko 1971, Salvi 1973, Smith, Michon and Brunelli in Michon and Moberg 1973).

The microsurgical technique of today is founded partly on the development of better optical aids and surgical instruments and partly on the detailed anatomical studies of Sunderland (see Sunderland 1968) and the studies on suture technique of Edshage (1964, 1966). Edshage showed the shortcomings of the traditional macrosurgical technique, and also defined a method to obtain a better intrafascicular apposition.

The adequate revascularization of free peripheral nerve grafts is of importance. It is true that a nerve graft may be without blood circulation for a short period of time. According to Weiss and Taylor (1946) this period may be up to 3 days. After that time there will always be necrosis. Necrosis of the graft means ingrowth of connective tissue and the formation of a scar, which is a hindrance for the axon growth.

The anatomy of the peripheral nerve vessels as earlier mentioned (page No. 9) was clarified by Adams (1941) and Sunderland (1945). Several other authors also have studied the effect of devascularizing dissections of peripheral nerve stems (Bacsich and Wyburn 1945, Blunt and Stratton 1956, Lundborg 1970, Kline et al 1972) and have shown that extensive dissection may be carried out without or with only

temporary disturbances of nerve function. Other authors (Smith 1966, Nobel 1968) have stressed the importance of the extrinsic vascular system (see page No. 9) for a normal nervous function and Erhart and Rezze (1966) found lasting changes in nerve stems, in which the extrinsic vascular system was excluded. In his works Lundborg (1970, Lundborg et al 1973), using modern vital microscopic technique, showed the most important capacity of the intrinsic vascular system. He also studied the effect of ischemia and stretching of the nerve on the endoneural circulation.

Concerning free nerve grafts there is no conclusive information in the previous literature studied. Different sources of blood supply are reported. Either anastomosis are found at the graft ends (Tarlov and Epstein 1945, Hassler 1969) or anastomosis with vessels surrounding the grafts are shown (Sanders and Young 1941, Davis and Ruge 1950). Hassler (1969) observed a difference between autologous grafts deriving their blood supply from the nerve ends and homologous grafts deriving their supply from the surrounding vessels. The growing knowledge about the peripheral nerve system and its reaction to trauma, the refined techniques available for studying and the refined techniques used for surgery justifies further detailed studies on the conditions valid for nerve grafting.

AIM OF THE PRESENT STUDY

This study is primarily diverted to the revascularization process in free peripheral nerve grafts. In four series the following types of grafts are studied:

- SERIES 1 Autologous grafts
- SERIES 2 Homologous grafts
- SERIES 3 Homologous grafts beforehand treated with freezing
- SERIES 4 Homologous grafts beforehand treated with freezing and irradiation

Using a standardized operation technique and different methods to visualize the vessels, the answers to the following questions are searched.

- When is the blood flow re-established in the graft?
- How is the vessel contact established?
  Anastomosis between nerve end vessels and graft vessels?
  Ingrowth of vessels from the nerve ends into the graft?
  Anastomosis between vessels in the surrounding tissues and vessels in the graft?
- Are there differences in the mode of vascularization between different types of graft?
- What influence have factors such as inflammation and connective tissue ingrowth on the vascularization in different types of grafts?
- Considering all factors studied - which types of graft are to be preferred for further investigation?

TABLE 1. COMPILATION OF SERIES 1-4.

Group	Series (no of grafts)	Type of graft	Method	No of grafts observed after different periods in days												
				1	3	5	7	10	14	21	42	90	180	360	other periods	Total
A	1 (84)	Autologous	F	2	2	2	6	3	3	5	3	3	5	3	–	37
			S	2	5	2	6	3	5	6	3	3	6	2	4	47
	2 (130)	Homologous	F	2	2	2	5	2	2	4	3	3	5	3	–	33
			S	2	5	2	6	3	5	5	3	3	4	2	4	44
B		Homologous	F	2	3	2	2	–	2	5	4	2	1	2	1	26
			S	2	2	2	2	2	2	2	3	4	–	4	2	27
	3 (108)	Freezed homologous	F	2	3	2	3	3	2	5	3	3	5	4	1	36
			S	2	2	2	2	2	2	3	3	3	2	2	2	27
C		Freezed homologous	F	2	2	3	2	2	1	1	1	1	3	1	1	20
			S	2	2	2	3	2	2	2	2	2	2	–	4	25
	4 (52)	Freezed and irradiated homologous	F	2	2	3	2	2	2	2	2	2	3	1	1	24
			S	2	2	2	3	2	2	2	2	2	2	2	5	28
															Total	374

F = Fluorescence microscopy S = Stereomicroangiography and light microscopy

TABLE 2. MORTALITY IN THE MATERIAL.

Group	No of animals	Dead within 24 hrs.	Dead during planned observation period (specimens obtained)
A	94	14	2 (4)
B	63	3	3 (2)
C	50	—	6 (10)

OWN INVESTIGATIONS

Material

374 preparations from the tibial nerve of 207 rabbits were studied (Table 1). The rabbits were albinos of both sexes, weighing about 3 kg. As donors of homologous nerve grafts rabbits of different colours were used. In some cases albino rabbits were used as donors, in which cases the albino hosts were taken from a different breeder to avoid near relations between donor and host.

After the operation the rabbits lived in separate boxes. They were fed on pellets and got water ad libitum. The animals were sacrificed after different time periods - 1, 3, 5, 7, 10, 14, 21, 42, 90, 180, 360 days. Some animals had to be killed earlier than intended. The reason was decreased general condition, due to trophic ulcers or paresis of hind legs.

During the trials 28 animals died, 17 of them within 24 hours after operation. The other 11 cases died of intercurrent diseases (Table 2). Preparations could be taken in 8 of the last 11 cases.

Comments

There are many reasons for the use of the rabbit as an experimental animal. It has a handy size, yet its tibial nerves are big enough to make the operation practicable. The animal can be used for long time studies at a reasonable cost. Several authors (Sanders and Young 1941, Edshage 1964, Lundborg 1970, Millesi 1972) have used the rabbit - thus comparison with their results is possible.

Of the 207 animals used 17 died within 24 hours after the operation

(Table 2). 14 of these belonged to the first group investigated, none to the last group. The cause of death was an overdose of anasthetic, causing decreased respiration. 11 animals died later during the observation period. In this time an epizootia was pevalent among the animals, causing a pneumonia and rapid death. In 8 cases the post-mortem investigation showed no apparent changes in the peripheral nerves. The author chose to use the specimens from these animals for further studies. In 3 cases the cause of death was more obscure and the animals were not used.

Methods

Surgical procedure

The animals were anesthesized with a solution, containing 10 g of diemal sodium and 3.5 g of mebumal sodium in 100 ml of aqua sterilisata. 1 ml of this solution per kg bodyweight was given intravenously.

The rabbits were prepared for operation. The fur was removed in the operation zone and the skin was cleansed, using 70 percent ethyle alcohol. The operation was performed under sterile conditions. A Zeiss operation microscope was used, giving both the surgeon and his assistent the opportunity to observe the operation field in an appropriate magnification (about 10 times as a rule). After incision in the skin the fascia was opened immediately medial to the anterior border of the tibia. The tibeal nerve then was easily accessible by blunt dissection. The nerve runs alongside the tibeal artery and veins and is enveloped in a thin fascia which also holds the segmental arteries to the nerve stem. The nerve was freed for about 20 mm length.

In each animal both hind legs were used and two grafts were inserted. In order to avoid tension the grafts always were 5 mm longer than the resectates. The autologous grafts were obtained from one hind leg, 15 mm was resected. The

graft then was inserted in the other leg - here only 10 mm was resected. In the first leg a homologous graft of 20 mm length was inserted. In some animals homologous grafts were inserted in both sides. In these cases the length of the grafts were both 15 mm inserted in 10 mm gaps. In one group a homologous graft was inserted in one leg and a freezed homologous graft in the other. In another group a freezed homologous graft was inserted in one leg and a freezed and irradiated homologous graft in the other, all grafts being 15 mm and the defects 10 mm.

The different grafts were adapted to the tibeal nerve, using nylon suture 9-0 (Ethicon) on an atraumatic needle. Two stitches were placed at each end of the graft. The wound then was irrigated with saline, and the skin was sutured with 5-0 stainless steel. The operation time was about 45 minutes. The animals woke up after another hour and were then kept in their cages.

Preparation of the different grafts

The autologous grafts were taken from the animals' hind leg and were then stored for only 10-20 minutes in saline. The fresh homologous grafts were removed from the donor immediately before the operation on the host. They also were kept in saline for a maximum of 30 minutes. The freezed homografts were prepared in two ways. In group B (see Table 1) the grafts were taken from the donors and mounted on foil, enclosed in polythene wrapping and as fast as possible frozen in fluid nitrogen. The grafts then were kept in fluid nitrogen for a minimum of 3 weeks up to 6 months.

In group C (Table 1) the nerve grafts were mounted and enclosed as in the previous group. They were then kept in small glass tubes and frozen in carbon dioxide snow. After freezing they were kept in an ordinary freezer at -20° C for at least 3 weeks and up to 6 months.

The freezed and irradiated grafts were prepared as just described, freezed in carbon dioxide snow and then placed in a cobolt source for irradiation. A total dose of 2 million r.e.p. was given. The preparations then were kept at -20° C for 3 weeks to 6 months.

Approximately half the number of animals were used for stereomicro-angiography. They were killed by an overdose of anesthetic and then prepared as described on page No. 38.

The rest of the animals were used for fluorescencemicroscopic studies. Before they were killed they got a light anesthesia and intravenous injection of labelled albumin solution. After 30 minutes they got an overdose of anesthetic and were then prepared as described on page No. 35.

Comments

The surgical technique used allows the comparison between two grafts in the same animal. If none of the grafts causes a general reaction in the animal and so influences the other graft, the two grafts can be compared under almost identical conditions.

As shown by Table I the four series of grafts are distributed in 3 groups of animals. In group A autologous and fresh homologous grafts are inserted, in group B fresh homologous and freezed homologous grafts are inserted and in group C freezed homologous and freezed and irradiated homologous grafts are inserted.

The two different freezing techniques, fluid nitrogen used in group B and carbon dioxide snow used in group C, are used to make comparison to earlier works possible. The use of fluid nitrogen was advocated by Weiss and

Taylor (1943), who reported promising results. The second method was used by Marmor (1967). Some grafts also were prepared with irradiation, and in those cases it was necessary to use carbon dioxide snow for practical reasons.

The dose of irradiation was also chosen in accordance with Marmor's work to make direct comparison easier. However, Marmor had the opportunity to use a van den Graff accelerator - the author had access to a cobolt source. It is to be observed that there are differences both in the emission spectre and in the time used for giving the same dose.

The use of the operation microscope gives a very clear picture of the operating field and makes an accurate precision in handling the nerve and the graft possible. The stitches can be exactly placed in the epineurium, allowing the use of a minimum of foreign material. Edshage (1964) showed the importance of this for a diminished connective tissue reaction. The grafts always are made approximately 5 mm longer than the defects in order to avoid tension and to allow this sparse use of suture material. Millesi et al (1972) showed that diminished tension also gave a diminished amount of scar tissue.

The rabbit tibial nerve allows grafts up to 20 mm of length to be used with ease, with longer grafts techniqual difficulties arise. The use of 15 mm grafts is the rule in this study. The exception is the homologous grafts in group A, which were 20 mm of length necessitated by the created gap.

The tibial nerve is multifascicular and consists of 15-25 fascicles. In the region used it has an ovoid or triangular shaped cross-section with a maximal thickness of 1-1.5 mm. The three forearm nerves, in which grafting most frequently occurs in clinical work, are also of multifascicular type. In some experimental studies (Tarlov and Epstein 1945, Millesi et al 1972) the

sciatic nerve is used. It contains only 1 big and 1 - 2 small fascicles and must be supposed to react in a diverging way, for instance concerning revascularization and scar formation.

Techniques used for preparation of the specimens

After killing the animals the specimens were taken with the aid of the operation microscope. In connection with this procedure a macroscopic evaluation was made. The specimens taken from the animals, injected with labelled albumin, were studied by fluorescence microscopy. The other specimens at first were used for stereomicroangiography and after that they were studied by light microscopy.

The use of different methods gives the possibility of getting a more true picture of the problem. If labelled albumin is allowed to be distributed in the animal, a picture of the functioning vessel bed in sections is obtained. The stereoangiography gives a picture of the vascular bed in the unsectioned graft. The histologic methods can be used to confirm the results, but also makes it possible to observe changes in connective tissue, occurrence of inflammatory cells, degeneration of myelin and axon and signs of reinnervation.

Macroscopic observations

After dissecting the preparation an observation of the specimen was made in situ. The following facts were noted.

- Macroscopic vascular anatomy
- The occurrence of adherences and signs of inflammation
- The occurrence of swelling or atrophy of the graft

Comments

Blood vessels, filled either with Evans blue-albumin or barium contrast medium, are easily demonstrable giving a clear contrast to the tissues. The occurrence of adherences, however, often makes detailed observations difficult. Abscess formation, loose adherences and oedema are interpreted as signs of inflammation. Swelling of the graft is recorded, when the graft diameter is 50 percent greater than the original diameter. Swelling of the grafts often is most expressed at the suture regions and then there is also a swelling of the adjacent nerve ends. Photographs were taken in several cases.

Fluorescence microscopy

88 animals were used for these studies. The animals received an injection of labelled albumin solution intravenously. The solution contained 1 g of Evans blue (Merck) and 5 g of bovine albumin (Sigma Chemicals fraction V) in 100 ml of destilled water. The dose used was 1 ml per kg of body weight, given at a rate of 5 ml a minute. 30 minutes after the injection the animals were killed by an overdose of anesthetic. The grafts with adjacent nerve were freed and after the macroscopic observation mounted on corc plates and fixed in 10 per cent formaldehyde solution. Longitudinal frozen sections were made from the entire specimen. The sections were embedded in glycerine and studied in a Zeiss fluorescence microscope with a darkfield condenser and filter No. UG 5 before the condenser and filter No. 47 before the occular. On these assumptions the fascicles are coloured light green against a black background, the filled vessels are brilliant red. Leakage of albumin through the vessel wall gives a diffuse orange-coloured fluorescence. The fluorescence is rapidly diminishing and even if the sections are stored in cold and darkness, the slides must be studied within a week. The studies on these sections were directed to the following points:

- **The occurrence of filled endoneural vessels**
- **The occurrence of filled perineural and epineural vessels**
- The occurrence of "new vessels" and their anastomosis with vessels in the graft and with vessels in the nerve ends
- The occurrence of diffuse fluorescence, due to leakage of labelled albumin through the vessel wall
- The occurrence of grafts without filled vessels

Comments

The method of injecting labelled albumin earlier was used by Olsson (1966, 1969, 1971) and Lundborg (1970) for peripheral nerve studies. Albumin, labelled with Evans blue, is distributed through the vascular system. In peripheral nerve vessels there is a difference between the epineural and endoneural vessels. In the former albumin passes the intact vessel wall, in the latter there normally is no such passage. After damage of different kinds to the nerve the albumin does pass even the endoneural vessel wall.

If the labelled albumin is allowed to be distributed in the circulating blood for a sufficient period of time and the animal is handled carefully to avoid shock, the albumin is found in the entire functioning vessel bed. The finding of vessels, filled with labelled albumin in the grafts, therefore proves that blood circulation was established at the moment of death. There are other methods available for the study of the vessels in the grafts. Indian ink has been used, the clearance of radioactive isotopes and intravital microscopy. These methods require some special equipment and do not offer any advantages over the labelled albumin method for a study of this kind.

Basis of estimation

In a normal tibial nerve from a rabbit, treated with labelled albumin solution, there are the following types of vessels. One or two longitudinal epineural vessels are seen in one section - they are giving branches at intervals. The epineural vessels are surrounded by a zone of diffuse fluorescence. Perineural vessels are of lesser diameter and are often running obliquely penetrating the perineurium. The thin endoneural vessels are forming longitudinal stems with transverse anastomosis frequently occurring. Characteristic are the U-loops found by Edshage (1964). The endoneural vessels are only partly filled.

In this study the occurrence of endoneural vessels is reported if there are such vessels in the majority of fascicles in the section studied. Concerning epineural and perineural vessels, there must be at least a few millimeters of a typical vessel in the section, if a positive finding shall be noted.

The vessels, here named "new vessels", are primarily found in the suture regions. In the earliest observations they are thin, tortous vessels with varying diameter. Later they form a network of partly quite big channels, chiefly located in the suture regions. They make anastomosis with vessels in the surrounding tissue, in the nerve ends and in the graft. A positive report on such vessels is made, when found.

The diffuse fluorescence occurs in the epineurium, but also in the endoneurium of the grafts and the nerve ends. The fluorescence contrasts clearly against the black background and the light green nerve tissue. A positive report of fluorescence is made, when it is found in the endoneurium or in the suture zones. Grafts without filled vessels is reported in cases without any of the vessels, named above.

Stereomicroangiography

Preparing the animals for these studies the abdomen was opened and the abdominal aorta was freed some 15 mm distal to the renal arteries. A catheter was inserted in the aorta and fixed with a ligature. A suspension of barium sulphate (Mixobar suspension 0.4 g/ml, Astra) was administered under a constant pressure of 180 mm mercury. The suspension was tempered 37° C. The animal was now sectioned transversely just below the renal arteries. The inferior caval vein was left open and other vessels were ligated. The suspension was allowed to infuse for 10 minutes. After that it was exchanged by a mixture of equal parts of suspension and a 10 percent solution of formaldehyde and was still given under pressure and temperated. After 10 to 15 minutes it was possible to observe a mixture of suspension in the blood, pouring from the caval vein. The infusion then was stopped and the preparation placed in a bath of 10 percent formaldehyde and 70 percent alcohol in equal parts. The catheter was connected to a reservoir, containing the barium sulphate-formaldehyde mixture. To obtain a satisfactory pressure this reservoir was placed 200 cm over the preparation. After 48 hours the tibial nerves were freed bilaterally and the grafts with adjacent nerve were resected. The nerve preparations were mounted on cork plates and fixed in formaldehyde. The angiography was performed with a Philips' tube, type 25294, with 140 kV and 15 mAmp. The exposure time was 7-15 minutes. Two exposures were made, the preparations placed on the plate directly and the plate tilted about 7° between the exposures. The photo plates used were maximal resolution plates from Kodak or Ilford. After developing and fixing the plates were observed. In some cases there was a need for a further dissection to visualize the vasa nervorum. New exposures then were made. The plates were observed in a microscope and in a Zeiss mirror stereoscope. In some cases photographic enlargements were made. In the microangiograms the occurrence of different types of filled vessels and the occurrence of anastomosis was noted in the

following way:

- Endoneural vessels
- Perineural and epineural vessels
- Anastomosis chiefly between vessels in the proximal nerve end and graft vessels
- Anastomosis chiefly between vessels in the distal nerve end and graft vessels
- Anastomosis between vessels in both nerve ends and vessels in the graft
- Anastomosis between vessels in surrounding tissue and vessels in the graft
- Grafts without filled vessels.

Comments

The stereomicroangiographic technique is well-known and has been used by several authors both in peripheral nerve studies (Edshage 1964, Nobel 1968, Lundborg and Brånemark 1968, Lundskog et al 1968, Lundborg 1970, Sörensen and Asano 1971) and in studies of other tissues (Bellman 1953, Velander 1964, Bergljung 1968). Using a correct technique there is good filling of even minor vessels (Nobel 1968, Lundborg and Brånemark 1968). The use of the stereotechnique improves the possibilities of observing branching and anastomizing in the graft vessels. The method, however, cannot tell how large a part of the vessel bed observed really was in use for circulating blood. The injected contrast medium may open vessels which were closed in life. Thus there is the risk of getting a too optimistic picture of the vessel bed in the graft.

The angiographic technique leaves the specimen intact for further light microscopy studies, in which the findings can be confirmed.

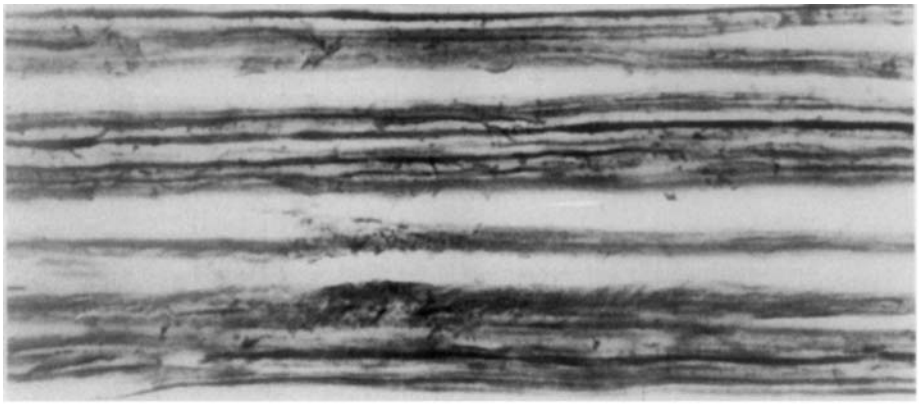

Fig. 4. Longitudinal section of normal tibial nerve. Palmgren.

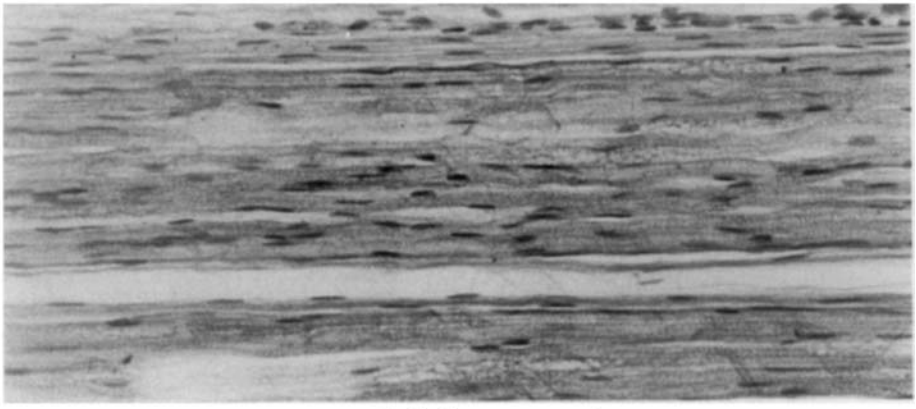

Fig. 5. Longitudinal section of normal tibial nerve. van Gieson.

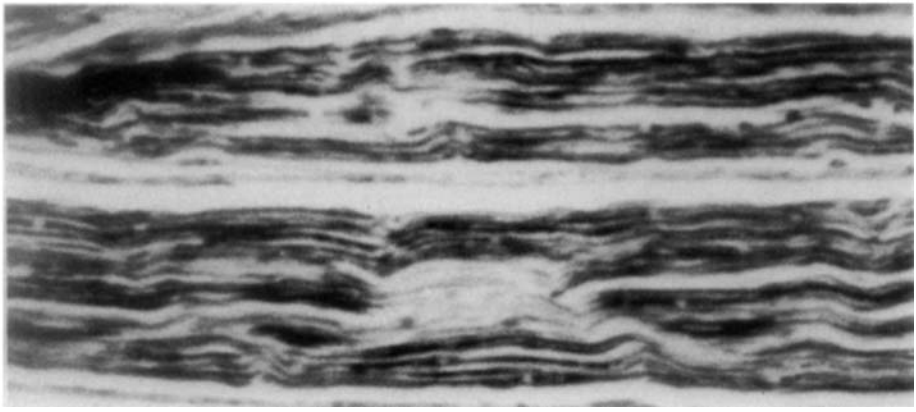

Fig. 6. Longitudinal section. Distal nerve 360 days after operation in a specimen with fresh homologous graft.

Basis of estimation

In a preparation of a normal peripheral nerve vessels of different kinds are filled. The major epineural vessels, running longitudinally, often constitute a hindrance for a closer view on the vessel bed, and they are therefore removed by dissection (using the operation microscope). The perineural vessels, giving obliquely running branches to the endoneurium, are easily recognized. The endoneural vessels appear as a meshwork of thin, longitudinal vessels with multiple transverse anastomosis. Especially if photographic enlargements are made, there is no difficulty of observing the capillaries.

Before studying the grafts an estimation of the degree of filling of the host nerve vessels was made. If the filling was not complete, the specimen was not used for further angiographic studies. Endoneural vessels are recorded, if they occur in a majority of the fascicles of the graft.

Epineural and perineural vessels are recorded, if there are typical vessels filled for a length of a few millimeters. Anastomosis from either proximal or distal nerve end are noted, if there is a clear predominance to one end. In other cases anastomosis to both ends are recorded. Anastomosis from vessels in surrounding tissue are recorded, whenever they are found. In many cases there exist anastomosis of all types.

When no filled vessels of the types described are seen in the graft, the case is recorded as a graft without filled vessels. In such cases sometimes small vessels are observed growing into the graft from the surrounding tissue vessels. These vessels as a rule only reach the outer layer of the epineurium.

Light microscopy

The specimens used for angiography again were kept in formaldehyde and after

that dehydrated in rising concentrations of alcohol. They were embedded in paraffine and cut in sections, 4 microns thick. Longitudinal sections were obtained from the suture regions and transverse sections from the middle of the grafts. The sections were stained according to three different methods. The van Gieson method stains collagen, connective tissue cells and inflammatory cells. The Luxol fast blue method stains the normal myelin clear blue, the degenerated myelin in different shades of light blue. The vessels containing barium contrast are stained clear red. The Palmgren method stains the axon dark violet.

Each specimen thus generated nine different slides, each containing three sections from different depths of the preparation. To evaluate these more than 5000 sections the author was enforced to list the number of findings to be recorded as follows:

Luxol fast blue	Filled endoneural vessels
	Filled epineural and perineural vessels
	"New vessels"
	Degenerated myelin
van Gieson method	Increased endoneural collagen
	Increased thickness of the perineurium
	Increased epineural collagen
	Increase of inflammatory cells
Palmgren method	Normal looking axons
	Degenerated axons or lack of axons
	Regenerated axons in distal nerve

Necrosis of grafts, if occurring, was also recorded.

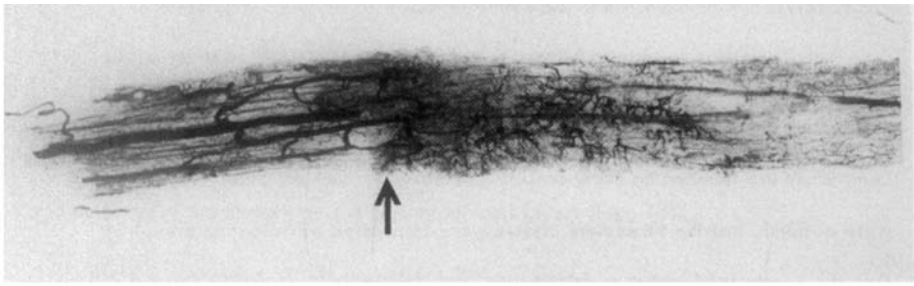

Fig. 7. Microangiogram from an autologous graft 7 days after operation. Arrow in fig. 7 - 10 indicates proximal suture region.

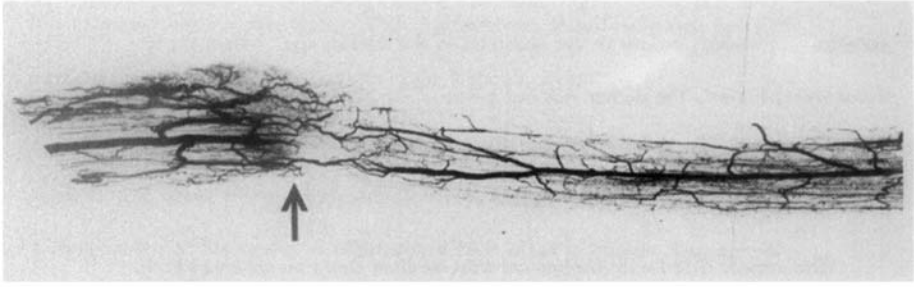

Fig. 8. Microangiogram from a fresh homologous graft 7 days after operation.

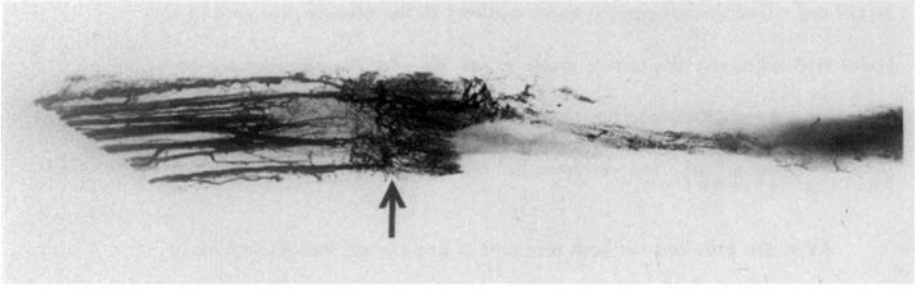

Fig. 9. Microangiogram from a freezed homologous graft 7 days after operation.

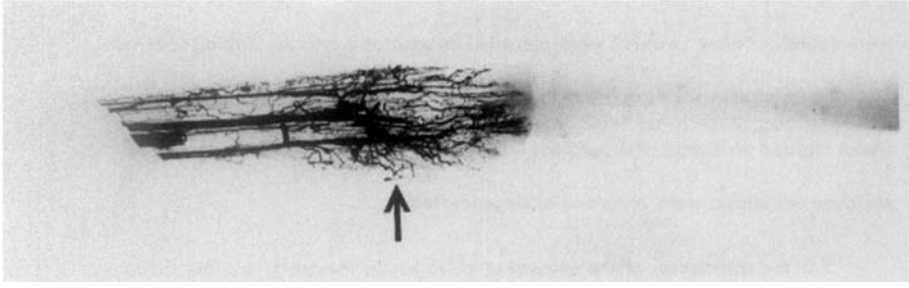

Fig. 10. Microangiogram from a freezed and irradiated homologous graft 7 days after operation.

Comments

All methods used for staining the sections are well-known standard methods used in clinical work. Their selective staining of different tissue compounds are founded on many earlier observations. Several other methods were at hand, but the three here chosen were considered sufficient to visualize the matters of interest to this study.

In order to make the observations as objective as possible the entire material was coded, before it was observed in the microscope. When the slides were studied, the author was not aware of which type of graft he was studying, nor the period of time gone after the operation. The code was broken only when all slides were observed.

The vessels filled with barium contrast medium could be observed both in the van Gieson stained sections and in the Luxol stained sections. As the bright red filled vessels gave a sharp contrast to the blue background in the Luxol stained cases, the author chose to use them for the observations on vessels.

Basis of estimation

As in the previous methods a record of endoneural vessels was made, if a majority of the fascicles in a section held such vessels. Perineural and epineural vessels were registered, if typical vessels of a few millimeters length were found. "New vessels" were recorded as soon as a typical finding occurred. The normal myelin staining bright blue has a homogenous appearance in the Luxol stained sections. All sections with vacuoles in the myelin and changed staining properties were recorded as degenerated.

For the estimation of the amount of collagen in the grafts and the thickness

of the perineurium, the observed section was compared to a section from a normal tibial nerve of the rabbit. A record of increased amount of collagen was made, when there was a clear difference to the normal preparation. The increase in thickness of the perineurium is partly due to an increase in collagen and partly to a hypertrophy of the perineural cell layers (Fig. 16).

For the estimation of inflammatory cell occurrence, a normal preparation also was used as a standard. The normal nerve contains none or only a few scattered cells of this type. When aggregations of inflammatory cells occurred in the grafts, a record was made (Figs 13, 14).

The normal axon is a distinct structure easily recognized (Fig. 4). In the early degeneration phase there is irregular swelling, later fragmentation and total disappearance of the axon. A regenerated axon often is thinner than normal ones and often shows an irregular diameter, but sometimes it looks nearly normal. For recording such axons must be seen in a majority of the fascicles of a section (Figs 6 and 17).

In grafts recorded as necrotic, there was a destruction of normal nerve structure and invasion of connective tissue and inflammatory cells in the major part of the graft.

RESULTS

For each of the four series the records made after observation are listed here. The records are arranged under four headings: macroscopic observations, fluorescence microscopy, stereomicroangiography and light microscopy. Under each heading the recorded observations are found in chronological order. The records are made according to the basis of estimation set in the foregoing chapter. Normal findings are as a rule not recorded here.

At the end of each series there is a short comment on the major findings in the series.

Figures, given in parenthesis, refer to the total number of grafts observed. The corresponding figures also are found in the tables 3 - 11.

Series 1

84 autologous grafts (Table 1).

Macroscopic observations

1 day (4)	Slight haematoma around the grafts and slight oedema at the nerve ends.
3 days (7)	Slight haematoma around the grafts and slight swelling of the nerve ends.
5 - 7 days (16)	Slight oedema in and around the grafts. Inflammatory reaction with adherences in 3 cases.
10 - 14 days (14)	Slight oedema in and around the grafts. Abscess formation in one case. Paresis of hind legs in one animal.
21 - 60 days (18)	Slight oedema in the grafts and the nerve ends. Adherences.
90 - 180 days (19)	Slight adherences in all cases. Paresis of hind legs in one of the animals.
300 - 360 days (6)	Slight oedema in the grafts. Adherences.

TABLE 3. FLUORESCENCE MICROSCOPY FINDINGS. GROUP A.

days	1		3		5		7		10		14		21		42		90		180		360	
	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H
Number of grafts observed	2	2	2	2	2	2	6	5	3	2	3	2	5	4	3	3	3	3	5	5	3	3
Grafts with filled endoneural vessels	-	-	2	2	2	2	6	4	1	2	3	2	5	-	3	2	3	3	5	5	2	1
Grafts with filled perineural and/or epineural vessels	-	-	-	2	2	2	-	4	-	2	-	-	5	1	3	3	3	3	5	5	3	3
Grafts with "new vessels"	-	-	-	-	-	-	1	1	-	1	-	-	3	-	1	1	-	1	2	2	2	3
Diffuse fluorescence outside the vessels	2	1	2	2	2	2	6	5	3	2	3	2	5	3	3	2	3	3	4	4	1	1
Grafts without filled vessels	1	1	-	-	-	-	-	1	2	-	-	-	-	3	-	-	-	-	-	-	-	-

A = autologous grafts H = homologous grafts

Fluorescence microscopy (Table 3)

1 day (2)	No endoneural vessels in the grafts. In the nerve ends filled vessels of normal appearance. Diffuse fluorescence in the suture regions.
3 days (2)	Endoneural vessels in both grafts. Diffuse fluorescence in the suture regions.
5 - 7 days (8)	Endoneural vessels in all grafts. Diffuse fluorescence at the graft ends. New vessels in the proximal suture region of one graft, a 7 day preparation.
10 - 14 days (6)	Endoneural vessels in 4 grafts. Diffuse fluorescence in all graft ends. Two grafts without filled vessels. In the above mentioned case with abscess, the graft showed no special changes.
21 - 42 days (8)	Endoneural vessels in all grafts. In one graft "new vessels" endoneurally. New vessels in the suture regions of 4 more cases. Diffuse fluorescence in all graft ends.
90 - 180 days (8)	Endoneural vessels in all cases. Diffuse fluorescence in the suture regions in 4 cases. New vessels in the same region in 2 grafts.
360 days (3)	Endoneural vessels in 2 grafts. "New vessels" in these cases, but no diffuse fluorescence. In the third case there was fluorescence.

Stereomicroangiography (Table 4)

1 day (2)	No endoneural vessels in the grafts, but well filled vessels in the nerve ends.
3 days (5)	Endoneural vessels in 4 cases, epineural vessels in all cases. In 2 cases anastomosis between vessels in the proximal nerve end and endoneural vessels in the graft, in one case between vessels in the distal nerve and vessels in the graft. In this case there also were anastomosis between graft endoneural

TABLE 4. STEREOMICROANGIOGRAPHIC FINDINGS. GROUP A.

days	1		3		5		7		10		14		21		42		90		120		180		360	
	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H
Number of grafts observed	2	2	5	5	2	2	6	6	3	3	5	5	6	5	3	3	3	3	2	2	6	4	2	2
Grafts with filled endoneural vessels	-	-	4	4	2	2	5	3	3	3	2	3	5	4	1	2	3	3	1	1	4	3	2	2
Grafts with filled perineural and/or epineural vessels	-	-	5	4	2	2	5	3	3	3	4	5	6	5	2	3	3	3	2	2	6	4	2	2
Graft vessels with anastomosis to proximal nerve end vessels	-	-	2	1	2	1	-	-	-	2	1	-	1	-	-	2	1	1	-	-	2	2	-	1
Grafts vessels with anastomosis to distal nerve end vessels	-	-	1	-	-	-	2	-	1	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-
Graft vessels with anastomosis to vessels in both nerve ends	-	-	-	3	-	1	3	2	2	1	-	1	2	1	1	-	2	1	1	1	2	1	2	1
Grafts with anastomosis between graft vessels and vessels surrounding the graft	-	-	2	2	1	2	2	1	3	2	2	3	3	4	1	1	3	3	1	1	4	1	2	2
Grafts without filled vessels	2	2	-	1	-	-	1	3	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-

A = autologous grafts H = homologous grafts

vessels and vessels in the surrounding connective tissue. Such anastomosis also were at hand in the 4th case.

5 - 7 days (8) — One case not contrast filled, due to technical error. Endoneural vessels in 7 cases. In 2 cases anastomosis between vessels in the proximal nerve and vessels in the graft, in 2 between distal nerve vessels and graft vessels and in 3 anastomosis in both ends. In 2 cases there also were anastomosis from vessels in the surrounding connective tissue (Fig. 7).

10 - 14 days (8) — The paretic case mentioned belonged to this group - no endoneural vessels. Endoneural vessels in 5 cases. Proximal vessel anastomosis in 1 case, distal in 1 case and anastomosis in both ends in 2 cases. In the 5th case there were anastomosis with vessels in the surrounding tissue. In one case the graft was without filled vessels, in 2 other cases there were epineural vessels only. The vessels in all the host nerves were well filled.

21 - 42 days (9) — Endoneural vessels in 6 cases. Proximal vessel anastomosis in 1 case, anastomosis in both ends in 3 cases. 2 of these also received anastomosis from the surrounding tissue vessels. In 2 more cases there were anastomosis with vessels in surrounding tissue.

90 - 180 days (11) — Endoneural vessels in 8 cases, epineural in all. Proximal vessel anastomosis in 3 cases. 2 of these also had vessel contact to the surrounding tissue. Anastomosis with both ends in 4 cases. All these also had contact to the surrounding tissue vessels. In one case anastomosis with the surrounding vessels was the only observed contact.

360 days (2) — Endoneural vessels in both cases and anastomosis of all listed types in both.

TABLE 5. LIGHT MICROSCOPY FINDINGS. GROUP A.

days	1		3		5		7		10		14		21		42		60		90		120		180		300		360	
	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H
Number of grafts observed	2	2	5	5	2	2	6	6	3	3	4	4	6	5	3	3	1	1	3	3	2	2	6	4	1	1	2	2
Grafts with filled endoneural vessels	–	1	4	2	2	2	4	3	3	3	2	2	5	5	2	2	–	–	3	3	2	2	5	4	–	–	2	2
Grafts with filled perineural and/or epineural vessels	–	–	3	3	2	2	5	3	3	3	3	2	6	5	2	2	–	–	3	3	2	2	6	4	–	–	2	2
Grafts with "new vessels"	1	1	3	3	2	2	5	3	3	3	4	3	5	5	2	3	–	–	3	3	2	2	6	4	–	–	2	2
Inflammatory cells	1	–	2	1	–	–	1	2	1	2	2	2	2	3	1	1	–	–	–	–	–	–	–	1	–	–	–	–
Increased endoneural collagen	1	–	2	1	1	–	4	–	2	1	2	1	2	2	1	3	–	–	–	3	–	–	6	3	–	–	2	1
Increased thickness of the perineurium	1	1	3	1	1	2	5	3	2	2	2	2	1	3	1	1	1	1	2	3	1	1	3	3	1	1	2	2
Increased epineural collagen	1	2	5	3	2	2	6	4	2	1	4	2	6	4	2	3	1	1	3	3	2	2	6	4	1	1	2	2
Degenerated myelin	–	1	3	4	1	1	5	4	3	3	4	3	5	5	2	3	1	1	3	3	2	2	6	4	1	1	2	2
Normal looking axons	1	1	5	4	1	–	3	–	–	1	2	–	3	1	–	1	–	–	1	1	–	–	5	3	1	1	1	2
Degenerated axons or lack of axons	1	1	–	1	1	2	3	6	3	2	2	4	3	4	3	1	1	1	2	2	2	2	1	1	1	1	1	–
Regenerated axons in distal nerve	–	–	–	–	–	–	–	–	–	–	–	1	1	2	1	2	–	–	1	1	1	1	4	3	1	1	1	1
Necrotic grafts	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

A = autologous grafts H = homologous grafts

Light microscopy (Table 5)

Observations on the vessels

1 day (2)	No endoneural vessels (Fig. 11).
3 days (5)	Endoneural vessels in 4 cases. The graft, here found to be without demonstrable vessels, had vessels according to the angiography and showed no abnormity otherwise. In one case vessels were found here, but not in the angiogram.
5 - 7 days (8)	One case was not contrast filled and thus not used for the angiographic study. No vessels were found in light microscopy either. Endoneural vessels in 6 cases. In the 7th case there were perineural vessels (but according to the angiogram endoneural vessels were observed) (Fig. 12).
10 - 14 days (7)	One angiographed case was not used here. Endoneural vessels in 5 cases, the same cases as in the angiography. The paretic case mentioned belonged to this group and showed no specific changes in the sections studied.
21 - 60 days (10)	One case was not contrast filled and consequently showed no vessels. Endoneural vessels were found in 7 cases. 2 of these showed no endoneural vessels in the angiograms. On the other hand 2 grafts had filled vessels in the angiograms, but showed no vessels in the sections used for light microscopy.
90 - 180 days (11)	Endoneural vessels in 10 cases, 7 of them already found in the angiograms. In 3 cases the angiograms only showed epineural vessels. One case, showing endoneural vessels due to the angiography, only showed perineural vessels here. The combination of methods, however, made it possible to find endoneural vessels in all cases. One paretic case in this group did not show any specific changes in the sections studied.
300 - 360 days (3)	Endoneural vessels in 2 cases. The 3rd case was never contrast filled (Figs 19 and 20).

Other observations

Invasion of inflammatory cells was observed in grafts from the first day up to 42 days.

An increase of endoneural and epineural collagen was observed in a majority of grafts from the first day on. A thickening of the perineurium was a frequent finding.

Degeneration of the myelin was observed from the third day, but for as long as 42 days there were cases with normal looking myelin. In no case normal looking myelin was again observed in the preparations during the observation time. Axons of normal appearance were found up to 7 days. Degenerated axons were found from the first day. Regenerating axons were observed from the 21st day on.

No necrotic grafts were found in this group.

Comments on Series 1

The macroscopic observations revealed only slight inflammatory reaction with oedema and adherence formation. In the late cases there were adherences in a majority of the grafts, indicating that an inflammation had occurred. In two cases there were signs of decreased function (paresis).

Fluorescence microscopy

No filled vessels in the first day specimens, but from the third day there were filled endoneural vessels in 32 of the 35 grafts observed. Diffuse fluorescence outside the vessels, indicating a leakage of albumin, was the rule not only in the early cases, but also in specimens from 180 and 360 days. "New vessels" are recorded from the 7th day on.

Stereomicroangiography

No filled vessels in the first day grafts. From the third day on there

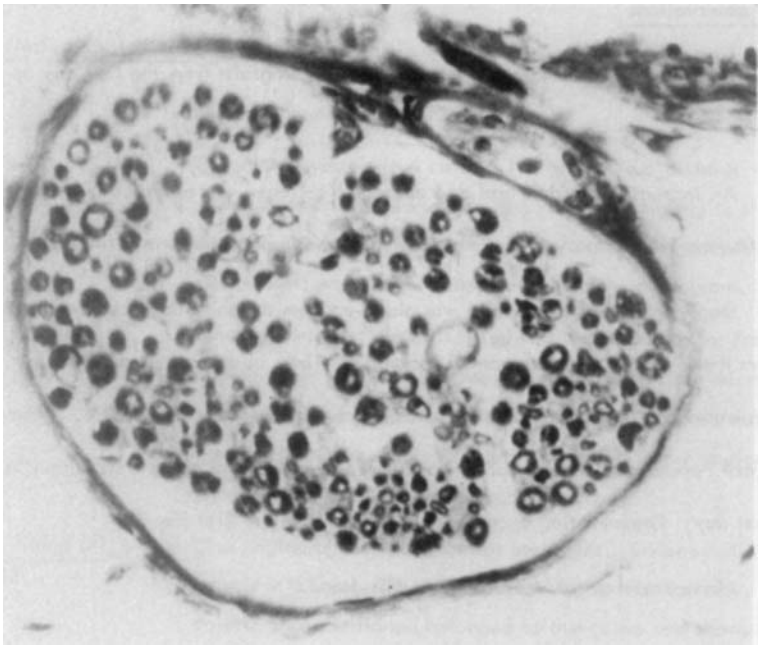

Fig. 11. Transverse section of a fascicle in an autologous graft 1 day after operation. No filled endoneural vessels observed. Luxol.

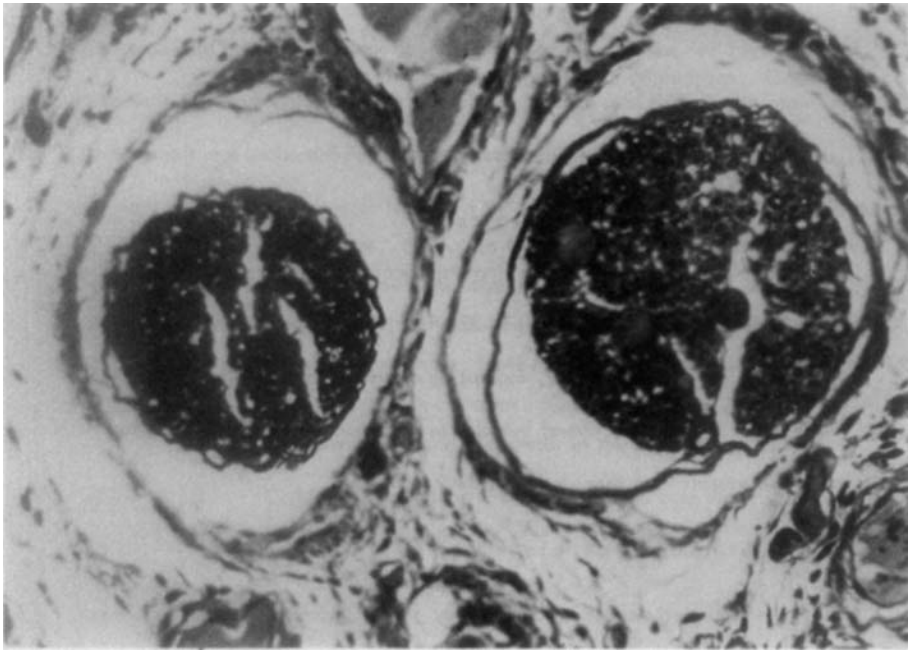

Fig. 12. Transverse section of two fascicles in an autologous graft 5 days after operation. Filled endoneural vessels. Luxol.

were filled endoneural vessels in 32 of 42 specimens observed. Anastomosis with nerve end vessels were found in 9 of the 11 grafts with endoneural vessels up to the 7th day. The frequency of anastomosis with the surrounding vessels was lower in the early cases - in the first week there were 4 grafts of 11 showing such anastomosis. In the specimens from 90 to 360 days there were 9 out of 10 grafts with such anastomosis. These observations suggest that anastomosis at first are established between nerve end vessels and graft vessels, later there are anastomosis with surrounding tissue vessels.

Light microscopy

Filled endoneural vessels from the third day on in 32 of 44 grafts. No vessels in the first day specimens. In a few cases there were endoneural filled vessels seen in the Luxol sections, vessels, which were not recorded in the angiograms. "New vessels" were recorded from the first day on. Increase in collagen amount was the rule, and increased thickness of the perineurium was a frequent finding. The degeneration of the myelin was observed from the third day. Normal looking myelin was found up to the 42nd day. No reappearance of normal myelin was found in the late specimens. The degeneration of axons started in the first day specimens. Regenerated axons in the distal nerve were found from the 21st day.

Series 2

130 fresh homologous grafts (Table 1).

Macroscopic observations

1 day (8)	Haematoma in one case. Slight oedema i 2 cases.
3 days (12)	Slight oedema i 6 cases, especially in the suture regions.
5 - 7 days (23)	Massive oedema i 2 cases, inflammation with pus surrounding

TABLE 6. FLUORESCENCE MICROSCOPY FINDINGS. GROUP B.

days	1		3		5		7		10		14		21		42		90		180		300		360	
	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F
Number of grafts observed	2	2	3	3	2	2	2	3	-	3	2	2	5	5	4	3	2	3	1	5	1	1	2	4
Grafts with filled endoneural vessels	2	1	3	1	2	1	2	1	-	3	1	1	1	2	2	-	2	-	1	4	1	1	2	4
Grafts with filled perineural and/or epineural vessels	2	1	3	1	2	1	2	1	-	3	1	1	1	2	2	-	2	-	1	4	1	1	2	4
Grafts with "new vessels"	-	-	1	-	-	-	-	-	-	2	1	-	1	2	3	3	1	-	1	5	1	-	2	4
Diffuse fluorescence outside the vessels	2	1	2	2	2	2	1	2	-	3	2	1	3	4	2	2	1	-	-	1	1	1	2	3
Grafts without filled vessels	-	1	-	2	-	1	-	2	-	-	1	1	4	3	1	-	1	3	-	-	-	-	-	-

H = homologous grafts F = freezed homologous grafts

the graft in one case, haematoma in one case, subcutaneous abscess in one.

10 - 14 days (18) — Inflammatory reaction in one case. Paresis of hind legs in one animal.

21 - 60 days (30) — Oedema in 3 cases, adherence formation in 3 cases, slight inflammation in 2, abscess formation in 2 cases.

90 - 180 days (24) — Adherences in 3 cases. Paresis of hind legs in two animals.

300 - 360 days (15) — Oedema in 2 cases, adherences in one case. One animal in bad general condition, due to paresis of hind legs.

Fluorescence microscopy (Table 3 and 6)

1 day (4) — Endoneural vessels in 2 grafts. The vessels in the host nerve ends looked normal. Diffuse fluorescence in the suture regions in these cases and in one more case.

3 days (5) — Endoneural vessels in all cases and also in the host nerves there were filled vessels. Diffuse fluorescence in 4 cases. In one case "new vessels" were observed and they also were seen between the fascicles. The endoneural vessels were observed just up to the end of the graft.

5 - 7 days (11) — Endoneural vessels in 10 cases. "New vessels" were found in the suture regions in one case. In all cases there was an intense diffuse fluorescence in the suture regions. The graft without filled vessels was the infected case observed macroscopically.

10 - 14 days (6) — Endoneural vessels in 5 cases. "New vessels" in the suture regions in 2 cases. Intense fluorescence in the suture regions in all cases. One graft without filled vessels.

21 - 42 days (15) — Normal endoneural vessels in 5 cases. In the two abscess cases (observed macroscopically) there were no filled vessels and the same picture was found in one more case. In 2 of the oedematous cases there were no endoneural vessels. In 5

TABLE 7. STEREOMICROANGIOGRAPHIC FINDINGS. GROUP B.

days	1		3		5		7		10		14		21		42		90		180		360	
	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F
Number of grafts observed	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	2	2	-	2	4	2
Grafts with filled endoneural vessels	-	-	1	-	2	-	2	2	1	-	2	1	-	1	-	-	-	1	-	1	1	1
Grafts with filled perineural and/or epineural vessels	-	-	1	-	2	-	2	2	2	1	2	1	2	3	3	3	-	-	-	2	3	2
Graft vessels with anastomosis to proximal nerve end vessels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Graft vessels with anastomosis to distal nerve end vessels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Graft vessels with anastomosis to vessels in both nerve ends	-	-	1	-	-	-	1	1	1	-	-	-	-	-	-	-	-	-	-	1	1	1
Grafts with anastomosis between graft vessels and vessels surrounding the graft	-	-	-	-	2	-	2	2	-	-	2	1	-	1	-	-	-	-	-	1	1	1
Grafts without filled vessels	2	2	1	2		2	-	-	-	1	-	1	-	-	-	-	2	1	-	-	1	-

H = homologous grafts F = freezed homologous grafts

cases there were no endoneural vessels in the graft. In one of these there were perineural vessels, in two other cases there were "new vessels". Such "new vessels" were also found in 3 cases with endoneural vessels. Diffuse fluorescence in 10 cases in the suture regions.

90 - 180 days (11) Endoneural vessels in 11 cases. "New vessels" at the sutures in 5 cases. Diffuse fluorescence in the suture regions in 8 cases.

300 - 360 days (6) Endoneural vessels in 5 cases. "New vessels" in the suture regions in 5, diffuse fluorescence in 4 cases. One of the 2 grafts without endoneural vessels had perineural vessels.

Stereomicroangiography (Table 4 and 7)

1 day (4) No endoneural vessels. The vessels in the nerve ends looked normal.

3 days (7) Endoneural vessels in 5 cases. In 2 grafts there were no vessels to be seen. The host nerves showed well filled vessels in all cases. Anastomozing was observed with the proximal nerve in one case, with both proximal and distal nerve in 3 cases and with the surrounding vessels in one case. The cases without filled vessels showed no specific macroscopical changes.

5 - 7 days (12) Endoneural vessels in 9 cases. Anastomosis with proximal nerve end vessels in one case, with both proximal and distal in 4 cases and with surrounding vessels in 7 cases. In 3 grafts there were no vessels. In one case of these there was no contrast filling in the host nerve vessels, and one case showed small vessels around the graft partly penetrating in it. Macroscopically there were no special changes in these 3 grafts (Fig. 8).

10 - 14 days (12) Endoneural vessels in 9 cases. The 3 other grafts showed epineural and perineural vessels and no other specific changes.

TABLE 8. LIGHT MICROSCOPY FINDINGS. GROUP B.

days	1		3		5		7		10		14		21		30		42		90		180		300		360	
	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F	H	F
Number of grafts observed	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	1	3	3	4	3	-	2	1	1	4	2
Grafts with filled endoneural vessels	1	1	1	-	1	1	2	1	2	1	2	2	-	3	-	-	3	2	2	2	-	2	-	-	2	2
Grafts with filled perineural and/or epineural vessels	1	-	1	-	2	1	2	2	2	1	2	2	1	3	-	-	3	3	2	2	-	2	-	-	2	2
Grafts with "new vessels"	-	1	2	2	2	1	2	2	2	2	2	2	2	3	-	-	3	2	2	2	-	2	-	-	3	2
Inflammatory cells	1	2	1	1	1	1	-	-	1	1	2	2	1	2	1	1	-	-	-	-	-	-	-	-	-	-
Increased endoneural collagen	-	1	2	-	1	1	-	2	2	2	-	1	1	2	-	-	2	1	3	3	-	2	1	1	3	2
Increased thickness of the perineurium	-	-	1	1	2	1	-	-	2	2	1	-	2	3	1	1	3	2	4	3	-	1	1	1	3	2
Increased epineural collagen	-	2	2	2	2	2	1	2	2	2	2	1	2	3	1	1	3	3	4	3	-	2	1	1	4	2
Degenerated myelin	-	1	1	1	2	2	1	2	2	1	2	2	2	3	1	1	3	3	4	3	-	2	1	1	4	2
Normal looking axons	2	2	1	2	-	2	-	-	1	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-	4	-
Degenerated axons or lack of axons	-	-	1	-	2	-	2	2	1	2	2	2	2	2	-	1	3	3	4	3	-	1	1	1	-	2
Regenerated axons in distal nerve	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	-	-	1	-	-	-	2	-	-	4	2
Necrotic grafts	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

H = homologous grafts F = freezed homologous grafts

Anastomosis with proximal vessels in 2 cases, with distal vessels in one case, with both proximal and distal in 3 cases. In 7 cases anastomosis with surrounding vessels. The paretic case belonged to this group, but the graft had no specific changes.

21 - 42 days (13) — Endoneural vessels in 6 cases. In the other 7 cases there were epineural vessels only. There were no specific characteristics for these grafts micro- or macroscopically. In the cases with endoneural vessels there were anastomosis with proximal vessels in 2 grafts, with both proximal and distal in one graft and with surrounding vessels in 5 cases.

90 - 180 days (11) — Endoneural vessels in 7 cases. Of the 4 cases without such vessels 2 had epineural vessels, 2 were without filled vessels. The host nerve in all cases showed contrast filled vessels. The anastomosis observed were with proximal vessels in 3 cases, with both proximal and distal in 3, and with surrounding vessels in 6 cases. The paretic cases showed well filled vascular systems.

360 days (6) — Endoneural vessels in 3 cases, of the rest 2 epineural vessels and one was without filled vessels. This case did not show any specific changes. Anastomosis with proximal nerve vessels in one case, with both proximal and distal in 2, and with surrounding vessels in 3 cases. In one case there were no vessels in the central part of the graft.

Light microscopy (Table 5 and 8)

Observations on the vessels

1 day (4) — Endoneural vessels in 2 cases. These vessels were not observed in the angiograms.

3 days (7) — Endoneural vessels in 3 cases. These also showed vessels in the angiograms (Fig. 14).

5 - 7 days (12) — Endoneural vessels in 8 cases, all with vessels in the angiograms. There were 2 necrotic grafts, both without filled

vessels in the angiograms.

10 - 14 days (11) — One of the angiographic cases was not studied. Endoneural vessels in 10 cases, 9 of these were also found in the angiography, the 10th showed endoneural vessels in the Luxol sections, but only perineural and epineural vessels in the angiogram. One more graft without filled vessels showed no other specific changes.

21 - 60 days (15) — 2 cases, not used for angiography, were added here. Both were without demonstrable vessels. Of the other 13 grafts endoneural vessels were observed in 10 grafts. Of the six grafts with endoneural vessels observed in the angiograms all show such vessels here. 5 grafts, showing only epineural vessels in the angiograms, also had endoneural vessels here. 2 cases with epineural vessels in the angiograms showed no vessels here (Fig. 13).

90 - 180 days (13) — 2 cases, not used for angigraphy, were added, both without demonstrable vessels. One of the angiography specimens was not used for histologic methods. Of the remaining 10 grafts endoneural vessels were observed in all cases. The paretic cases had vessels and showed no other specific changes.

300 - 360 days (8) — Two preparations, not used for angiography, were added here. They showed no filled vessels. Endoneural vessels in 4 cases. Of the other cases one had new vessels (the case without filled vessels in the angiogram). The other case without filled vessels here showed in the angiogram epineural vessels (Fig. 18).

Other observations

Invasion of inflammatory cells occurred in about the same frequency as in Series 1.

The increase of endoneural and epineural collagen was of the same proportion as in Series 1. Thickening of the perineurium was a frequent finding.

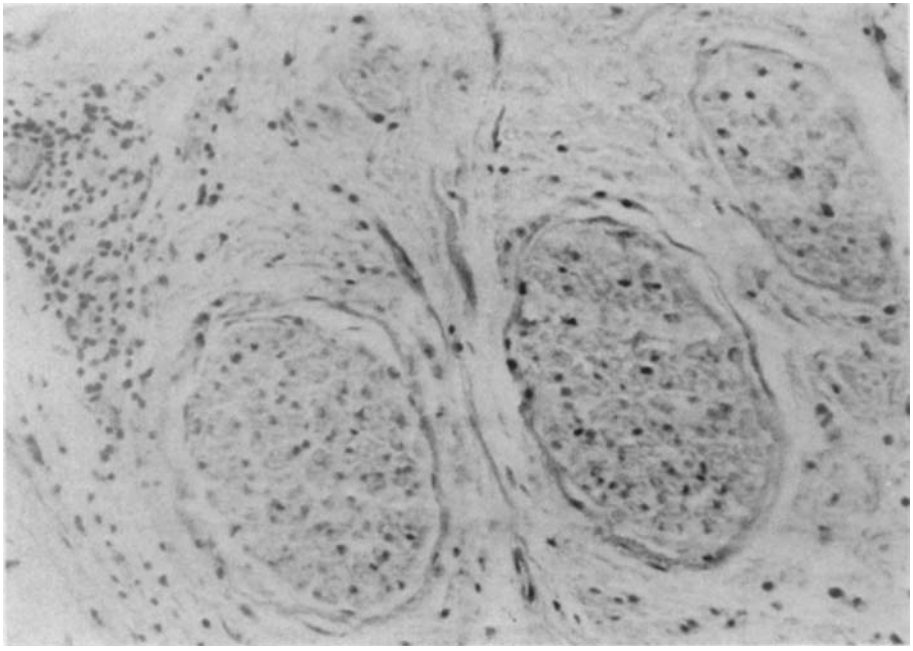

Fig. 13. Transverse section of homologous graft 42 days after operation. van Gieson. Increase of collagen and inflammatory cells.

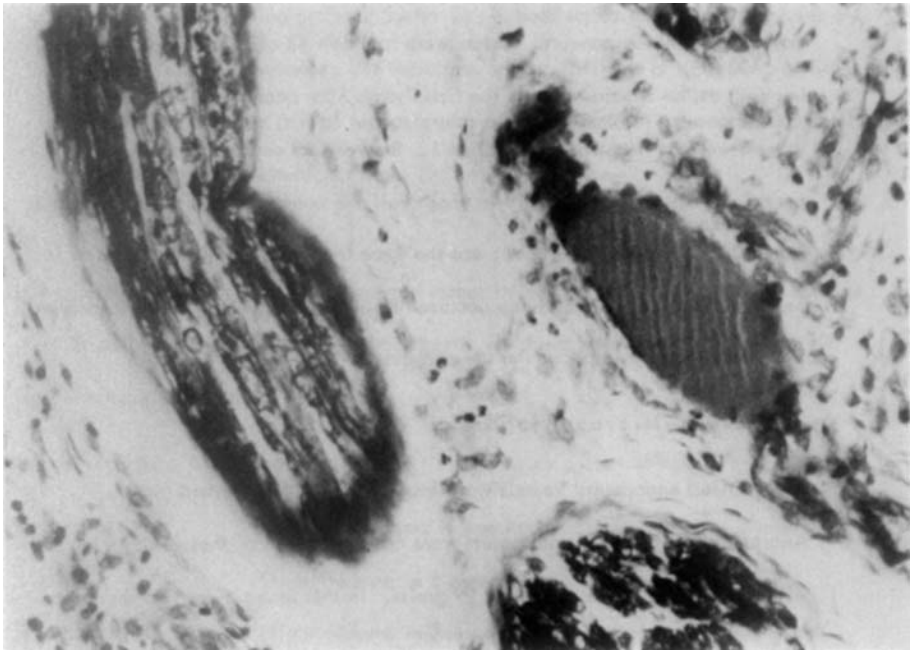

Fig. 14. Longitudinal section from proximal suture region in a specimen with a fresh homologous graft 3 days after operation. Filled endoneural vessels in graft (left) and inflammatory cells. Dark ovoid structure (right) is a transsected nylon suture.

Degeneration of the myelin was observed from the third day. Specimens, containing normal looking myelin, were found up to 14 days. No reappearance of normal myelin was observed. Degenerated axons were found from the first day. Normal looking axons were found up to the third day, then again from the tenth day. Regenerated axons were found in the distal nerve from the tenth day (Fig. 6).

Comments on Series 2

Macroscopic observations

In this series there was a more intense inflammatory reaction, sometimes with abscess formation. A pronounced oedema was more often seen. The adherences in the specimens from 180 and 360 days were not more frequent than in Series 1. Four animals had paresis of the hind legs.

Fluorescence microscopy

Endoneural vessels were recorded already in two specimens from the first day. After that endoneural vessels were found in 43 of 59 observed grafts. Looking at the specimens from the first 7 days, the occurrence of endoneural vessels were as frequent as in Series 1. Between 10 and 42 days filled endoneural vessels were found only in about half the number of grafts. In the grafts from 90 to 360 days there again was the same frequency of filled vessels as in Series 1. Diffuse fluorescence occurred as in Series 1. New vessels occurred from the third day in about the same frequency as in Series 1.

Stereomicroangiography

Filled endoneural vessels were found in 39 of 65 observed grafts. Anastomosis with nerve end vessels were observed in 27 of these 39 grafts and with surrounding tissue vessels in 29 grafts. In this series there was the same

frequency of anastomozing with nerve end vessels and with vessels in the surroundings, both in the early and late cases.

Light microscopy

Filled endoneural vessels were found in 47 of 68 cases, which means that some cases with filled vessels were not observed in the angiograms. The inflammatory reaction, increase in collagen and degeneration of myelin and axons did not differ from Series 1.

In Series 2, using homologous grafts, there were fewer grafts with filled endoneural vessels than in Series 1. This was most apparent in the grafts observed between 10 and 42 days. In the early and late observed grafts the frequency of grafts with filled vessels was about the same as in Series 1. There also was a more pronounced inflammatory reaction in the observations from 10 to 42 days. Looking at the preparations from 360 days, they were very similar to the corresponding grafts of Series 1. In both series there were fascicles of quite normal appearance, but there also was an increase of collagen, a thickening of the perineurium and no reappearance of normal myelin as recorded by the Luxol fast blue method.

Series 3

109 homologous grafts beforehand freezed either in fluid nitrogen or in carbon dioxide snow. The grafts thereafter were kept either in fluid nitrogen or in a freezer at -20° C for 3 weeks up to 6 months.

Macroscopic observations

1 day (8)	Slight haematoma in one case. Slight oedema i 3 cases.
3 days (9)	Slight oedema in 6 cases.
5 - 7 days (19)	Slight oedema in one case. Inflammation with pus in one.

TABLE 9. FLUORESCENCE MICROSCOPY FINDINGS. GROUP C.

days	1		3		5		7		10		14		21		42		90		150		180		360	
	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I
Number of grafts observed	2	2	2	2	3	3	2	2	2	2	1	2	1	2	1	2	1	2	1	1	3	3	1	1
Grafts with filled endoneural vessels	1	-	-	1	1	-	1	1	1	2	-	-	-	-	-	-	1	2	-	-	2	1	1	1
Grafts with filled perineural and/or epineural vessels	1	1	-	1	1	-	2	-	1	1	-	-	-	-	-	-	1	2	-	-	2	1	1	1
Grafts with "new vessels"	-	-	-	-	-	-	-	-	-	-	-	1	1	1	-	1	1	2	-	-	3	3	1	1
Diffuse fluorescence outside the vessels	2	1	1	1	2	3	2	2	2	2	1	2	1	2	1	1	1	2	1	1	1	3	-	1
Grafts without filled vessels	1	1	2	1	2	3	-	1	1	-	1	1	-	1	1	1	-	-	1	1	-	-	-	-

F = freezed homologous grafts · I = freezed and irradiated homologous grafts

10 - 14 days (17)	Oedema in 8 cases, total necrosis in one case.
21 - 42 days (23)	Oedema in 4 cases, abscess in one.
60 - 180 days (24)	Adherences in 7 cases, hind leg paresis in two animals.
300 - 360 days (9)	All normal looking.

Fluorescence microscopy (Table 6 and 9)

1 day (4)	Endoneural vessels in 2 grafts. The vessels in the host nerve ends were filled in all cases. Diffuse fluorescence in 3 cases.
3 days (5)	Endoneural vessels in one graft, the other grafts without filled vessels. The host nerve ends showed filled vessels in all but one case. Fluorescence in 3 cases.
5 - 7 days (10)	Endoneural vessels in 4 cases. All these showed an intense diffuse fluorescence not only in the suture regions, but in the entire grafts. Of the remaining grafts 4 were without filled vessels. Three of these showed diffuse fluorescence. The macroscopically observed graft with inflammation had well filled vessels.
10 - 14 days (8)	Endoneural vessels in 5 grafts. Of the remaining three one showed total necrosis (observed macroscopically), two were without filled vessels. All but the total necrotic case showed an intense diffuse fluorescence, especially in the suture regions. "New vessels" in this region were observed in 2 cases, both vascularized also by endoneural vessels.
21 - 42 days (10)	Endoneural vessels in 2 grafts only. The remaining grafts were without filled vessels (one was the case with abscess macroscopically). "New vessels" were observed in 6 cases. The host nerve showed normal vascular anatomy both proximally and distally. Diffuse fluorescence in the suture region in 8 grafts.
90 - 180 days (13)	Endoneural vessels in 7 cases. 6 grafts were without filled

TABLE 10. STEREOMICROANGIOGRAPHIC FINDINGS. GROUP C.

days	1		3		5		7		10		14		21		42		90		180		360	
	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I
Number of grafts observed	2	2	2	2	2	2	3	3	2	2	2	2	2	2	2	2	2	2	2	2	-	2
Grafts with filled endoneural vessels	-	-	-	-	-	-	2	-	-	-	2	2	2	1	1	1	-	1	2	1	-	-
Grafts with filled perineural and/or epineural vessels	-	-	-	-	-	-	2	1	2	1	2	2	2	1	2	2	1	2	2	2	-	1
Graft vessels with anastomosis to proximal nerve end vessels	-	-	-	-	-	-	-	-	2	-	1	2	2	1	-	-	-	1	-	-	-	-
Graft vessels with anastomosis to distal nerve end vessels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Graft vessels with anastomosis to vessels in both nerve ends	-	-	-	-	-	-	2	-	-	-	1	-	-	-	1	1	-	-	2	1	-	-
Graft with anastomosis between graft vessels and vessels surrounding the graft	-	-	-	-	-	-	-	-	1	-	-	-	1	1	1	1	-	-	1	1	-	-
Grafts without filled vessels	2	2	2	2	2	2	1	2	-	1	-	-	-	1	-	-	1	-	-	-	-	1

F = freezed homologous grafts I = freezed and irradiated homologous grafts

vessels. Diffuse fluorescence was observed in 4 cases, new vessels in 9 cases. Even the grafts with filled vessels had an appearance differing from the normal. 2 paretic cases - one graft showed endoneural vessels, the other not. No other specific changes in those two.

300 - 360 days (6) — Endoneural vessels in all cases. Diffuse fluorescence in 5 of the grafts, in one in the entire graft. In all cases there were new vessels in the suture regions.

Stereomicroangiography (Table 7 and 10)

1 day (4) — No endoneural vessels. In the host nerve ends normal looking vessels.

3 days (4) — No endoneural vessels. Normal vessel anatomy in the host nerve.

5 days (4) — No endoneural vessels. In one case ingrowth of vessels from the proximal nerve end was observed.

7 days (5) — Endoneural vessels in 4 grafts. In one case there were no vessels in the graft, but in the host nerve. Anastomozing both with proximal and distal vessels was observed in 3 cases, with surrounding vessels i 2 cases (Fig. 9).

10 - 14 days (8) — Endoneural vessels in 5 cases. Of the remaining three two were without filled vessels and one showed epineural vessels. In all cases the host nerve vessels were well filled. The grafts without filled vessels did not show any other specific changes. Anastomosis with proximal vessels occurred in 3 cases, with both proximal and distal vessels in one case and with surrounding vessels in 2 cases. In one graft, with both proximal and distal vessel contact, there was a central avascular zone.

21 - 42 days (10) — Endoneural vessels in 4 cases. The remaining 6 grafts all showed epineural vessels. Anastomosis was observed with proximal vessels in 2 grafts, both with proximal and distal vessels in one and with surrounding vessels in 3 cases.

TABLE 11. LIGHT MICROSCOPY FINDINGS. GROUP C.

days	1		3		5		7		10		14		21		42		60		90		120		180		300		360	
	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I	F	I
Number of grafts observed	2	2	2	2	2	2	3	3	2	2	3	3	2	2	1	1	2	2	1	1	2	2	1	1	2	2	-	2
Grafts with filled endoneural vessels	-	-	-	-	-	-	-	-	-	1	1	2	2	1	-	-	2	2	-	-	1	1	-	-	2	2	-	1
Grafts with filled perineural and/or epineural vessels	-	1	-	-	-	-	1	1	1	1	1	2	2	1	-	-	2	2	-	-	1	1	-	-	2	2	-	1
Grafts with "new vessels"	-	-	2	2	2	2	3	3	2	2	2	2	2	2	-	-	2	2	-	-	2	2	-	-	2	2	-	2
Inflammatory cells	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	-
Increased endoneural collagen	1	1	2	1	1	-	1	2	2	2	2	2	2	-	1	1	1	1	-	-	1	2	-	-	1	1	-	1
Increased thickness of the perineurium	2	2	2	2	2	2	2	2	2	2	2	3	2	-	1	1	2	2	1	1	2	2	1	1	2	2	-	2
Increased epineural collagen	2	2	2	1	2	2	3	3	2	2	3	3	2	1	1	1	2	2	1	1	2	2	1	1	2	2	-	2
Degenerated myelin	2	2	2	2	2	2	2	3	2	2	3	3	2	1	1	1	2	2	1	1	2	2	1	1	2	2	-	2
Normal looking axons	1	2	2	2	1	1	1	1	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1	1	-	2
Degenerated axons or lack of axons	1	-	-	-	1	1	2	2	2	1	3	3	2	1	1	1	2	2	1	1	2	2	1	1	1	1	-	-
Regenerated axons in distal nerve	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	1	1	2	-	2
Necrotic grafts	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

F = freezed homologous grafts I = freezed and irradiated homologous grafts

90 - 180 days (8) Endoneural vessels in 4 cases. Of the rest 2 showed epineural vessels, 2 were without filled vessels. Anastomosis with distal vessels in one case, both with proximal and distal in 3 cases and with surrounding vessels in 2 cases. The cases without filled vessels did not show any other specific changes.

360 days (2) Endoneural vessels in one graft with anastomosis with both proximal, distal and surrounding vessels. In the other graft there were epineural vessels.

Light microscopy (Table 8 and 11)

Observations on the vessels

1 day (4) Endoneural vessels in one case.

3 days (4) No endoneural vessels.

5 days (4) Endoneural vessels in one case.

7 days (5) Endoneural vessels in one case - this case also showed vessels in the angiogram. Perineural vessels in 2 other cases. One of these did not show any vessels in the angiogram, the other had both endoneural and epineural vessels.

10-14-16 days (9) Endoneural vessels in 5 cases, 3 had such vessels in the angiogram, 1 only showed epineural vessels in the angiogram and in one case no angiogram was made. Of the remaining 4 cases 3 showed vessels in the angiogram and one was without filled vessels also then. This case did not show any other special characteristics.

21 - 42 days (13) 3 cases from animals, that died during the observation period, were not used for angiography. No vessels were found in these cases. Of the 10 remaining cases endoneural vessels were found in 9 cases and perineural vessels in the 10th (this case showed no vessels in the angiogram).

60 - 180 days (11) 3 grafts were not used for angiography. They did not show any filled vessels. Of the 8 grafts used for angiography, endoneural

vessels were found in 6 cases in the Luxol sections. 2 grafts showed no vessels with any technique. They showed no other special characteristics.

300 - 360 days (3) One case was not used for angiography and showed no filled vessels. The other two both showed endoneural vessels.

Other observations

Invasion of inflammatory cells was recorded in the cases from group B (Table 1) from the first day up to 30 days. In the cases from group C there were only inflammatory cells recorded in one case (a 3 days specimen).

Increase of endoneural and epineural collagen and thickening of the perineurium occurred in almost every case.

Degeneration of myelin was recorded from the first day in all grafts. No appearance of normal myelin was observed. Axons of normal appearance were found up to 7 days. In some specimens normal looking axons again were seen after 180 days. Degenerated axons were found from the first day. The degeneration seemed to be more slow than in Series 1 and 2. Regenerated axons in the distal nerve were found from the 90th day and later in a few specimens.

No necrotic grafts were recorded.

Comments on Series 3

Macroscopic observations

The inflammatory reaction observed was not very marked. There were one necrotic case and two cases of abscess formation. The adherences found were insignificant. There was paresis of the hind legs in two cases.

Fluorescence microscopy

Endoneural vessels were found in 27 of 56 grafts which was considerably

less than in the previous series. Diffuse fluorescence was intense and frequently occurring also in the long period cases. In many grafts there were changes observed, the normal fascicular pattern was disturbed, a dense tissue staining more grey was found in greater or smaller parts of the grafts. "New vessels" were observed from the 10th day.

Stereomicroangiography

Endoneural vessels were found in 18 of 45 grafts. In the grafts observed from 1 to 7 days only about 1/4 showed filled vessels. In the later observed grafts about half the grafts showed such vessels. The number of observed anastomosis with nerve end vessels was greater than the number of anastomosis with surrounding tissue vessels.

Light microscopy

Endoneural vessels in 25 of 53 grafts. Also in this series a few vessels were shown in the Luxol stained sections, but not in the angiograms.

The inflammatory reaction was not marked, the adherences found were fewer than in earlier series. The observations on collagen, perineurium and myelin were as in the previous series. The degeneration of the axon seemed to proceed more slowly. Regenerated axons in the distal nerve were found after 90 days and thereafter.

In Series 3, using freezed homologous grafts, the inflammatory reaction evoked seemed to be smaller than in the previous series. Among the grafts which had been freezed in carbon dioxide snow and then kept in a freezer at -20° C, there was only one case recorded with invasion of inflammatory cells. There were considerably fewer filled endoneural vessels. The anastomosis found were chiefly with nerve end vessels.

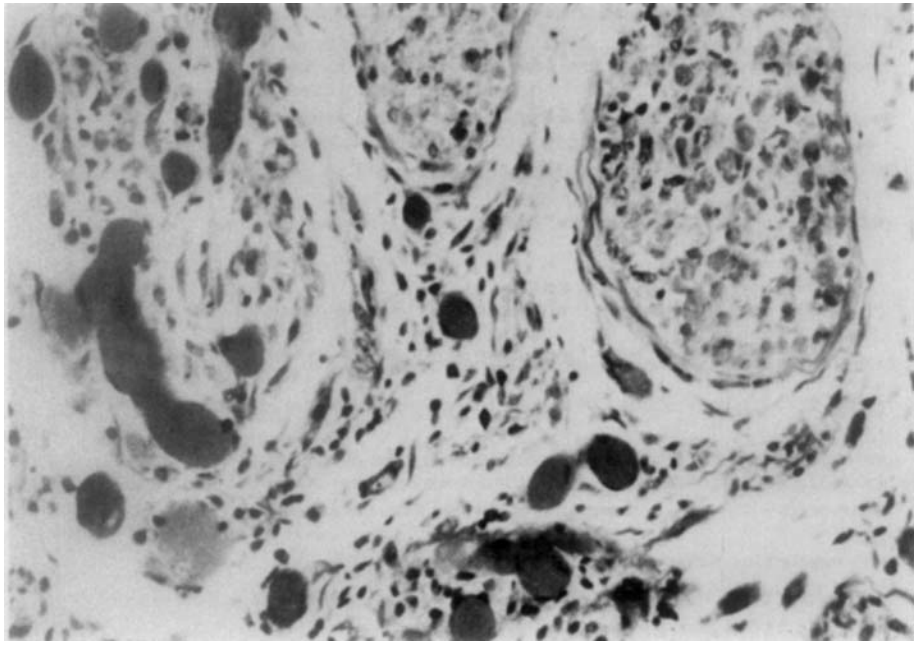

Fig. 15. Transverse section from a freezed and irradiated homologous graft 21 days after operation. Note "new vessels". Luxol.

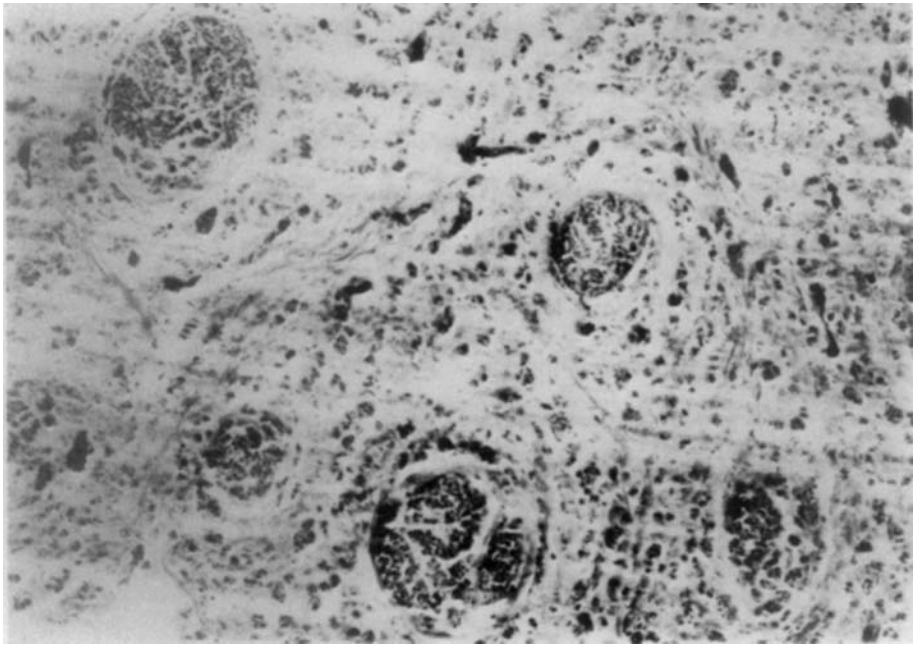

Fig. 16. Transverse section of a freezed and irradiated homologous graft 180 days after operation. Note increase in collagen. Luxol.

Series 4

52 homologous grafts beforehand freezed in carbon dioxide snow, then irradiated and kept in a freezer at -20° C for 3 weeks up to 6 months.

Macroscopic observations

1 day (4)	Haematoma in 2 cases, both also with slight oedema in the suture regions.
3 days (4)	Oedema in the graft ends in one case.
5 - 7 days (10)	No changes observed.
10 - 16 days (9)	Slight oedema in 2 cases.
21 - 42 days (10)	Slight oedema in 4 cases, abscess in one case.
60 - 180 days (12)	No changes observed.
360 days (3)	No changes observed.

Fluorescence microscopy (Table 9)

1 day (2)	No endoneural vessels, but in one case epineural vessels. Diffuse fluorescence in the entire grafts. The host nerve appeared normal.
3 days (2)	Endoneural vessels in one case. Intense diffuse fluorescence in the suture regions. Only partly normal fascicular structure. The host nerves appeared normal.
5 - 7 days (5)	Endoneural vessels in one case (7 days). Diffuse fluorescence in all grafts, especially in the suture regions. The host nerves were normal looking.
10 - 14 days (4)	Endoneural vessels in two cases. Diffuse fluorescence in all grafts, especially in the suture regions. "New vessels" were found in the suture region in one case.
21 - 42 days (4)	No endoneural vessels. Diffuse fluorescence in all grafts. "New vessels" were found in 2 grafts. The host nerves normal looking.

90 - 180 days (6) — Endoneural vessels in 3 cases. These showed diffuse fluorescence, especially in the suture regions and in one case in the entire graft. "New vessels" were found in the suture regions. 3 cases without vessels. The host nerves were normal looking.

360 days (1) — Endoneural vessels in the graft. A few normal looking fascicles were passing the entire graft. No fluorescence in the proximal suture zone, but in the distal there was a light diffuse fluorescence. "New vessels" in the suture regions.

Stereomicroangiography (Table 10)

1 day (2) — No endoneural vessels. In the nerve ends normally filled vessels.

3 days (2) — No endoneural vessels. The host nerve showed normal vascular anatomy.

5 days (2) — No endoneural vessels. In one case there was ingrowth of vessels from the proximal nerve end.

7 days (3) — No endoneural vessels. Epineural vessels in one case. In this case and one other ingrowth of vessels from the proximal end was observed (Fig. 10).

10 days (2) — No endoneural vessels. Epineural vessels in one case. In one case ingrowth of vessels from the proximal end.

14 days (2) — Endoneural vessels in both grafts. Both showed anastomosis with vessels in the proximal nerve end.

21 - 42 days (4) — Endoneural vessels in 2 grafts - one had proximal anastomosis, one anastomosis to both ends and both had additional anastomosis with surrounding vessels. One graft was without filled vessels. In one graft epineural vessels were found.

90 - 180 days (4) — Endoneural vessels in 2 grafts, proximal anastomosis in one and with both ends in the other. The remaining grafts showed epineural vessels.

360 days (2) — No endoneural vessels. In one case epineural vessels.

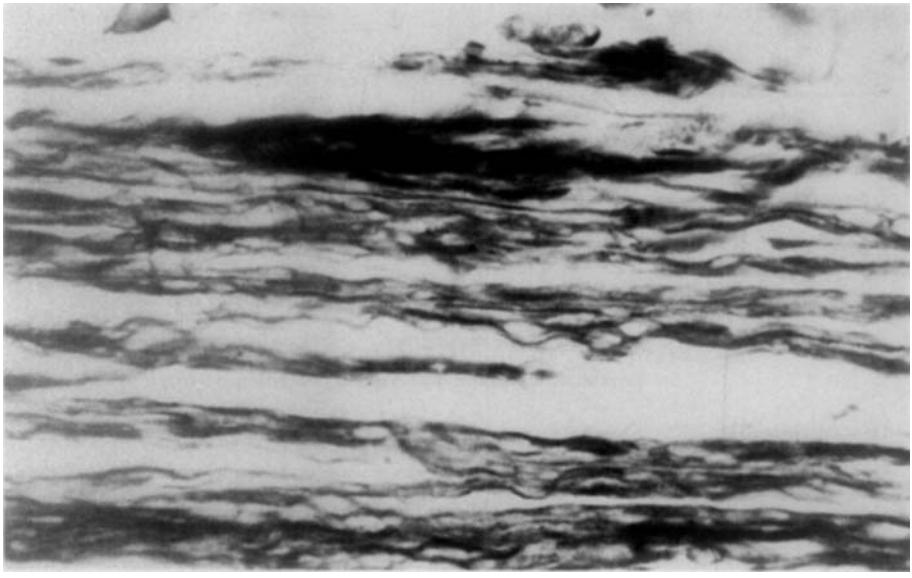

Fig. 17. Longitudinal section from the distal nerve in a specimen from series 4. 180 days after operation. Palmgren.

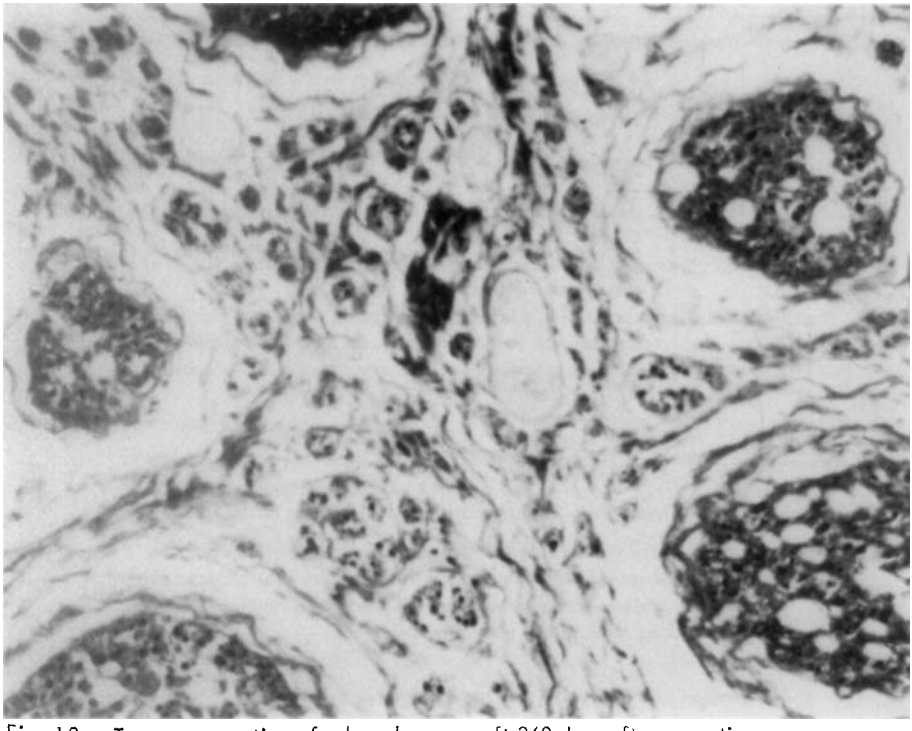

Fig. 18. Transverse section of a homologous graft 360 days after operation. Palmgren. Several axons outside the original fascicles.

Light microscopy (Table 11)

Observation on the vessels

1 day (2)	No endoneural vessels.
3 days (2)	No endoneural vessels.
5 days (2)	No endoneural vessels.
7 days (2)	No endoneural vessels. Epineural vessels in one case (not observed in the angiography). Ingrowth of vessels from the proximal nerve was observed in two cases.
10 - 16 days (5)	One case, not used for angiography, showed no vessels. Of the 4 remaining cases there were endoneural vessels in 3, the same cases that showed endoneural and epineural vessels in the angiogram. One case without filled vessels - in that case ingrowth from the proximal nerve end vessels was observed.
21 - 42 days (6)	One case of 30 days and one of 42 was not used for angiography and did not show any filled vessels. Of the remaining 4 one was necrotic (avascular in the angiogram), the other 3 showed endoneural vessels (Fig. 15).
60 - 180 days (6)	Of these cases 2 were not used for angiography and did not show any vessels. Of the 4 earlier angiographed, 3 showed endoneural vessels and one (with epineural vessels in the angiogram) was without filled vessels (Fig. 16).
360 days (2)	Endoneural vessels in one case, the case showing epineural vessels in the angiogram.

Other observations

Invasion of inflammatory cells occurred only in a few cases.

Increase in endoneural and epineural collagen, and the increase of the perineurium was more marked than in the previous series.

Degeneration of the myelin was observed from the first day. No appearance

of normal myelin was observed.

Normal looking axons were found in the grafts up to three weeks. Degenerated axons were observed from the first day, regenerated axons in the distal nerve from 90 days and later.

One graft was recorded as necrotic.

Comments on Series 4

Macroscopic observations

There was only a faint inflammatory reaction in the majority of cases. One case of abscess formation was recorded.

Fluorescence microscopy

Endoneural vessels were found in 6 of 24 grafts from the third day on. Only 1/4 of the grafts had filled vessels, which was less frequent than in Series 3 and considerably less frequent than in Series 1 and 2.

During the first 7 days only 1 of 6 grafts showed endoneural filled vessels. In the grafts observed longer there were such vessels in about half the number of grafts. The diffuse fluorescence was intense and frequently observed. As in Series 3 there were changes in the grafts. In some fascicles the normal nerve tissue seemed to be replaced by a homogenous tissue with changed staining properties.

Stereomicroangiography

Filled endoneural vessels in 6 of 23 grafts were found from the 14th day. Anastomozing is chiefly observed from nerve end vessels.

Light microscopy

Endoneural vessels were found in 10 of 21 cases from the 10th day. As in earlier series, a few specimens showed vessels in the Luxol stained sections, but not in the corresponding angiograms.

The inflammatory reaction was very sparse. The observations on collagen, perineurium and myelin were the same as in previous series. Normal looking axons were found up to 21 days, regenerated axons in the distal nerve from 90 days and later.

In Series 4, using freezed and irradiated homologous grafts, the inflammatory reaction was insignificant as in Series 3. The occurrence of grafts with filled endoneural vessels was frequent, especially during the first 7 days. The anastomosis found were chiefly with nerve end vessels.

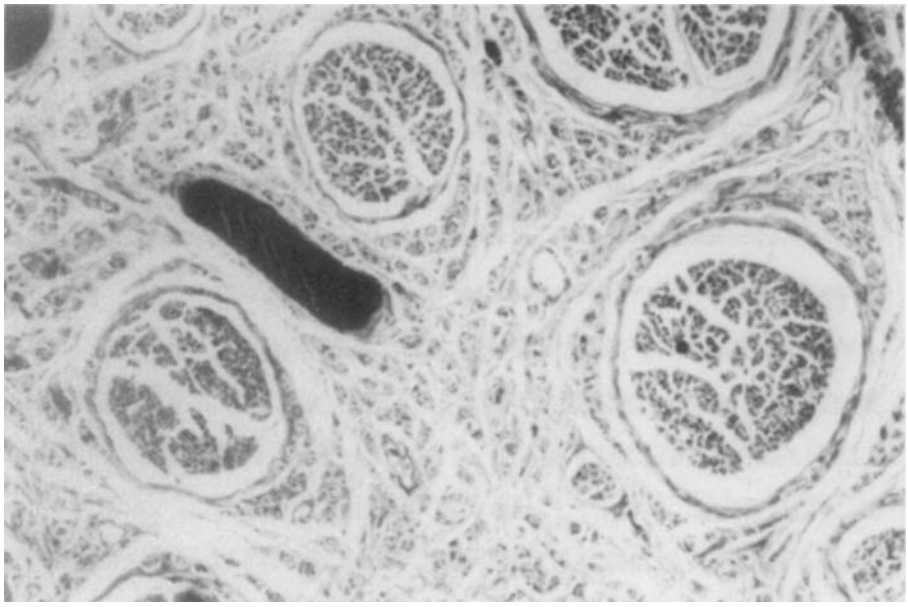

Fig. 19. Transverse section of an autologous graft 360 days after operation. Luxol. Note increase in collagen and thickened perineurium.

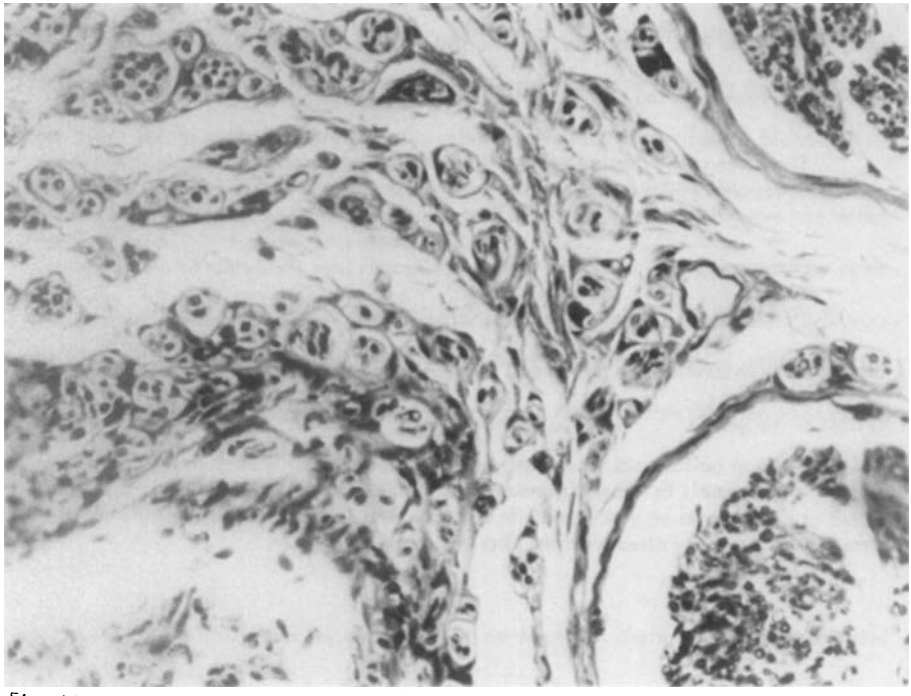

Fig. 20. Transverse section of an autologous graft 360 days after operation. Luxol. Numerous axons outside the original fascicles.

DISCUSSION

The circulation in a free nerve graft must be re-established within a few days, if the graft shall survive (Weiss and Taylor 1943, 1944). During the first days the basic demands of the graft are satisfied by diffusion of oxygen, but after this period of time a revascularization is neccessary. If a vessel contact is not established, there will be necrosis of the graft.

There are several possibilities of reopening of the graft circulation. After placing the graft in the appropriate manner, there is a coagulation of fibrinogen at the the junctions. In this coagle there soon are fibroblasts, collagen fibres and capillaries to be found (Millesi et al 1972). In the nerve ends there is hyperaemia, and an increase of collagen and fibroblasts is also seen. From the cut endoneural capillaries in the nerve ends new vessels can grow into the coagle. The epineural vessels also produce ingrowing vessels, of which some bridge the gap to the grafts' epineural vessels, others grow into the coagle. In the tissue, surrounding the graft, there are also several cut vessels giving new branches into the fibrincoagle there formed.

There are now different possibilities of revascularization. Vessels from the nerve end may bridge the fibrincoagle in the junction zone and reach the graft end, there anastomozing with vessels of the graft. Vessels from the epineural vessel system may also penetrate the junction coagle and anastomose with graft vessels. Vessels from the tissues, surrounding the graft, may in a similar fashion anastomose with epineural vessels of the graft.

If the vessels in the graft remain open, there may in this manner be a rapid recirculation, probably already in the first post-operative day.

If the vessels in the graft are closed, e.g. filled by trombi, there must be an ingrowth of vessels in the graft, and revascularization then would occur at a considerably later time.

The early established circulation in the graft is modified by the occurrence of oedema. In his studies on the permeability of the endoneural vessels Olsson (1966) shows that after damage of a peripheral nerve, an increased permeability of the endoneural vessels occurs. The result is an exsudate with a high concentration of albumin. Lundborg (1970) showed that ischaemia of more than 8 hours endurance caused a transient oedema. Mellick and Cavanagh (1968), who studied the vessel permeability, found two waves of increased permeability, primarily one within the first 24 hours, then another 1 - 4 days after the injury. The nerve grafts here studied have been in an ischaemic situation of several hours duration. An oedema therefore is formed as soon as circulation starts. As the perineurium is of a rather tough structure, the result may be an increase of the endoneural pressure, which may diminish the circulation intrafascicularily. The oblique passage of the perineural vessels through the perineurium also gives the theoretical possibility of a valve mechanism, hindering the blood flow. As Olsson (1966) also observed, the proteinrich oedema may activate fibrocytes giving fibrosis later and in that way may modify the circulation.

The intense inflammatory reaction that repeatedly is reported as a cause of the failure of the use of homologous grafts (see page No. 21) consists of oedema, hyperaemia, increase of collagen and macrophages. As far as this reaction is occurring in the epineurium, it may have the effect of increasing the circulation in the graft in the short time perspective. Later the induction of fibrosis may be devastating to the the graft.

In earlier studies on the circulation in nerve grafts, there are different opinions

on the revascularization. Sanders and Young (1941) and Davis and Ruge (1950) found an ingrowth from nearby vessels. Tarlov and Epstein (1945) observed an ingrowth both from the nerve end vessels and vessels in the surrounding tissue. Hassler (1969) reported a difference between autologous grafts, which received vessels from the nerve ends, and homologous grafts, receiving blood from the surrounding tissue vessels. However, most of the literature on peripheral nerve grafts does not deal with this subject.

The literature on the circulation of the peripheral nerves is earlier reviewed. Most studies show that the intrinsic vascular system of the nerve is enough to produce an adequate circulation in the nerve even after extensive dissection of the extrinsic system (Adams 1942, Sunderland 1945, Bacsich and Wyburn 1945, Blunt and Stratton 1956, Lundborg 1970, Kline et al 1972).

Methods

The fluorescencemicroscopy method basicly is a study on the distribution of labelled albumin in and around the vessels at the moment of death. The presumption for a uniform distribution of the albumin is that there was an adequate peripheral circulation. To secure this, the animal received a very light anesthesia, when the albumin was injected. The albumin was allowed to circulate for a sufficient period of time, and the animals were then rapidly killed by an overdose of anesthetic. Under these circumstances it seems to be right to conclude, that the method gives a picture of the functioning vessels in the sections studied.

The stereomicroangiography is based on a postmortem injection of barium contrast medium under artificial pressure. The method gives a good filling of even small vessels. Theoretically there is the possibility that even vessels, which are not functioning, may be filled, which means that a too optimistic view of the vessel bed

may be obtained. The study of Lundskog et al (1968), however, shows that compared with a biomicroscopic study, the microangigraphy gave an underestimation of the vascular system.

A possible cause of fault is an incomplete filling, due to technical error. Using the method for studies of grafts, the findings can be controlled by observing the filling of the host nerve. If that filling is not satisfactory, it is better to exclude the specimen from the study.

The chief advantage with the stereomicroangiography is the excellent view it gives on branching and anastomozing of vessels. As the specimens are unchanged after the angiography, they may be used for further observations in light microscope. This offers a possibility of confirming the observations made.

Endoneural vessels

Using the fluorescence microscope, filled endoneural vessels at first are seen 3 days after the operation in the autologous grafts and after that in 32 of the 35 grafts observed. In the fresh homologous grafts, filled endoneural vessels are observed already after one day, and then in 43 of the 53 grafts observed. Comparing the results in these series, there is the same frequency of filled vessels during the first 7 days. Between 10 and 42 days there are endoneural vessels in only half the number of homologous grafts, but in the greater part of the autografts. In the grafts from 90 to 360 days the frequency of endoneural vessels again is the same.

The results thus show that the inflammatory reaction is more intense in the fresh homologous grafts in the time period 10 - 42 days, and that in the same time period about fifty percent of the grafts have an insufficient endoneural circulation. The findings are in line with the supposed effects of an endoneural oedema. In grafts, studied 90 days and later after operation, the inflammatory reaction is not so intense

and the frequency of filled endoneural vessels also is the same as in autologous grafts.

In the freezed homologous grafts filled endoneural vessels are found from the third day in 27 of the 56 grafts. The frequency of filled vessels is considerably lower than in the fresh homologous grafts. In this series the inflammatory reaction is less pronounced, compared to the reaction of the fresh homologous grafts, and even compared to the autologous grafts it is small.

The low frequency of filled vessels therefore must have reasons other than inflammation. There is the possibility, that the freezing procedure caused damage to the graft vessels of such kind as to hinder the revascularization. If this is correct, the diminished inflammatory reaction is caused by a damaged vascular system, and must not be regarded as a positive effect, which often is done in earlier studies.

In the freezed and irradiated homologous grafts, endoneural vessels are found in only 6 of the 24 grafts studied. The inflammatory reaction is insignificant. The same conclusions are drawn as in Series 3.

The stereomicroangiography confirms the findings from the fluorescence microscopy. The autologous and fresh homologous grafts have a high frequency of filled endoneural vessels from 1 - 3 days after operation. The grafts previously freezed or freezed and irradiated have a considerably lower frequency of filled endoneural vessels.

The observations on anastomosis formation in autologous grafts show anastomosis with nerve end vessels in 9 of 11 grafts studied up to 7 days after operation. In the same period of time anastomosis with vessels in the surrounding tissues are observed in 4 of the 11 grafts. In grafts from 90 to 360 days after operation anastomosis with vessels in surrounding tissues are found in 9 of 10 grafts observed. This indicates,

that in autologous grafts the earliest revascularization occurs from the nerve ends in most cases. Anastomosis with vessels in the surrounding tissues follow soon, and after longer observation periods such anastomosis are found in most cases. A nearly normal vascular pattern is early found in the grafts. After 360 days the grafts still have less vessels than a normal nerve.

In the fresh homologous grafts, anastomosis with nerve end vessels and surrounding vessels occur with the same frequency even in the early specimens observed.

In the homologous grafts, previously freezed or freezed and irradiated, anastomosis with nerve end vessels were more often found than anastomosis with surrounding tissue vessels. In these series there also are observations on ingrowth from the nerve end vessels in grafts without filled vessels (Figs. 9 and 10). Both findings give further support to the theory, that the freezing and irradiation procedures cause a damage of the graft vessels.

A study of the endoneural vessels in light microscope again shows the same frequency of filled vessels as in the previous methods. Of interest are the observations of filled endoneural vessels in Luxol-stained sections from specimens, which according to the angiography hold no vessels. The only conclusion of this is that tiny vessels may be overseen in the angiograms in spite of the use of maximal resolution film and a high grade of magnification, and that the control with light microscope is of value.

Other vessels

The recordings of perineural and epineural vessels in no way change the conclusions, drawn by the study of the endoneural vessels.

The vessels, here called "new vessels", are of some interest, since they in some grafts doubtless contribute to the circulation. The new vessels are first observed in the vicinity of the sutures. In early cases they are small and tortuous with varying

diameter, resembling vessels found in granulation tissue. In later cases the vessels sometimes are quite large, and anastomosis with vessels in the nerve end, in the graft and in surrounding tissue, are found. The new vessels probably belong to the ingrowing connective tissue, at first observed around the sutures, which always is referred to as an obstacle for axon regeneration. From the vascularization point of view, however, they are a support to the circulation in the graft.

The new vessels also are of interest in connection with the observations of diffuse fluorescence outside the vessels. This finding is a sign of leakage of albumin through vessel walls and in peripheral nerves it is normally found in epineural but not in endoneural vessels. As earlier mentioned, different types of trauma to the nerve changes the permeability of the endoneural vessels and the albumin passes the vessel wall.

In the present study this phenomenon is observed not only as expected in the specimens studied within short time periods after the operation, but also after long periods of time, in some cases even after 360 days. There are two possibilities to explain this - either the new vessels intruding into the endoneural milieu, but originating in the epineural, allow the albumin to pass the wall, or else the explanation is that the barrier function never is restored after grafting. The restoration of the barrier function is probably connected to the regeneration of the perineurium. This question has caused some discussions. Some authors have found signs of regeneration (Thomas and Jones 1967), others have not (Sunderland 1968). Morris et al (1972) in an electron microscopy study of the sciatic nerve of the rat conclude that in regeneration bundles of axons get enclosed in a regenerated perineurium, the original fascicles are only partly used for regenerating axons. The result is a multitude of small bundles of axons, each bundle with a perineural sheath. The same observations have been made by several authors (Millesi et al 1972, Meier et al 1972) and are also made in this study (Fig. 20).

The work of Morris et al (1972) shows that the regenerating axon sprouts tend to combine in groups enclosed in a separate perineural wrapping. These groups do not respect the original fascicular pattern. The authors conclude that the cause of this grouping is the disturbance of the endoneural evironment, caused by the rupture of the perineurium. They further criticize the present concept of anatomical thinking and recommend that the goal for future peripheral surgery must be the separation of the endoneural milieu from the mesodermal surroundings.

The findings in this study of a persistent leakage of albumin is a sign of an incomplete separation of the two milieus, irrespective of which explanation is chosen and the improvements in grafting technique to come may lie in the early restoration of the endoneural milieu.

Other observations of interest

The inflammatory reaction evoked by homologous grafts, is in the literature repeatedly reported as the chief cause of the bad results noted. In the present study the occurrence of inflammatory cells is observed in the different types of grafts used. In the autologous grafts an increase of inflammatory cells is observed from the first day up to 42 days. This finding is interpreted as an evidence of the degeneration process going on. In fresh homologous graft sections there was about the same occurrence of inflammatory cells (the macroscopic observations suggested an increased inflammatory reaction), in the homologous grafts pre-treated the invasion was considerably smaller. These findings are in contrast with most of the earlier studies. Especially Marmor et al (1970) have stressed that there always is an intense reaction, when fresh homologous grafts are used. It seems that the grafts observed by Marmor were placed subcutaneously, not in contact with a peripheral nerve. This will diminish the possibilities of revascularization and the risk of necrosis is great.

In order to investigate the effect of placing nerve grafts without contact to a

peripheral nerve, the following experiments were made.

In the hind leg of a rabbit one homologous graft was inserted in the usual way, and one graft of the same length was placed on the muscles but without contact with the tibial nerve. After 14 days the specimen was observed. A difference in the inflammatory reaction evoked was noted. The graft without nerve contact showed a more intense invasion of inflammatory cells. The inflammation observed by Marmor and the inflammation observed in grafts placed under the kidney capsule (Verhoog and van Bekkum 1971) thus may be a reaction, caused by necrotic tissue and the relative influence of immunologic mechanisms is not clearly shown.

On the other hand nerve tissue must be supposed to have immunological properties as well as other tissues (Gibson and Medawar 1942, Medawar 1944, 1945, Ahrons and Ehlers 1970, Wickremesinghe and Yates 1972). The clinical experiences with homografts are not encouraging (Seddon 1954, Sunderland 1968).

According to the present study the inflammatory reaction caused by fresh homologous grafts is not so intense as might be expected. Using these relatively short and thin grafts, the amount of foreign protein introduced is small. As Sanders (1954) showed, there is a relationship between the amount of tissue inserted and the inflammatory reaction evoked. The surgical technique used may also be of importance for the modest inflammation.

An increased amount of collagen is frequently observed both endoneurally and epineurally. An increased thickness of the perineurium also is frequently observed. There is both increase in collagen and a hypertrophy of the perineural cells. These findings are in earlier studies mentioned by Sanders and Young (1941) without comments and by Millesi et al (1972). As the observed increase occurs even in the autologous grafts, an immunological reaction as a sole cause is not probable. It is possible

that the findings are a result of hypoxaemia.

The degeneration of the myelin is observed to start as expected, but regeneration of myelin is not observed. Not even in the late autologous grafts there are normal staining myelin to be found. There is a sheath, but it is vacuoulized and not normally staining.

The degeneration of axons also starts as expected. Regenerated axons are observed in the autologous and fresh homologous grafts after 7 and 10 days, which is in line with previous figures of time. In the distal nerve regenerated axons are found from 10 - 21 days. In the series of pre-treated grafts there only are a few grafts with observed regenerated axons in the distal nerve from 90 days and later. In Series 4 the findings are the same as in Series 3.

CONCLUSIONS

The results of the observations made on different types fo grafts give the answers to the questions raised.

In the autologous grafts filled endoneural vessels, indicating that blood circulation had occurred, were observed from the third post-operative day and after that in 32 of 35 grafts. In specimens, studied by stereomicroangiography, these results are confirmed. Up to the 7th post-operative day the majority of anastomosis observed occurred at the nerve ends, but anastomosis to surrounding vessels also were found. Later anastomosis of the latter type were found in a rising frequency.

In the fresh homologous grafts filled endoneural vessels are found from the first day to the 7th at the same frequency as in the autologous grafts. Between 10 and 42 days after operation there are filled endoneural vessels in about half the number of grafts. In the grafts observed after 90 to 360 days there again are filled vessels, observed at the same frequency as in the autologous grafts. The stereomicroangiography confirms these findings. Anastomosis with nerve end vessels and vessels in the surrounding tissue were recorded in about the same frequency. The inflammatory reaction evoked was more intense than in the autologous grafts as observed macroscopically. In light microscopy studied sections invasion of inflammatory cells occurred in both series equally often.

In grafts - autologous or fresh homologous - observed 360 days after operation, there is an increase of collagen both endoneurally and epineurally. There are regenerated axons, some within the original fascicles, many in small newly formed bundels. The total number of axons is less than in a normal nerve. A myelin sheath is observed,

but it never resumes normal staining to Luxol fast blue.

In homologous grafts, beforehand treated with freezing or irradiation, filled endoneural vessels are found in a considerably lower frequency than in the previous series from the third post-operative day. Anastomozing was observed primarily at the nerve ends. The inflammatory reaction observed in sections or macroscopically was insignificant.

In the present study the difference between autologous and homologous grafts is not so pronounced as in earlier investigations. There is an increased inflammatory reaction in the homologous grafts, but the final results are very similar in the two series. The cause of this may be the use of an atraumatic technique, the avoidance of stretching in the sutures and a minimum of foreign material used.

The freezing and irradiation of grafts does diminish the inflammatory reaction. The revascularization process is, however, disturbed and the findings of filled endoneural vessels few. This indicates that the diminished inflammatory reaction is caused by the damages vessels in the graft and thus is not a sign of a good result. According to the observations here made, the freezing and irradiation procedures do not seem to offer any advantages and can so far not be recommended for clinical use.

As initially stated, there is a need of homologous grafts. The findings in this study indicate that further investigations, concerning homologous grafts, are justified. Furthermore, the influence of the perineural barrier function on axon regeneration ought to be investigated.

SUMMARY

In an experimental study on free peripheral nerve grafts 374 tibial grafts in 207 rabbits were used.

With a standard operation technique autologous grafts, fresh homologous grafts, freezed homologous grafts and freezed and irradiated homologous grafts were inserted in the tibial nerve of rabbits.

After different periods of time - from 1 to 360 days - the rabbits were sacrificed and the specimens were used either for fluorescencemicroscopy (after intravitally administration of albumin labelled with Evans blue) or for stereomicroangiography. The latter specimens were also used for light microscopy studies.

The results show that in 84 autologous grafts 32 of 35 had filled endoneural vessels after injection of albumin-Evans blue from the third post-operative day. 32 of 42 grafts showed filled vessels according to the stereomicroangiography, of these anastomosis to nerve end vessels were found in a higher frequency (9/11) in the first post-operative week, anastomosis to vessels in surrounding tissues were found in 4/11 cases in the same time period. Later the frequency of anastomosis was the same for both types. Out of 130 fresh homologous grafts, albumin-Evans blue filled endoneural vessels were found from the first day on in 43 of 59 observed grafts. In stereomicroangiography 39 of 65 observed grafts were contrast filled. The anastomosis with nerve end vessels were as common as anastomosis with vessels in the surroundings.

The macroscopically observed inflammatory reaction was more intense than in the autologous grafts. The invasion of inflammatory cells in sections studied was about the same.

In 109 homologous grafts beforehand freezed (with either fluid nitrogen or carbon dioxide snow, thereafter kept freezed for 3 weeks up to 6 months) endoneural vessels, filled with albumin-Evans blue, were found in 27 of 56 observed grafts from the first day on. According to the stereomicroangiography contrast filled endoneural vessels were found in 18 of 45 grafts. The inflammatory reaction was smaller than in the fresh homologous grafts.

In 52 homologous grafts beforehand freezed (with carbon dioxide snow and kept in a freezer for 3 weeks to 6 months) and irradiated (in a cobolt source 2 million r.e.p.) there were albumin-Evans blue filled endoneural vessels in 6 of 24 grafts and in the stereomicroangiography in 6 of 23 grafts. The inflammatory reaction was very sparse.

After discussion of the results it is concluded, that the difference between the autologous grafts and the fresh homologous grafts in this study is rather small. The cause of this is discussed. Possibly the surgical technique (the use of operation microscope, avoidance of stretching by the use of longer grafts than the gap to be bridged and the use of a minimum of foreign material) may be of some importance. The amount of foreign tissue introduced also was rather small.

The freezing and irradiation procedures cause a considerable hindrance for the revascularization. There is a diminished inflammatory reaction, but that is probably the effect of the vascular damage, and must not be interpreted as a favourable condition.

Certain other observations are recorded. The finding of increased permeability of the endoneural vessels seems to occur not only in connection with the operation, but even after 360 days in some cases. The cause of this is discussed and the incomplete restoration of barrier function may be a possible factor. New vessels of mesodermal origin are frequently found, chiefly in the suture regions in all types of grafts. An

increased amount of collagen both endoneurally and epineurally is observed and the perineurium is often thickened. All these findings also occur in autologous grafts and both the increase of collagen and the thickening of the perineurium may be caused by a hypoxaemia.

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