HISTOCOMPATIBILITY MATCHING AND PRESERVED NERVE ALLOGRAFTS IN DOGS

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
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OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
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ROTTERDAM, 1983

PROMOTOREN: PROF. DR. S. A. DE LANGE
PROF. DR. D. L. WESTBROEK
CO-REFERENT: PROF. DR. D. W. VAN BEKKUM

"If we are to reach real peace in this world and if we are to carry on a real war against war, we shall have to begin with children; and if they will grow up in their natural innocence, we won't have to struggle; we won't have to pass fruitless idle resolutions, but we shall go from love to love and peace to peace, until at last all that peace and love for which consciously or unconsciously the whole world is hungering".

(M. K. Gandhi)

To my parents
To Anjali and Sandeep

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CHAPTER I

1.1. Introduction

Lesions of peripheral nerves, especially those serving important motor effectors and sensitive areas, such as those in the upper extremities, can be extremely crippling. Not surprisingly, surgical repair of such lesions has been attempted for many decades, but it was not possible to claim consistent succes until many lessons had been learned by trial and error. One of the more important facts emerging was that end-to-end sutures could only be expected to yield functional recovery if the anastomosis was not subjected to axial tension.
Extreme measures, including extensive nerve mobilisation, re-routing, limb flexion, nerve stretching and even bone shortening were applied to facilitate nerve suturing. Using these measures, Seddon (1947, 1954) achieved success in 71% of his cases and Woodhall and Beebe (1956) in 74.8% of their cases drawn from their Second World War Experience, although the anastomosis was not always free from tension. The cases described included a number in which the post-traumatic defect between the two ends of the nerves exceeded 20 centimeters. In such cases, end-to-end anastomosis without tension is out of the question and recovery is not always possible. Seddon (1954) pointed out, therefore, that the number of cases in which nerve suturing was indicated - not merely technically possible - was appreciably less than 70%.
Analysis of nerve suture results of Second World War series showed that in cases where the gap between the nerve ends was of the order of 10 centimeter or more, the postoperative stretching, however carefully carried out, generally damaged the nerve to such an extent as to

exclude recovery (Highet and Holmes, 1943; Highet and Sanders, 1943).
Consequently, the suture was doomed to failure in almost all cases in which segments of nerves were destroyed and the ends could not be approximated without exerting traction on them. The idea, therefore, to substitute the destroyed part of the nerve with a graft may be regarded as entirely logical.
Comparative experimental studies have shown better results with end-to-end nerve suture, without tension, in comparison to those where nerve suture was put on tension. Results of nerve grafting even were found to be much superior to those of nerve suture under tension (Miyamoto, 1979; Rodkey et al., 1981). Interfascicular autogenous nerve grafting results were found to be as good as epineural repair without appreciable tension in adult Rhesus monkeys by Bratton et al. (1979).
Trials to this effect were already undertaken more than a century ago. Philipeaux and Vulpian (1870), pioneers in this field, reported their first success using a segment of the lingual nerve as an autotransplant to bridge a defect in the hypoglossal nerve in the dog. Thereafter, a great deal of energy was invested into further investigations to establish suitable bridging grafts which would restore the continuity between the two nerve ends. With the basic objective of providing a scaffolding for regenerating axons in order to guide them to the distal stump, Assaky (1886) and Huber (1895) used catgut and silk threads to bridge the nerve ends in experimental studies, but without success. The same method was used clinically in 10 cases by Stopford (1920) - again without success. Using fresh and formalinized artery and vein (Bungner, 1891; Foramitti, 1904) also failed. Only Swan (1941) reported some motor and sensory recovery by closing

a gap in the ulnar nerve using a vein graft. In addition to non-biological and non-neurogenic material, as mentioned above, various types of neurogenic material have also been used to bridge nerve gaps. Huber (1895) reported some success in grafting the sciatic nerve of a cat into a dog; but later, after carrying out 17 autotransplants of sciatic nerve in dogs, he suggested that the allografts and the xenografts were much less successful than the autografts. Attempts to improve the results of allografts and xenografts by storage in ice (Bethe, 1916), in saline (Tello, 1914), in a 3% aqueous solution of borax and formalin (Huber, 1920) were unsatisfactory. The classical work of Sanders and Young (1942) and Gutmann and Sanders (1942), showed that all types of allografts provoked a considerable lymphocytic reaction leading to necrosis, subsequent fibrosis and thus failure. This reaction was found to be more pronounced in longer rather than shorter grafts. This explains the occasional success with allografts not exceeding 3-4 centimeters in length (Seddon and Holmes, 1944). Longer grafts thus failed invariably. According to the above mentioned investigators, these failures were thought to be caused by the immune response to foreign tissue. The host's reaction to the grafted foreign tissue seemed suggestive of an active acquired immune reaction and consequently seemed to depend quantitatively upon the amount of foreign tissue transplanted.

Many techniques have since been used to reduce the immunogenicity of allografts. Marmor (1964a, b), Ikeda (1966), Hirasawa et al. (1974), Hiles (1972), and Pollard et al. (1973) used irradiation to reduce the immunogenicity of allografts in dogs, apparently with favourable results. The results in clinical practice, however, remained disappointing. Poor results were obtained in 18 patients

in whom such grafts were used (Marmor, 1972). Ducker and Hayes (1970) and Gye et al. (1972) shared the views of Marmor (1972), commenting that the success of irradiated nerve allografts in man and animals is nowhere near that obtained with autografts.

Pre-operative irradiation of nerve allografts has been found to have adverse effects on the nerve regeneration in experimental studies of Stearns (1982). McGuirt (1976) and McGuirt and McCabe (1977) have reported no harmful effects of irradiation with facial nerve autografts in human beings. Irradiation of peripheral nerve grafts is, however, no more used in clinical practice.

Lyophilization of the nerve allografts has been attempted by Jacoby et al. (1971) and Singh and De Lange (1975), Pollard and McLeod (1981), Kalopissis et al. (1971) and Hirasawa and Inoue (1981). Jacoby et al. (1971) reported very encouraging experimental as well as clinical results, but re-examination of his reported success in seven patients proved that his results were largely based on overenthusiasm. None of these seven cases showed definite signs of functional recovery (Kuhlendahl et al., 1972). Reinnervation was, however, recorded in few short nerve allografts reported by Singh and De Lange (1975), although the results were very unsatisfactory. Immuno-suppression by, for example, modification of the host with azathioprine (Immuran^{R}), is an alternative method for preventing the immune reaction. This has recently been carried out in animal experiments, either in combination with irradiation of the graft (Marmor, 1967) or alone, the latter giving better results (Pollard et al., 1971). Use of systemic steroids along with irradiation or systemic chloroamphenicol and irradiation or a combination of the two has been found to reduce the rejection response of the host in variable degrees in the experimental

studies of Parekh (1982). So the solution of the problem has to be searched in the immunological behaviour of the host and donor.

Immunosuppression of the host has, however, many harmful side-effects. As the adverse effects of this therapy may outweigh the advantages, its use in man is not advisable.

Experimental studies by Dasgupta (1967) and Verhoog and Van Bekkum (1971) have shown that the immunological reaction towards nerve transplants is similar to that found in organ transplants, and follows laws similar to those laid down for the other organ transplants, e.g. kidneys.

Marmor (1964) and Dasgupta (1967) suggested myelin to have major antigens responsible for immunological reaction, while Aguayo (1979) and Pollard and McLeod (1981) believed that major antigens carried on Schwann cells are more important than those in myelin. Lennon et al. (1978) have also suggested that within the central nervous system HLA-antigens are not expressed by myelin. Contrary to the above reports Levinthal et al. (1978) have suggested that major transplantation antigens may reside mainly in the epineurium and the surrounding connective tissues.

During the last decade no definite solution has been found for the immunological rejection of nerve allografts, and so attention has been focussed on improving the feasibility and results of autogenous nerve transplantation.

Improvements in the instrumentation and techniques of microsurgery have opened up the possibilities of using thin, peripheral sacrificable sensory nerves to bridge nerve defects, in the form of cable grafts (Millesi et al., 1972).

Good functional recovery of ulnar and median nerves with interfascicular nerve grafting in human beings, have also been reported by Moneim (1982), Millesi (1981), Young et

al. (1981) and Simesen et al. (1981). Burger and Millesi (1979) reported good clinical functional results of 82% median nerve, 80% ulnar nerve and 92% of radial nerve by using interfascicular autologous nerve grafting technique. Similar is the experience of Haase et al. (1980, 1981) with 43 peripheral nerve lesions of 37 children of 5-16 years using the interfascicular autologous nerve grafting technique. These results were found to be superior to those obtained by conventional methods. Recently Narakas (1981) has reported good results even in the surgical repair of brachial plexus in 100 cases, mainly involving three upper roots, by using autologous nerve grafts. Similar good results of brachial plexus nerve grafting are also reported by Jamieson and Hughes (1980). Autologous nerve grafts have also been tried with good results in digital nerves by Wilgis and Maxwell (1980) and McFarlane and Mayer (1977).

Although more than 84% success has been reported in patients using this microsurgical interfascicular autogenous nerve transplantation technique, the procedure is still far from ideal. It has the following major disadvantages:

1.: Cable grafts of peripheral sensory nerves do not replace the normal anatomical neurolemmomal pattern of the parent nerve, which might be very important in achieving the desired result.

2.: One might not have sufficient donor autologous sensory nerves to be able to bridge large gaps in thick nerves or larger gaps in multiple nerves.

3.: Sensory loss produced by the removal of such sensory nerves does produce some discomfort in most of the patients. This is fairly readily accepted, with the hope of regaining some function of the damaged nerve which is functionally more important. There are, however, some

patients who do suffer the disabling sequelae of such surgery. Fourty two percent of the patients complained constant irritating tenderness at the donor site of sural nerve in series of nerve grafting reported by Staniforth and Fischer (1979).
4.: Finally the patient is exposed to the risk of tedious and time consuming surgery.

Thus the search for a better transplant still continues and has led to the objectives of the present study.

1.2. Objectives of the present study

The basic aim of this study was to find a better transplant which could fulfil the following conditions:
1.: Grafts of the most frequently required nerves should be readily available in all lengths and thicknesses.
2.: Grafts should be easily accepted by the host tissue without inducing any untoward rejection in the case of allografts and thus lead to good functional recovery.
3.: There should be no disabling untoward effects on the donors of the grafts.
4.: The anatomical neurolemmal structure of the graft should be more or less the same as the parent nerve so that functional recovery of the nerve is as complete as possible.

Thus, under ideal circumstances a segment in a particular nerve should be replaced by a segment of the same nerve, removed at approximately the same site from the donor. As man is not equipped with spare nerves, these conditions can only be fulfilled by using cadaveric specimens and by banking of preserved nerve allografts.

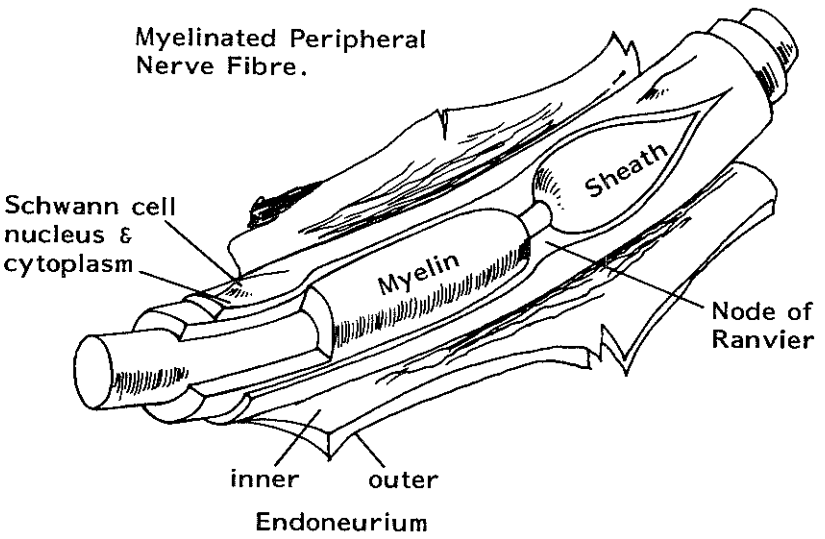
With the ultimate goal of achieving a "better nerve allograft", we tested the possible role of:
a.: Prolonged cryopreservation of cadaveric nerve allografts with a view to nerve banking.
b.: Matching for the Major Histocompatibility Complex antigens, an accepted successful procedure in other organ transplantation situations in order to minimize immunological rejection of preserved nerve allografts.
c.: Irradiation of cryopreserved nerve allografts in addition to matching for Major Histocompatibility Complex antigens in order to reduce immunogenicity.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Nerve anatomy

The complex anatomical and functional structure of the peripheral nerve can best be explained by describing its individual components separately, as follows.



Diagrammatic reconstruction of the essential histological features of a myelinated nerve fibre.

Figure 1. By the courtesy of S. Sunderland (Nerve and Nerve Injuries. Churchill Livingstone, Edinburgh/London, 1978, 2nd edition).

2.1.1. Nerve fibre

The nerve fibre is composed of a central core of axoplasm surrounded by a multilayered sheath. The axoplasm is a viscous fluid containing molecules organized to form neurofibrils; these extend out from the cell body into the flow of axoplasm, and maintenance of their connection with the cell body is a prerequisite on which the life of the axon depends. This vital relationship appears to be associated with an intracellar pressure which promotes a proximo-distal flow of the axoplasm and plays an appreciable role in nerve regeneration and trauma (Young, 1945; Causey, 1948; Weiss, 1943).
The nucleus and perinuclear area of the neuron are known to contain important energy producing mechanisms which involve the breakdown and synthesis of nucleoproteins. The materials essential to survival and efficient functioning of the axon are provided by the cell body and transported by centrifugal flow. Droz and Leblond (1963) demonstrated that proteins are synthesized in the nucleus and perikaryon of nerve cells and then move along the axon at the rate of $\pm$ 1.5 millimeter a day to replace proteins broken down in the axoplasm. Later studies of this very complex process indicate the presence of a bi-directional pattern of streaming in the axons (Lubinska, 1964). The axon is surrounded by a multilayered sheath known as the myelin sheath. The complex features of this sheath vary in myelinated and non-myelinated nerve fibres. The longitudinal segmental arrangement of the myelin sheath in myelinated nerve fibres outlines the nodes and internodes of the nerve fibre.
There is a very wide variation in the axon-myelin relationship in myelinated and non-myelinated nerve fibres (Sunderland and Roche, 1958).

Motor, sensory and sympathetic nerve fibres contribute to the formation of the peripheral nerve trunk. Motor nerve fibres originate in the anterior horn neurons of the spinal cord and terminate in the neuromuscular endings of the skeletal muscles. Sensory nerve fibres carry the peripheral dendrites of the posterior root ganglion neurons. They are represented by both non-myelinated and myelinated types. Sympathetic fibres of the peripheral nerves are the postganglionic processes of the neurons of the ganglionated sympathetic trunk. In general, these fibres are non-myelinated.

2.1.2. Funiculi

The funiculus is a bundle of nerve fibres invested by a thin but strong sheath of connective tissue, the perineurium. Each funiculus usually contains motor, sensory and sympathetic fibres in varying numbers and combinations. These funiculi repeatedly unite and divide along the full length of the nerve, thus forming a funicular plexus (Sunderland, 1945; Sunderland and Ray, 1948; Sunderland et al., 1959). The function of the interneural plexuses is to assemble the requisite afferent and efferent fibres for each branch from the appropriate segmental sources (Goldberg, 1924; Sunderland, 1945b).
Sunderland and his associates (1945b, 1948, 1959) in their detailed histological study on the peripheral nerves and their branches, showed that despite the changing plexiform character of the funicular pattern, fibres from peripheral branches pursue a localized course in the nerve for variable though often considerable distances above the site of branching.

2.1.3. Connective tissue

A peripheral nerve trunk consists of nerve fibres, each enclosed in an endoneurial sheath and bound together into bundles or funiculi by perineurium. These bundles are, in turn, embedded in a loose areolar connective tissue framework - the epineurium. Thus the endoneurium, perineurium and epineurium constitute the connective tissue of peripheral nerves and each possesses individual structural and functional peculiarities.
The epineurium contains collagen and elastin fibres which run longitudinally between the funiculi and hold them together. The epineurium is relatively thicker at junction points in the nerve, probably acting as a protective covering to nerve fibres. The elasticity of the epineurium also allows slight lengthening of the nerve without the funiculi being strained.

2.1.3.1. Perineurium

This is a relatively thin but dense and distinctive sheath of fibrous tissue, covering each funiculus, in which elastin and collagen fibrils are disposed about the bundle circularly, obliquely and longitudinally. Its function is to protect the nerve fibres which it encircles. Circular and oblique fibres of the perineurium resists and maintain intrafunicular pressure, thus promoting a proximo-distal flow of the axoplasm, an essential process during nerve regeneration. The tensile properties maintain the integrity of nerve fibres under stress.

2.1.3.2. Endoneurium

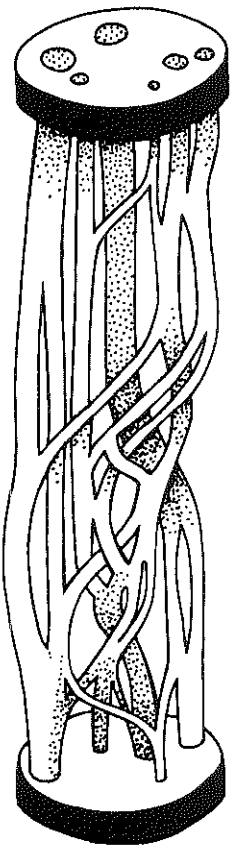
This is the supporting connective tissue inside the funiculus containing mostly collageous tissue. It forms intrafunicular septa that separate it from the perineurium and partly subdivide the fibres inside the funiculus into smaller groups. These septa carry the intrafascicular blood capillaries. In this way the endoneurium forms the supporting wall of what is commonly called an endoneurial tube; this contains a cylinder of tissue composed of the axon, the Schwann cells and the myelin. The endoneurium, just as the perineurium, has a protective role although to a lesser extent.

2.1.4. Blood vessels

Peripheral nerves are abundantly vascularized throughout their length by a succession of vessels which, by repeated division and anastomosis within the nerve, form an unbroken intra-neural vascular net (Sunderland, 1945a). The maintenance of the normal structure and function of peripheral nerve fibres depends on an adequate blood supply to nerve trunks. The importance of the blood supply is illustrated by the observation that, for the first six to eight hours after sectioning the nerve, the metabolic activities associated with recovery from fatigue induced by stimulation, are more dependent on the blood supply to the fibre than on its connection with the cell body (Causey and Schoepfle, 1951; Causey and Stratmann, 1953).

2.1.5. Lymphatics and tissue spaces

There is a lymphatic capillary network in the epineurium



Funicular plexus formations
in a 3 cm segment of a
specimen of the musculocutaneous
nerve of the arm.

Figure 2. Diagramatic reconstruction of funicular plexus formation. By the courtesy of S. Sunderland (Nerves and Nerve Injuries. Churchill Livingstone, Edinburgh/London, 1978, 2nd edition).

which is not connected to the perineurium or endoneurium spaces. It is drained by lymphatic channels which accompany the arteries of the nerve and drain to regional lymph nodes. It is generally believed that there are no true lymphatic capillaries inside the funiculi. Knowledge concerning the anatomical features and central relationships between these pathways and the movement of material along them is still incomplete.

2.1.6. Nervi nervorum

These constitute sympathetic and sensory fibres, originate from the fibres in the nerve and form the perivascular plexuses (Hromada, 1963). These are distributed to the epineurium where they form plexuses and to the perineurium and endoneurium.

2.2. Nerve regeneration

The process of nerve regeneration and functional recovery after nerve suture or implantation of a nerve graft primarily involves the outgrowth of an axon to replace the damaged portion. This process is lengthy, and can be divided roughly into the following periods:

2.2.1. Initial delay

This is the time required for the neuron to recover, for the axon to commence growth, to grow and to reach the injured zone. Recovery of the neuron from the retrograde effects which depress or arrest its activity is marked by

the correction of disorganized physiochemical processes, reversal of the transient depression of protein synthesis and the associated chromatolysis, and the accumulation of nucleoproteins which are reformed into the characteristic pattern of the Nissl material. The protein manufacturing apparatus of the nucleus becomes extremely active to meet the growing demand of the regenerating axons (Hyden, 1943; Thomas, 1966, 1970). Associated chromatolysis and hyperactivity of R N A (Ribo Nucleic Acid) help to reorganize the disturbed physiochemical process. Further growth of the axon and production of axoplasm depend on the nerve cell itself (Bodian and Dziewiatkowski, 1950; Weiss et al., 1945). The rate of progress of the axon tip along the endoneurial tube depends on the joint effort of the propelling force of the axon tip in the distal direction (called the histodynamic impulse of Heidenhain, 1911), the turgor force of Young (1945) and the activity of an especially organized growth cone which forms the axon tip itself. The axon tip as it descends leads to elongation of the axoplasm. This activity depends on the dynamic impulse from the cell body. The peripheral resistance against which the central force of the growth acts, plays an important role in facilitating or obstructing the pathways of growing axons. The time of this initial delay varies enormously. In general the delay ranges from 2 to 15 days in the cases of injuries where the endoneurial wall is unchanged, and even longer in cases where the continuity of the endoneurial tube is broken.

In severe injuries involving severance or tearing of the nerve at proximal levels, superimposed by other associated injuries and infection, the recovery of normal structure of the neuron may be delayed for periods exceeding 136 days (Barr and Hamilton, 1948; Sunderland, 1946, 1947a, b, c; Bowden and Sholl, 1959). The axon tip retains the capacity

for growth even one year after injury, as observed by Holmes and Young, 1942); Duncan and Jarvis (1943) even postulated that this capacity was inexhaustible. In man this capacity is retained for several years (Sunderland, 1950).

2.2.2. The scar delay

The time required for the growing axon to traverse the injured zone depends on all the factors responsible for initial delay together with the formation of scar tissue joining the ends of the severed nerve fibre, which must be traversed if the axon is to reach the distal section of the endoneurial tube according to one of the hypotheses. This movement of the axon sprouts through the scar tissue is determined by resistance gradients. The sprout that meets with the least resistance advances more easily and rapidly than the others. The specific power of axonal attraction of the distal stump helps to guide the growing axons to their proper pathways and to overcome the resistance of the scar tissue (Sunderland, 1953). Regenerating axons are known to bridge small nerve gaps without any guidance (Gutmann and Sanders, 1942; Pollock et al., 1947; Erhart and Reeze, 1965). Generally, regeneration axons under favourable conditions may cross the suture line and enter the distal stump within 3 to 20 days. Their entry may be delayed by as much as 40 to 50 days depending on the nature and extent of scar tissue. This resistance also results in the formation of multiple sprouts at a single axon tip. A single axon tip may give rise to as much as 50 sprouts (Ranson, 1912; Weddell, 1942).

2.2.3. The distal segmental delay and maturation process

The time required for the axon to grow from the site of injury to its peripheral termination is dependent on several factors: patency of the distal endoneurial tube, the distance to be travelled and the rate of advancement of the axon tip. The velocity of the growing axon is 14 to 16 times greater in the peripheral stump than in the scar tissue. The rate of regeneration is usually greater when the distal tube escapes injury. Once the regenerating axons have entered endoneurial tubes in the distal stump, regeneration follows the same course regardless of the period of denervation (Sunderland, 1949; Holmes and Young, 1942). Myelination proceeds centrifugally along the fibre. During regeneration, the axon may enter not its old tube, but one that conducts to a functionally unrelated end organ. In such cases, the fibre, though remyelinated, does not regain its original diameter or degree of myelination (Weiss et al., 1945; Aitken, 1949; Rajkovits, 1953). Even when the axon reoccupies the original tube, its development into a mature fibre will be incomplete if it is prevented from reinnervating the end organ, because of changes that have developed in the peripheral tissue as a result of prolonged denervation. Functional recovery requires more than a re-establishment of anatomical continuity and the restoration of those morphological features of the nerve fibre which collectively represent the maturation process itself, on which a normal conduction in individual nerve fibres depends. Further changes at the periphery directed towards restoration of nerve terminal--end-organ connection and transmission mechanism, the density and pattern of reinnervation, are also necessary. Functional readjustments required to correct residual deficiencies in axonal growth and the reversal and

correction of atrophic changes in the skin and muscle fibres must be carried out at the same time. The time required to effect these improvements in peripheral tissues is called the terminal delay period; this increases with the duration of denervation.

Histological studies of sutured and grafted primate peripheral nerves have been carried out with the help of light and electron microscope by Hudson et al. (1979), fascicular architecture through the course of graft was found to be variable, fascicular structure was frequently absent in the central part of the graft segments and in segments close to the second suture site. Bratton et al. (1979) with similar studies in primates compared the histological findings of nerve grafts with nerve suture. Non-graft repairs were found to be superior at 4 and 6 months of regeneration. However, by 9 and 12 months the graft repairs had caught up and were equal to the non--graft repairs. Histologically, it was observed that many axons missed the graft segments and were present in extrafascicular connective tissues, nonetheless, enough axons regenerated to the distal nerve to explain the success of these relatively short graft. Stromberg et al. (1979) studied the effect of change in the nerve graft polarity on nerve regeneration and function with sciatic nerve of rats. They found no difference in nerve conduction velocity and amplitude of reversed and non--reversed nerve grafts. They were identical after 4 months. Histological sections showed the grafted nerve to be indistinguishable from the control, whether or not the segments were reversed.

2.2.4. *Conduction velocity of regenerating nerve fibres*

Berry et al. (1944) and Berry and Hinsey (1946) in a study of electrical activity of regenerating nerves in rats, concluded that the conduction velocity of regenerating nerve fibres is directly proportional to the rate of axonal growth, as it gradually increases in diameter and myelination.

From this study it was expected that during regeneration, the growing fibres would conduct the impulse slowly at first and then with increasing velocity, as regeneration continued. Normal nerve conduction velocity was achieved at the time that the regeneration was complete.

From a combined electrical and histological study, it was concluded that during regeneration the change in both the threshold of excitability and the maximum conduction velocity did follow the change in diameter, including myelin thickness (Cragg and Thomas, 1961).

The complexity of this regenerating process and its relationship to conduction velocity was demonstrated by the observation of Weiss and Davis (1943) and Denny-Brown and Benner (1944a, 1944b). They revealed that interruption of conduction may occur without Wallerian degeneration or any significant morphological change in the nerve fibres. They also remarked that the nerve fibre might continue to conduct and produce a normal response at the periphery when the axon is preserved, even although the remaining architectural features of the nerve fibres are grossly disorganized.

After an analysis of the available controversial data, it could be concluded that the growth of the axon and the maturation of the restored axonal pathways, on which the functional efficiency of the new fibres depends, occur as two separate but related events during recovery. Both

proceed centrifugally down the fibres but the complex morphological changes that constitute maturation advance at a slower rate than does the axon tip, and continue long after the axons has been reconstructed. Cragg and Thomas (1964) in an extensive experimental study, found that most fibres recovered normal conduction velocity by 200 days when regeneration was permitted to proceed normally along the distal stump. Both axonal and total fibre diameter were also found to be near normal at the same time. Sanders and Whitteridge (1946), however, commented that the conduction velocity is more closely related to myelin sheath thickness than to axonal diameter.
However, according to the experience of Erlanger and Schoepfle (1946), Cragg and Thomas (1964) and Berry et al. (1944), normal conduction velocity is never fully regained after regeneration. They reported residual defects varying from 20 to 30%. Cragg and Thomas (1964) could not correlate the residual conduction velocity defects either to internodal length or to the axonal diameter: myelin thickness ratio. This discrepancy remains unexplained. Recently Dolenc et al. (1979) measured motor and sensory conduction velocities to evaluate function of grafts bridging defects in median, ulnar and radial nerves in 73 patients and reported values only moderately lower than the normal limits. Tallis et al. (1978) after performing similar studies with autogenous sural nerve grafts, had reported that by 2 years motor conduction velocity across the graft itself reached in most cases 40 to 80% of the conduction velocity in the contralateral normal limb. Sensory nerve action potentials were obtained in 44% of nerves after 18 months, although in all cases the amplitude of the potentials and in most cases their velocity, was greatly reduced. As there seems to be a reasonable correlation between conduction velocity and

nerve regeneration, measurement of nerve conduction is a good reliable criterion for the degree of nerve regeneration and functional recovery. In normal dogs the maximum conduction velocity of the peroneal major nerve ranges between 30 m/s and 50 m/s.

2.3. Nerve grafting

There is already an extensive literature on nerve grafting which demonstrates the intricacy of the problem and further illustrates the continuing efforts to find a better solution. Biological as well as non-biological tissues have been used to bridge nerve defects. The existing literature is summarized under the following headings:

2.3.1. Use of non-biological materials

a.: "Suture a distance": the continuity of the nerve was restored by bridging the gap with a series of catgut or silk threads, which were thought to provide a scaffolding for the down growth of regenerating axons. Experimental studies carried out by Assaky (1886), Huber (1895) and Alexander et al. (1948) suggested that the results were no better than those obtained on spontaneous regeneration which can occur when nothing is interposed between the nerve ends. Complete failures were reported by Stopford (1920) who used this method in 10 clinical cases.

b.: "Tubulation": nerve gaps have been closed by using a bridging tube which fitted onto the two nerve ends and thus restored anatomical continuity. For this purpose, both empty tubes made of magnesium (Payr, 1900), casein

treated with formalin (Auerbach, 1915, 1916), gelatine and agar (Lotheisen, 1901; Edinger, 1916; and Spitzy, 1917) and one made of rubber (Steinthal, 1917) proved complete failures. Millipore and silastic tubulation of nerve gaps have been used by Noback et al. (1958), Campbell and Bassett (1957) and Campbell et al. (1961), but these too have given disappointing results, because of the excessive scarring caused by these materials.

Recent experimental studies of Millesi et al. (1972) revealed that collagen, millipore and silastic membranes all increased connective tissue proliferation and thus led to poor functional results. Thus, non-biological materials are of no use in the bridging of gaps in nerves and may even introduce new complications in the form of infection, foreign body reaction and extensive fibrosis, so that they are now only of historical interest.

2.3.2. Use of non-neural tissue

The use of non-neural tissue grafts was limited to the tubulation method only (described in Chapter 2.3.1.) with the objective of protecting the regenerating axons from the obstructive interference of extraneural scar tissue. The following autologous tissues have been used: decalcified bone (Gluck, 1880; Vanlair, 1882; and Huber, 1895), fresh and formalinized artery or vein (Bungner, 1891; Huber, 1919, 1920; Stopford, 1920; and Swan, 1941), and fascia lata (Denk, 1914; Platt, 1919; and Stopford, 1920). Swan (1941) closed a gap in the ulnar nerve using a segment of vein and found there was some motor and sensory recovery. Most other authors have found that this method fails. The graft invariably becomes involved in a

connective tissue reaction, thus leading to extensive fibrosis and failure of the regenerating process. Weiss and Taylor (1946) and Matson et al. (1948), however, used fresh as well as frozen arterial sleeves for grafting purposes. They obtained some favourable results in their experiments with monkeys, but in the clinical situation this method failed entirely. Hence, this method is also of purely historical interest.

2.3.3. Use of neural tissue

2.3.3.1. Xenografting

Nerve grafts belonging to various species have been used in the past. These were mostly tried on experimental animals, and only a very few reports are availabel of their use in man. In 1880, Gluck was the first to implant a fresh xenograft using a 3.5 centimeter section of the sciatic nerve of a rabbit to bridge a defect of 3 centimeter in the sciatic nerve of a hen. He reported return of function within eleven days, a finding which is now much debated. In 1886 Assaky reported four transplants including one successful 3 centimeter sciatic graft from a turkey to a rabbit with return of function in 35 days. In 1895 Huber reported five satisfactory transplants of the sciatic nerve of a cat to the ulnar nerve of a dog with return of function within 120 days. Later, Huber (1919, 1920) reported that xenografts are much less successful than allografts or autografts.
Attempts have also been made to treat xenografts in order to improve their success rate. In 1911 Duroux placed a cat's nerve, which had been kept on ice for 24 hours, into

a defect in a dog's sciatic nerve. The result was even worse than that with a fresh xenograft. In 1915, Ingebrigtsen reported that xenografts were unsuitable for bridging nerve defects. He reviewed the cases published to date and concluded that only one xenograft could be considered successful. In 1917 (c), Nageotte conceived the idea of using a xenograft that was fixed in alcohol and kept in 50% alcohol until 24 hours before use, when it was transferred to Ringer's solution. Although he claimed functional recovery, no details were given in his reports. In 1971 (a, b) Nageotte, using alcohol-fixed calf xenografts in sciatic nerve of the dog, observed return of electrical excitability in 138 days. In 1922, Policard and Leriche published the results of a two-year follow-up of a transplant of the sciatic nerve from a calf to a human. The nerve elements had grown into the implant from below, but the upper end was blocked with fibrous tissue. In 1929, Sweet used alcohol-fixed fetal calf nerve to bridge 1.5 centimeter to 3 centimeter gaps in the sciatic nerve of the dog. A significant amount of scar tissue developed at both suture lines hampering the growth of the axons, and the success rate was very small. In 1942, Sanders and Young reported their work on xenografts using both fresh and alcohol-fixed dog and rat nerves, transplanted to rabbits. The grafts were attacked by mononuclear white blood cells, which at 25 days had almost destroyed the graft. Weiss and Taylor stated in 1942 that most devitalized xenografts behave like foreign bodies and are completely useless. With the idea of reducing the inflammatory response as well as sterilizing the nerve grafts, Marmor et al. (1966) used irradiation in their experiments. They used 3 centimeter long peroneal nerve of a mongrel dog to bridge a similar defect in the calf. In another experiment he also used irradiated human

peroneal nerve to bridge a similar defect in a dog. He reported good results with irradiated nerve xenografts. Similarly, Hirasawa et al. (1974) found better results using irradiated rather than non-irradiated grafts of human cadaver nerve in rabbits. Although irradiation helped to reduce the inflammatory response and thus achieved better revascularization of these grafts compared to non-irradiated ones, the final results remained so unsatisfactory that a clinical trial was felt not to be justified.

2.3.3.2. Nerve tissue allografting

Nerve grafts belonging to the same species as the recipient have been investigated regularly since Forssmann's first report (1900). Such grafts have been used fresh and after storage, both experimentally and clinically. The results of clinical trials are very puzzling. Most of the investigators, after comparing the results of such allografts with autografts, have demonstrated clear superiority of the latter (Bentley and Hill, 1936; Samii and Kaje, 1972; Kuhlendahl et al., 1972; Millesi et al., 1972; Singh and De Lange, 1975; and Hausamen, 1976). On the other hand, Pinner-Poole et al. (1966) reported that such allografts provide a scaffold that is just as satisfactory as autogenous nerve. Lewis and McLauring (1966, 1969) also concluded that slowly frozen, predegenerated and irradiated allografts compared favourably with autografts. Nerve allografts have been used in the following forms:

a.: Fresh allografts. Experimental studies carried out in cats by Bentley and Hill (1936), in rabbits by Sanders

and Young (1942), demonstrated a massive invasion of such grafts by macrophages leading to necrosis within a few days. Sanders (1954) concluded from experiments in rabbits that the severity and rapidity of onset of the rejection depended "both upon the amount of tissue transplanted and on the genetic relationship between the donor and host". In general the intensity of the immunological reaction tended to be proportional to the size of the transplant; short thin allografts faring better than long thick grafts. Bentley and Hill (1936) reported that gaps up to 3 centimeter could be bridged satisfactory by allografts but longer ones invariably failed. Dickson et al. (1978) have reported good results with delayed (degenerated) interfascicular nerve grafting. Fascicular nerve grafts were allowed to undergo endoneurial tube emptying in situ before transfer. Ultrastructure and electrophysiological studies of these grafts were superior to those of fresh grafts in rats. Recently Levinthal et al. (1978) have compared the results of fascicular nerve allografts with whole nerve allografts in rats with known histocompatibility antigens. After completing histological studies with electron microscope, he concluded that whole sciatic nerve allografts demonstrated a gradual rejection response dependent upon the degree of tissue histocompatibility differences. The fascicular grafts, one fascicle of sciatic nerve of the rat, however, had evidence of greatly decreased rejection and no stigma of graded response dependent on degree of variability of tissue typing.
The clinical use of fresh allografts has been a failure. In a pooled series of cases from several sources analyzed by Sanders and Young (1942), only 8 of 42 showed some recovery. Amongst the improved group, three were facial nerve repairs; the remainder was described as regaining useful recovery. The other reports also showed

disappointing results (Seddon and Holmes, 1944; Woodhall, 1955; and Whitcomb, 1959). Similar was the experience of Kassakowski (1981) with his comparative study of cell--mediated immunity response in rats, using fresh and preserved nerve allografts.

b.: Stored allografts. If allografts could be stored without sacrificing any of their potential biological usefulness, this could improve the chances of the selection of grafts on the basis of their specific anatomical features, so that they can be matched more closely to the missing segment. A comparative study of cell-mediated immunity following with allogenic nerve allografts preserved by freezing, lyophilization and irradiation did not show any appreciable difference in their cell reaction. The reaction was delayed and weak in the latter two groups. The sensitivity of the recipients could be demonstrated both by using allogenic and synergic antigens, thus seemingly indicating the presence of the organ specific antigens of peripheral nerve tissue (Kossakowski, 1979). The fate of allografts stored in a variety of ways has been examined experimentally in dogs by Huber (1919,1927), in cats and rabbits by Sanders and Young (1942), in rabbits by Gutmann and Sanders (1942), and the results were not found to be far better than those using fresh allografts. Their use in the clinical situation has resulted in repeated failure (Seddon and Holmes, 1944; Lyons and Woodhall, 1949; and Whitcomb, 1959).

c.: Freeze-dried allografts. Lyons and Woodhall (1949) reported 18 cases in whom freeze-dried nerve allografts were used; they found reinnervation of only the proximal one or two centimeters of the graft. Davis and Ruge (1950b) reported similarly discouraging results in 10

patients.

d.: Freeze-dried irradiated allografts. The essential problem to be overcome in order to ensure survival of the allografts is its antigenicity, as this causes an immune response in the host, leading to destruction of the graft and its replacement by fibrous tissue.
In animal experiments, irradiation has been found to reduce the antigenicity of nerve allografts, so that irradiated allografts allowed the regeneration of axons (Bohler, 1962, 1967; Campbell et al., 1963a, 1970; Marmor, 1964a, 1964b, 1967; Ducker and Hayes, 1968b, 1970; Pollard et al., 1971; and Verhoog and Van Bekkum, 1971). Bucko and Steinmuller (1973), however, found reinnervation in only 2 of 125 such allografts over a period of eleven months. Pre-operative radiation of the nerve grafts in nerve grafting experiments using sciatic nerve grafts has been reported by Stearns (1982) with adverse effects on nerve regeneration. Success clearly depends on the length of the graft; greater lengths having higher failure rates. Irradiation after facial nerve autografting in cats had no statistical difference with those who did not have it in the experimental studies of McGuirt and McCabe (1977) and McGuirt (1976). Roberts (1967) found regeneration axons penetrated only 2 centimeter into 8 centimeter grafts. Ducker and Hayes (1968b, 1970) concluded that the maximum length for allografts to allow any regeneration was 4 centimeter. Also, the clinical success rate of irradiation grafts has remained far below expectation. Only Campbell and his associates (1963a, 1963b, 1970) have reported reasonable success with such grafts in man. Marmor's (1964a, 1964b) early enthusiasm for such grafts was later tempered by his failure in clinical practice (Marmor, 1967, 1972). Hiles (1972) also reported complete

failure in two patients with such grafts, as did Comtet et al. (1972) in his cases. Jacoby and his associates (1970, 1971) used de-antigenized, lyophilized grafts in 57 patients and initially reported good recovery in 84% of their cases. Some time later, these results were proved to have been misinterpreted because of the surgeon's overenthusiasm (Kuhlendahl et al., 1972). Penzholz (1973) reported similarly negative results from a review of another 66 cases and concluded that the use of such grafts to bridge peripheral nerve defects was no longer justified.

e.: Pre-degeneration of the graft. Myelin is supposed to be responsible for the host's reaction to a transplanted allograft. Attempts were made to remove this strong antigenic component prior to insertion of the graft. Experimental results with such pre-degenerated allografts were encouraging according to Lewis and McLaurin (1966, 1969) and Dasgupta (1967). However, Ducker and Hayes (1970) and Pollard and McLeod (1981) concluded that pre-degeneration in combination with irradiation dit not reduce the antigenic properties of the allografts to a greater extent than irradiation alone (Hirasawa and Inoue, 1981). Pollard and McLeod (1982) with their experiments with Trembler mouse concluded that the Schwann cells and not the myelin constitutes the principal antigen within nerve allografts and it is Schwann cell rejection that limits the succesful use of nerve allografts. Contrary to the above statement, Levinthal (1980) reported that the principal antignes are located in the epineurium and the surrounding connective tissue of the nerve allografts, with his study using one fascicle of sciatic nerve of rats as a graft. Kalopissis et al. (1978) studied the histomorpholgy of preserved peripheral nerve allografts in human beings. Posterior tibial nerve was lyophilized and

irradiated with X-rays doses varying 1-4 Mrd. The main alterations were found in the re-arrangement of the epineurial connective tissue of the interperineurial spaces in a reticulum of varying regularity. Condensed endoneural connective was destroyed and converted into irregular tubular spaces. They, however, concluded that the hope for suitability of histological samples as a means of quality control of lyophilized and irradiated grafts does not yet seem justified.

f.: Immuno-suppressive therapy. The mechanism of rejection in peripheral nerve allografts has been studied with the help of the light and electron microscope and compared with the rejection reaction with other organ transplants in experimental animals (Dasgupta, 1967). It was concluded that the rejection process, in cases of peripheral nerve transplants, followed rules similar to those for rejection of other organ allotransplants. With other organ, e.g. kidney transplants, immuno-suppressive drugs have been used to depress the immunological reaction to transplant antigens which cannot be adequately modified by freeze drying and irradiation. Immuno-suppressive drugs have also been tried out in nerve transplantation. Marmor et al. (1967) demonstrated that azathioprine (Immuran^{R}) provided sufficient protection against rejection. Pollard and Fitzpatrick (1972, 1973) demonstrated that azathioprine was more effective than irradiation in reducing the immunological reaction. On the basis of neurological, electrophysiological and histological studies, regeneration was judged to be more superior in animals given immuno-suppressive therapy. In contrast, Gye et al. (1972) and McLeod et al. (1975) reported no appreciable beneficial effect of immuno-suppressive therapy carried out on the frozen irradiated allografts in man, compared

with patients who had received no additional therapy. Pre-implantation irradiation of nerve allografts in rabbits, along with systemic administration of chloramphenicol have been found to suppress the tissue rejection completely, and the method has been found to be considerably more effective than the combination with steroids in the experience of Parekh (1982). Ikeda (1966) thought that the use of immuno-suppressive drugs was not only unnecessary, but might even interfere with the regeneration of axons. Ducker and Hayes (1970) were of similar opinion: immuno-suppression might endanger the host by its general toxic effects. Nerve grafting is not a life-saving procedure. Thus the use of immuno-suppressive agents, which have adverse effects if administered over the long-term and whose advantages are even dubious, is not justifiable in human beings undergoing nerve allografting (Sunderland, 1978).

2.4. Transplantation immunology

2.4.1. Major Histocompatibility Complex

Over the last decade, studies in transplantation immunology have provided insight into the genetic control of immune reactivity against allogenic tissue in a number of mammalian species. Graft rejection is dependent on the recognition of foreign histocompatibility antigens on the graft. The most important histocompatibility antigens, the so-called "major" histocompatibility antigens, have their code on a highly polymorphic chromosomal area. This area has been labelled the Major Histocompatibility Complex (MHC). Matching for structures controlled by the

MHC is beneficial for the survival of allogenic tissues of diverse histological types. This has been demonstrated in many experimental animals, e.g. dogs (Vriesendorp, 1973). A variety of different histocompatibility antigens is, however, present on the cells of each individual, and some of these are coded for by other chromosomal areas. These so-called "minor" histocompatibility antigens are likely to elicit a weeker immune response, and are probably only relevant in the absence of major histo-compatibility differences, at least in rats and in immuno--suppressed dogs (Graff et al., 1973; Bijnen et al.,1979). The genetic code for all these antigens resides in many genes on different chromosomes.
Zalewsky and Silvers (1980), however, concluded that minor antigens alone are as potent as major and minor antigens together in evoking an immune response, with their experiments with nerve allografts in normal and tolerant rats.
Extensive knowledge of the genetics of the MHC has been gathered in many species including man (Gotze, 1977). The MHC contains many, perhaps hundreds, of loci. At present, the gene products of only some of these loci can be determined. In families, the genes of the MHC are inherited as genetic entities which segregate en bloc. The genes of the MHC of one chromosome which segregate together within a family is called a haplotype (Ceppelini et al., 1967). Thus, within a family, matching for one or just a few MHC antigens generally results in matching for all MHC antigens of a haplotype. Within a family, three MHC matches are possible: two individuals can differ by two haplotypes, by one haplotype, or they can be MHC--identical if they share the determinants of both haplo-types. In frequently, this simple pattern of inheritance is disturbed by the occurrence of a so-called crossing-

-over between the two autologous chromosomes carrying the MHC, and consequently a new haplotype arises.

2.4.2. Histocompatibility testing

Traditionally,the oldest way of testing histocompatibility was by means of allo-antisera which contain specific antibodies, directed against histocompatibility antigens. These can be obtained either by deliberate immunization of volunteers or from pregnant females, i.e. post-partum sera, (Payne and Rolfs, 1958; Van Rood, 1958). A complement--dependent lymphocytotoxicity assay is generally used to determine whether a lymphocyte preparation will contain antigens that are defined by those sera. Individuals who show an identical pattern of reactions with an established panel of all antisera are said to be histocompatible. Antigens defined by this lympho-cytotoxicity assay are called Class-I antigens. Serological histocompatibility testing was developed for dogs by, amongst others, Vriesendorp et al. (1971, 1972, 1973). Later, a new, more direct test was developed to assess histocompatibility, the so-called Mixed Lymphocyte Reaction (MLR).
In this test, lymphocytes of two individuals are incubated. together in culture and the degree of the reactivity as measured by radioactive thymidine incorporation, is indicative of the degree of histocompatibility. Genetic analysis of serological testing and the mixed lymphocyte reactions in all species investigated so far, showed that both tests define different series of allo-antigens.
The genes that determine the outcome of these tests are, however, closely linked on the chromosome. The chromosomal area which carries the genetic information for these and other histocompatibility antigens, also belongs to the

Major Histocompatibility Complex (MHC), and the antigenic system for which it codes, the Major Histocompatibility System (MHS). The recombination frequency between the two sets of allo-antigens is low (0.007 in dogs; Bijnen et al., 1977); hence, for experimental purposes, from a practical viewpoint, serological testing alone is adequate, when organ donor and recipient are sibs.
Extensive studies in the dog showed that the canine MHC closely resembles the MHC in man; this makes the dog an excellent model for transplantation research.
Transplantation experiments with various organs, such as skin, kidney, small intestine, pancreas, lung, heart, liver and bone marrow, clearly demonstrated the relevance of matching for the serologically defined antigens of the canine MHC for graft prognosis (Vriesendorp et al., 1977a).

2.5. *Nerve tissue preservation*

The routine use of nerve allografts necessitates the availability of a larger number of allografts of different size. The development of a nerve bank would be a great asset in the reconstructive surgery of nerve transplantation. Availability of such a bank would facilitate more acceptable selection of the nerve allografts.
Preservation of nerve grafts below the temperature of dry ice following irradiation has been tried by Marmor (1972) but with unsatisfactory results. Lyophilization and dry storage of nerve grafts have also been tried unsuccessfully (Jacoby, 1970). Immunological rejection was supposedly the reason for failure. Recently, using cryogenic temperatures, it has been possible to extend the storage period of viable cells and fine tissue from days or weeks to months and years.

Owing to the use of glycerol and other cryoprotectants (dimethyl sulfoxide), considerable advances have been made in the long term preservation of mammalian cells in suspension (blood and serum) and thin organs (such as skin, cornea and intestine). Skin grafts, have, however, been stored at temperatures below that of ice without the addition of cryo-protectants (Santoni and Rugio, 1962). Survival of mammalian skin autografts stored in culture medium at deep freeze temperatures have been reported for as long as 2 years (Pepper, 1954).
No successful method of nerve preservation has yet been standardized. Kossakowski (1981) reported that freezing of peripheral nerves at -20°C for 14 days had practically no effect on the time of occurrence and the intensity of cellular response of the recipient, in comparision to lyophilization, which greatly decreased the response. In the experience of Zalewski and Gulati (1982), however, freezing destroys the Schwann cell of the nerve grafts and therefore is not suitable for nerve preservation, in their experiments with rats.
Verhoog (1978) preserved some nerve grafts of rats and monkeys for a short period only. As yet, long-term preservation of nerves has not been achieved.

2.6. Histological examination

Regeneration of a cut nerve, following either nerve suture or nerve transplant, is a very complex process. The primary feature of nerve regeneration is outgrowth of the axon to replace the perished portion. The entire process can be summarized as follows:

1.: recovery of the neuron from retrograde effects and the

onset of regeneration at the cut end of the axon,
2.: growth of the axon to the site of the injury,
3.: passage of the axon through the site of the injury,
4.: growth of the axon down the endoneural tube below the injury,
5.: restoration of appropriate end organ relatonships.

A further increase is the number of these axon fibres and maturation of the newly innervated endoneural tube will produce gradual functional recovery. The time required for completion of these regenerative processes can be divided into the following periods:

a.: the time required for the neuron to recover, for axon growth to commence and for the axon to reach the injured zone; this period usually varies from 2 to 4 weeks.
b.: the scar delay, time taken for the growing axons to traverse the injured zone - in case of grafting this is transplantation of the graft.
c.: the period of axon growth distal to the site of injury. This is the time required for the axon tip to reach the periphery.
d.: the period of functional recovery. This is the time required to complete those changes in the restored axonal pathways which determine normal conduction properties of the individual fibres, e.g. normal conduction velocity response.

Thus, during the regenerating process, histological evidence including cellular reaction, macrophage response, connective tissue proliferation, distal myelinization and axonal regeneration can easily be determined. Hence, histological criteria including the above responses can be used to establish the degree of nerve regeneration.

Distinctive histological features of nerve degeneration and immunological rejection in peripheral nerve allografts have been studied by Comtet and Revillard (1979). Sequential sections were prepared from isografted and allografted sciatic nerves in adult female DBA/2 mice and were compared with the Wallerian degeneration in the same inbred strains. However, few striking features permitted the distinction between allografts and isografts. The cellular infiltrate was always scattered in an irregular manner in allografts, contrasting with the homogenously dispersed cellular infiltration of isografts. Furthermore, a few pyroninohilic cells were found around but never inside isografts, whereas numerous pyroninophilic cells could be observed both inside and outside the allografted nerves.
In addition numerous vessels appeared inside the isografts and to a lesser extent in allografts. Thus the distinctive features of allograft rejection are therefore, both quantitative (cell infiltrating the epineurium) and qualitative (proportion of large pyroninohilic cell in the infiltrate).
Levinthal et al. (1978) studied cellular response, macrophage reaction, connective tissue reaction, myelinization and distal/proximal axon ratio, in a comparative study with nerve autografts and fascicular nerve allografts of inbred antigenically identified rate. They could not find any difference between allografted and autografted fascicles after two months. In their experiments no graded rejection phenomenon was found, depending on the difference of tissue typing, when only fascicular grafts were used. They explained this phenomenon by the hypothesis that major transplantation antigens reside predominantly in the epineurial and surrounding tissues.

CHAPTER 3

MATERIAL AND METHODS

3.1. Experimental animal

The dog was chosen as the experimental animal. Seventy beagles, divided into 12 groups as shown in table I, were used for this study. They were obtained from the "Central Proefdieren Bedrijf TNO".

3.2. Tissue typing

The dogs were typed for serologically defined leucocyte antigens belonging to the canine MHC, - called DLA - by a battery of approximately 80 antisera. Typing for MLR--antigens was not carried out. Littermate dogs were grouped in pairs with no, one or two differences in DLA haplotype on the basis of the segregation patterns of the serological reactions. When unrelated dogs were used, they were selected in such a way that all known serologically defined antigens were mismatched.

3.3. Nerve grafting

Peroneal nerves of the dogs were selected for this experiment. This nerve is of suitable size for transplantation studies. Its removal leads to minimal discomfort, which is tolerated easily during the long period of follow-up. Segments of the peroneal nerves, 4 centimeter or 7 centimeter in length, were removed from all 70 dogs, under general anaesthesia. In six dogs the nerves were removed

Table I. Experimental Groups

Group no.	No. of recipients	Kinship	DLA match	Cadaveric or alive	Irradiation	Preservation time in weeks	Length of nerve graft (cm)
1	5	related	identical	alive	irradiated	6	4
2	5	related	one haplotype difference	alive	irradiated	6	4
3	6	related	two haplotypes difference	alive	irradiated	6	4
4	6	unrelated	mismatched	alive	irradiated	6	4
5	6	related	identical	alive	non-irradiated	6	4
6	6	unrelated	mismatched	alive	non-irradiated	6	4
7	6	related	identical	alive	non-irradiated	6	7
8	6	unrelated	mismatched	alive	non-irradiated	6	7
9	6	related	identical	cadaveric	non-irradiated	6	7
10	6	unrelated	mismatched	cadaveric	non-irradiated	6	7
11	6	related	one haplotype difference	alive	non-irradiated	6	7
12	6	related	identical	alive	non-irradiated	56	7

3 hours after death and in another six dogs, 6 hours after death. All these grafts were then preserved, as mentioned in chapter 3.4. These nerve grafts then were sutured between the transected segments of the peroneal nerve on both sides. Nerve anastomosis was carried out orthotopically, using standard microsurgical techniques with 16 times magnification and using 10-0 atraumatic monofilament nylon for suturing, under standard operating theatre discipline. Removal of these transplanted nerve grafts was carried out about 9 months later after completion of the electro-physiological studies (see chapter 3.7.), under the same conditions but without the operating microscope.

3.4. Nerve preservation

Nerves were preserved in tissue Medium 96 in sterile, sealed bags at minimum constant temperature of -70°C for a period of about 6 weeks in all the experimental groups. Nerves in group 12 (table I) were preserved for about 56 weeks. An intermediated cooling rate (Griffiths and Beldon, 1978) was used in the freeze-thaw cycle. Sealed bags containing nerve grafts were removed from a deep freeze chamber temperature about an hour prior to surgery and kept at normal operating room temperature for about 45 minutes. These grafts were then removed from the sealed bags and kept in a standard normal saline solution for about 15 minutes at a temperature of approximately 30°C prior to implantation. Roughly an hour after removal from the deep freeze chamber temperature, the nerve grafts felt and looked like normal fresh nerve allografts, ready for transplantation.

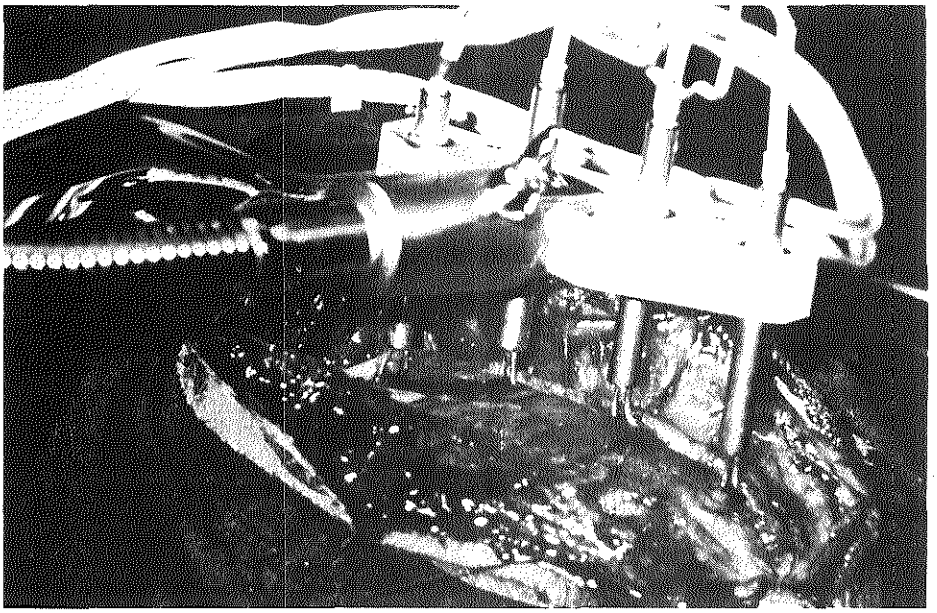


Figure 3. This figure shows electrodes at fixed points.

3.5. Nerve graft irradiation

In one set of experiments (groups 1, 2, 3, and 4 of table I) 23 of the frozen nerve grafts were irradiated with 1.66 Mrad for 10 seconds at the temperature of dry ice in a Van de Graaff generator at the Reactor Centrum Nederland, Petten. Irradiation was carried out within a few hours following removal of these grafts.

3.6. Follow-up

The neurological examination of a dog is not an objective procedure and can lead to biased results. Therefore, electromyographic (EMG) and histological techniques were used to evaluate the results obtained about 9 months after transplantation. This is the period required for the growing axons to reach the denervated muscle through the transplanted and autogenous nerve sheath.

3.7. Electromyographic examination

After about 9 months the grafts were re-exposed surgically under general anaesthesia without the use of muscular relaxants. The nerve was stimulated directly with bipolar platinum electrodes. Four electrodes were placed at fixed distances of 2 centimeter along the nerve (fig. 3). The first electrode was placed proximal to the graft and the last electrode distal to the graft. The compound action potential of the anterior tibial muscle was recorded through a concentric needle electrode. The nerve was stimulated supramaximally with a square wave of duration 0.3 m/sec. The temperature of the nerve as measured ranged

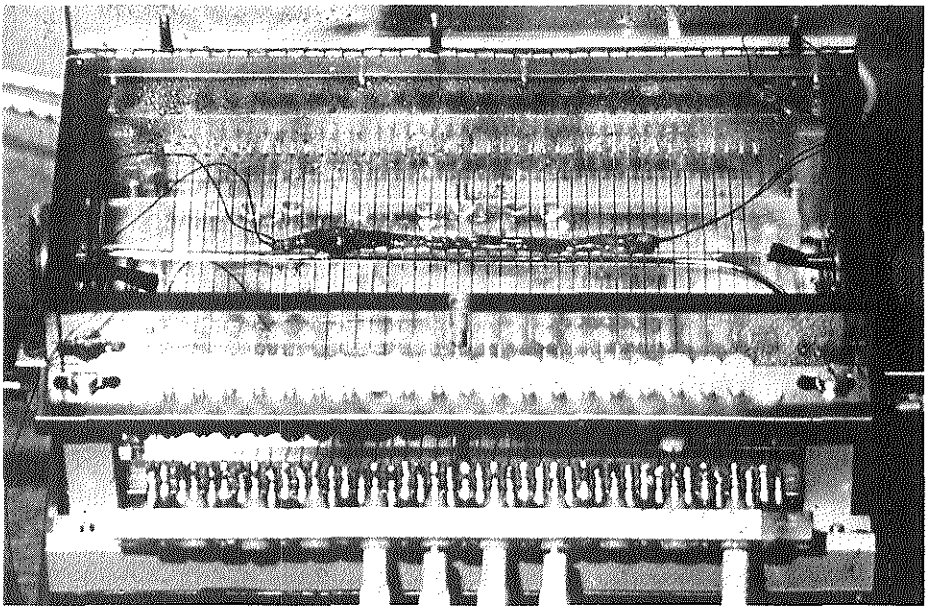


Figure 4. Temperature controlled multiple electrode chamber.

between 27 and 30°C. The maximum conduction velocity of the graft was estimated from the latency of the proximal and distal stimulating points to the anterior tibial muscle and the conduction distance. The nerve graft was removed together with one centimeter of normal nerve proximal and distal to the graft and placed on a mesh of multiple electrodes in a closed temperature controlled chamber, connected by a computer system (fig. 4). The same stimulation studies were repeated in this test system and final maximum conduction velocity determined. The results were grouped, on the basis of maximum conduction velocity, into four EMG grades:

Grade I : normal conduction velocity greater than 30 m/sec.
Grade II : reduced conduction velocity between 20 and 30 m/sec.
Grade III: markedly reduced conduction velocity between 15 and 19 m/sec.
Grade IV : no response.

This grading of electromyographic findings was confirmed by an independent expert in the field of electromyography, not involved in the research scheme. In normal dogs, the maximum conduction velocity of the peroneal nerve ranges between 30 m/sec and 50 m/sec.

3.8. Histological studies

The transplanted nerve, together with 1 centimeter of the host nerve, both proximally and distally, was removed for EMG studies, then used for histological studies after the nerve conduction velocity had been determined. Longitudinal sections at proximal and distal transplant suture sites and transverse sections of the proximal and distal segments of

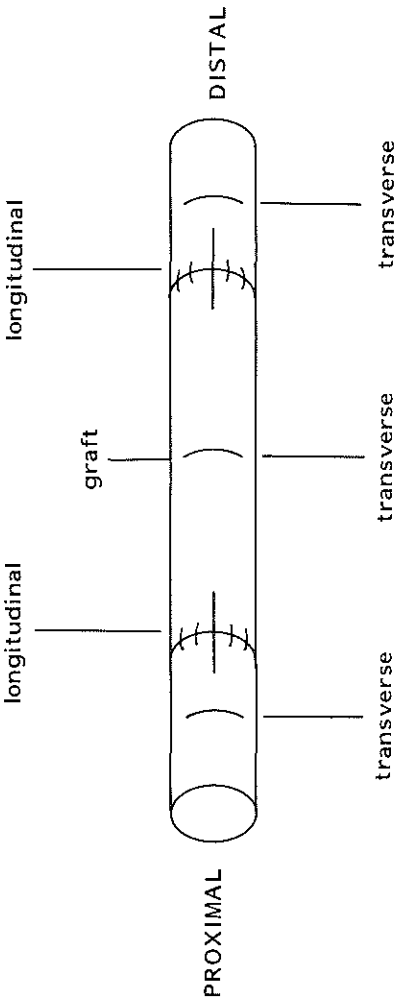


Figure 5. Diagrammatic representation of the sites of various sections made, in relation to the graft.

the host nerve and of the middle of the transplanted nerve were taken (fig. 5). Longitudinal sections at the proximal and distal nerve suture sites were later prepared with specific stains. To evaluate the degree of axonal regeneration the Bodian method was employed (Romeis, 1968); Kluever's method was used to visualize the late stage of myelin regeneration (Romeis, 1968). Special attention was paid to qualitative pictures of the nerve sheaths. Van Gieson and Haemotoxylin-Eosin methods were used to visualize the connective tissue (Romeis, 1968).
To facilitate differential evaluation, care was taken to obtain transverse as well as longitudinal nerve sections at the same level in relation to the proximal and distal nerve suture sites. Transverse sections were taken at the proximal and distal segments of the normal host nerve and in the middle of the graft to obtain an impression of the number of axons which succeeded in traversing through the nerve graft. The qualitative and quantitative histological findings, received after a thorough study of the histological preparation, prepared according to the above mentioned methods, were graded as follows:

Grade I (figure 6a, b, c, d)
Good regeneration with commencement of myelinization, parallel bundles of nerve fibres, no fibrosis, no inflammatory reaction.

Grade II (figure 7a, b, c, d)
Good regeneration, little irregularity in parallelism of nerve fibre bundles, slight fibrosis, no inflammatory reaction.

Grade III (figure 8a, b, c, d)
Incomplete regeneration, slight interlacing of the nerve

fibres, evident fibrosis, slight inflammatory reaction.

Grade IV (figure 9a, b, c, d)
Poor regeneration, marked interlacing of nerve fibres, extensive fibrosis, evident inflammatory reaction.

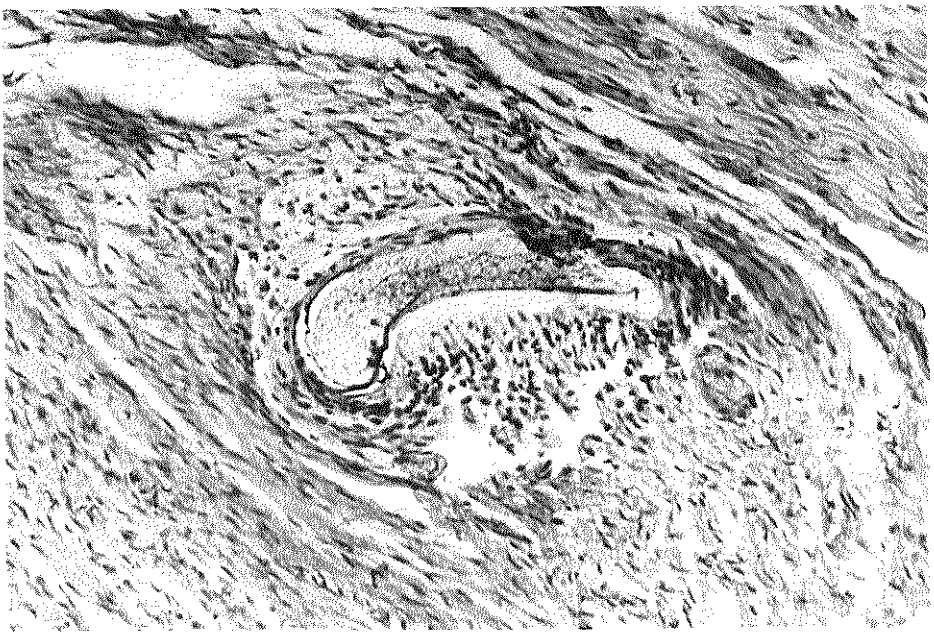


Figure 6a. Site of nerve suture. Slight lymphocytic reaction close to the suture (H&E, X 235).

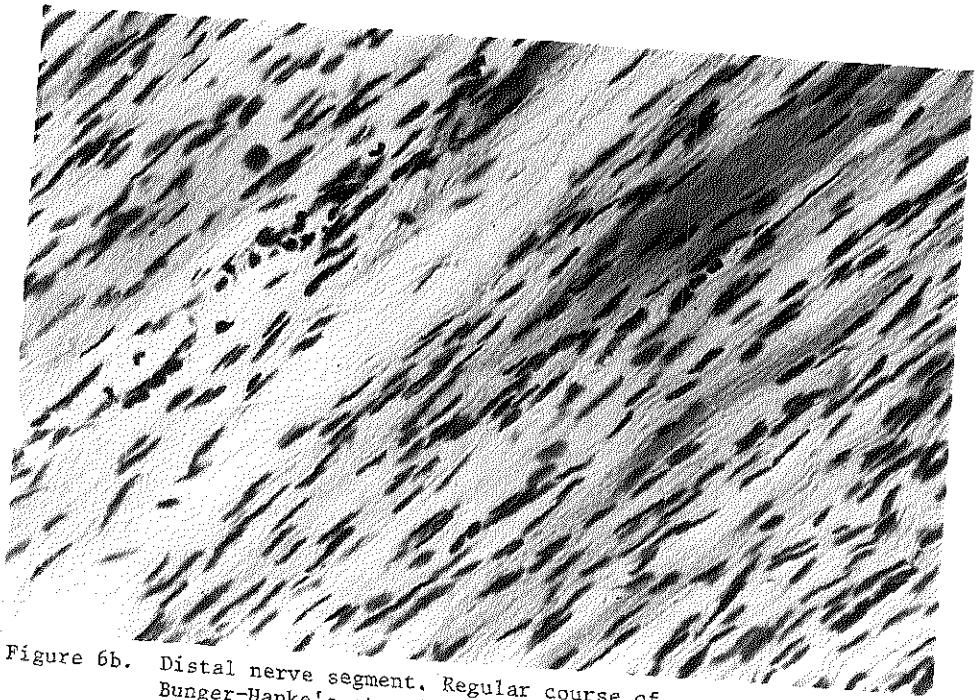


Figure 6b. Distal nerve segment. Regular course of Bunger-Hanke's strands (proliferation of Schwann cells) (H&E, X 380).

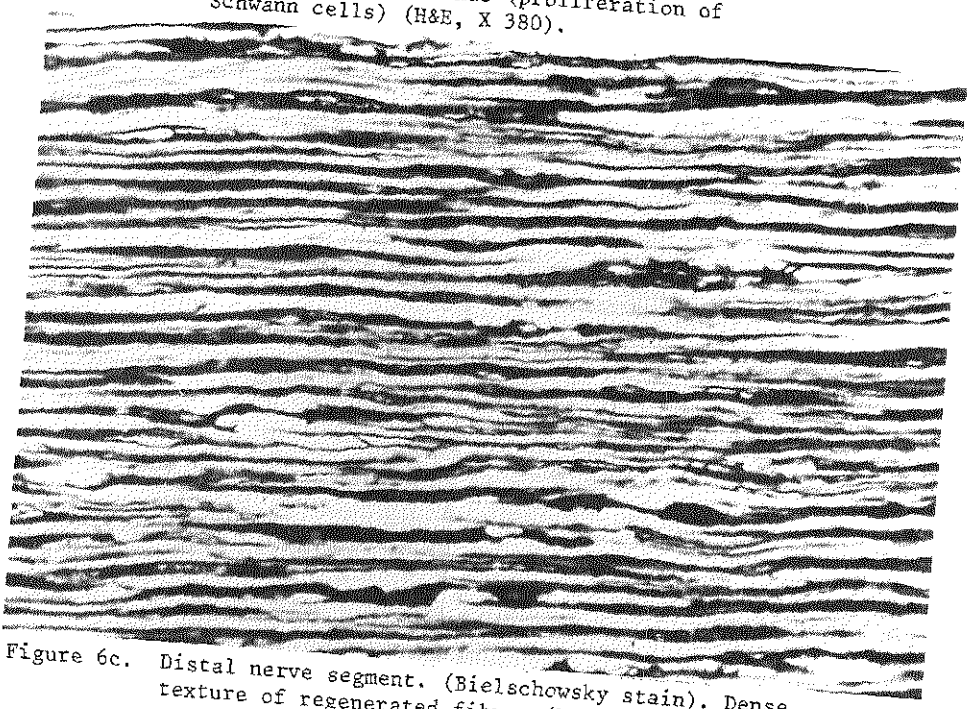


Figure 6c. Distal nerve segment. (Bielschowsky stain). Dense texture of regenerated fibres (X 380).

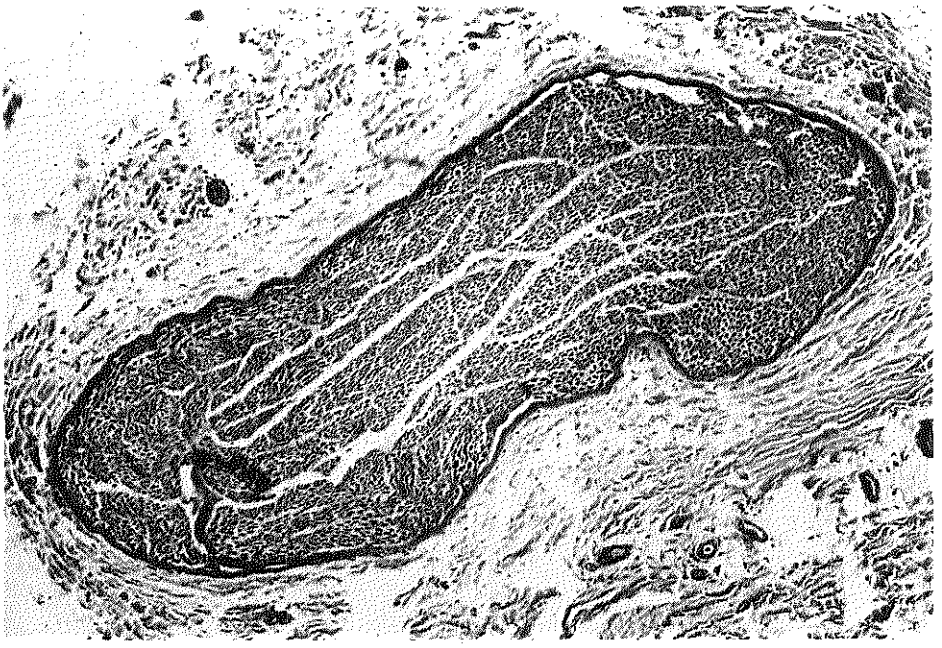


Figure 6d. Distal nerve segment (transverse section). Compact and dense pattern of the axons. Slight proliferation of the epineurium. No aberrant fibres (Bodian, X 235).

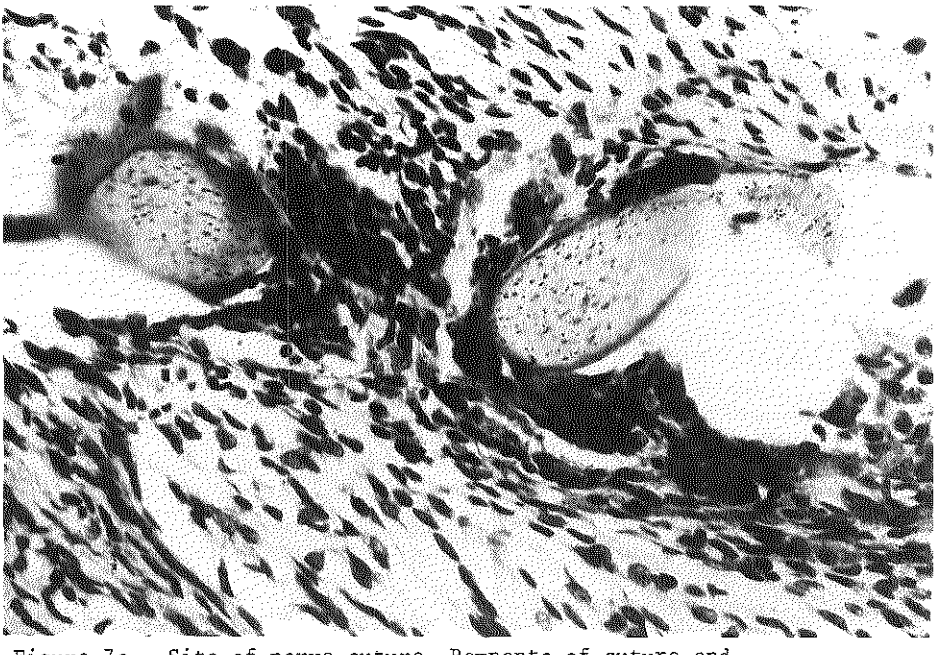


Figure 7a. Site of nerve suture. Remnants of suture and polymorhic inflammatory reaction with foreign body giant cells (H&E, X 380).

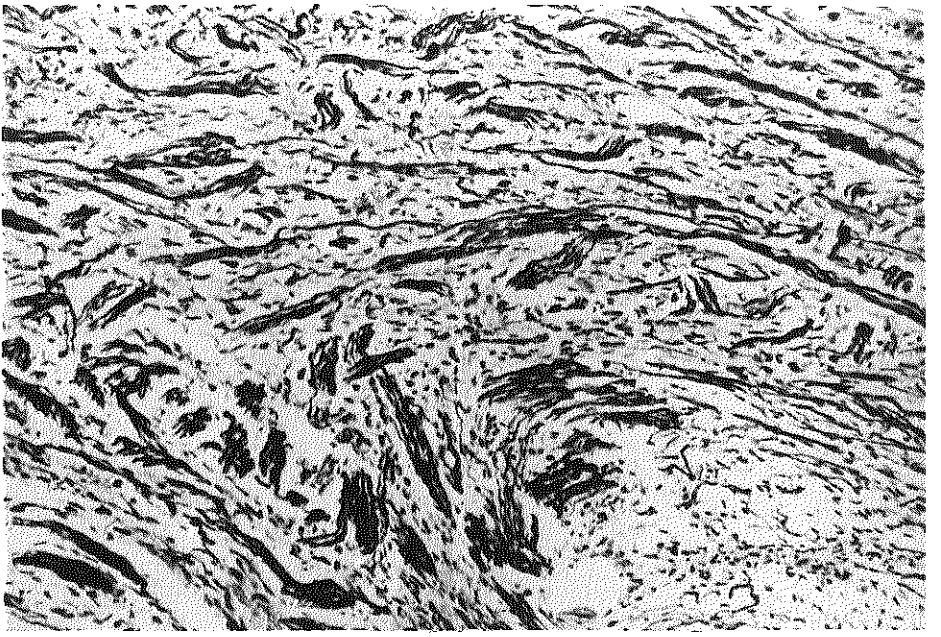


Figure 7b. Distal nerve segment. Slight irregularity of nerve fibre bundles (Bielschowsky, X 235).

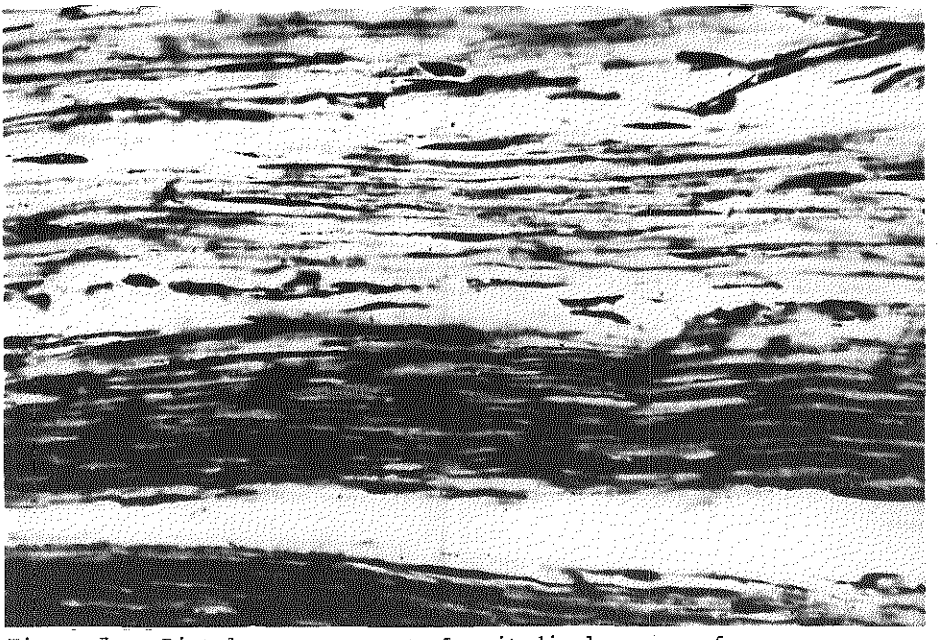


Figure 7c. Distal nerve segment. Longitudinal course of nerve fibres with uneven texture of bundles (Bielschowsky, X 235).

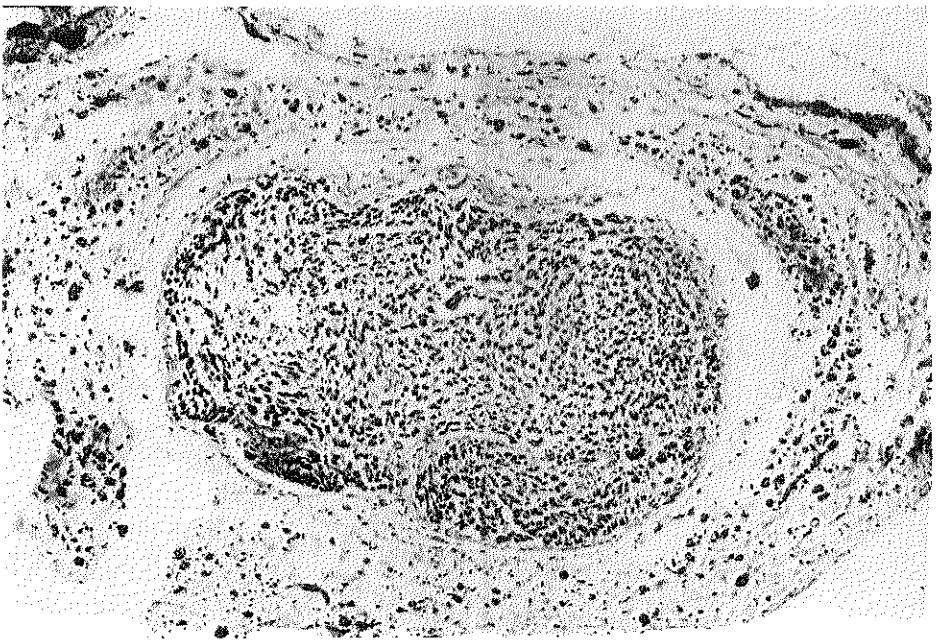


Figure 7d. Distal nerve segment (transverse section). Less dense axonal pattern in the nerve trunk. Mild proliferation of the connective tissue of epineurium with aberrant fibres (Bodian, X 235).

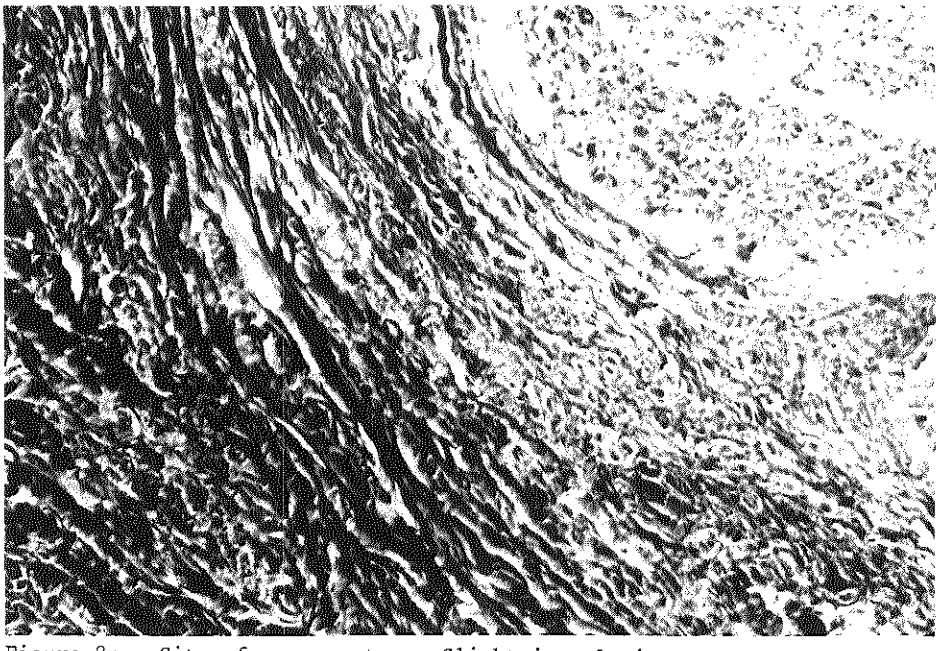


Figure 8a. Site of nerve suture. Slight interlacing of the nerve fibres (Van Gieson, X 235).

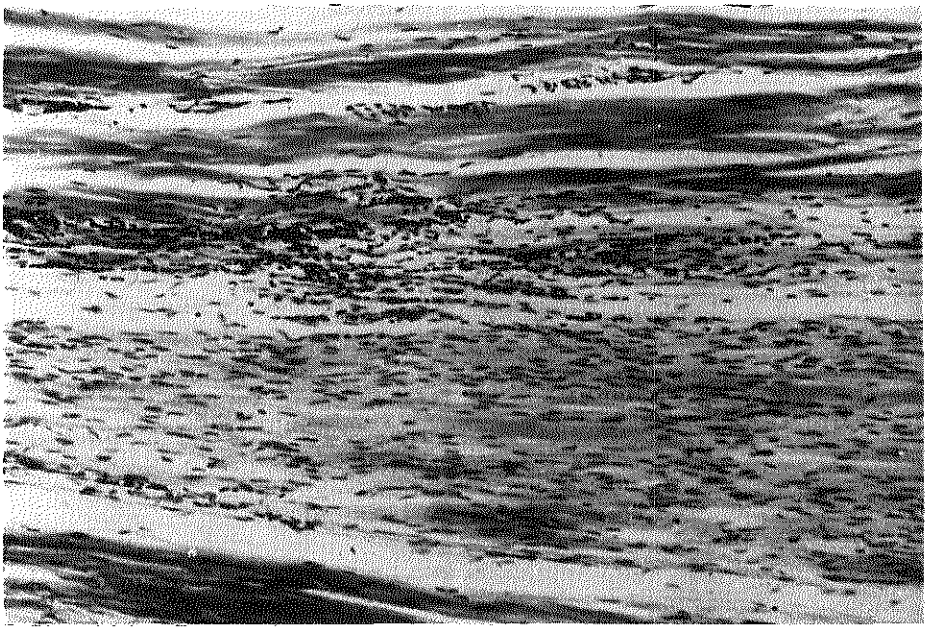


Figure 8b. Distal nerve segment. Inflammatory reaction in epineurium (H&E, X 235).

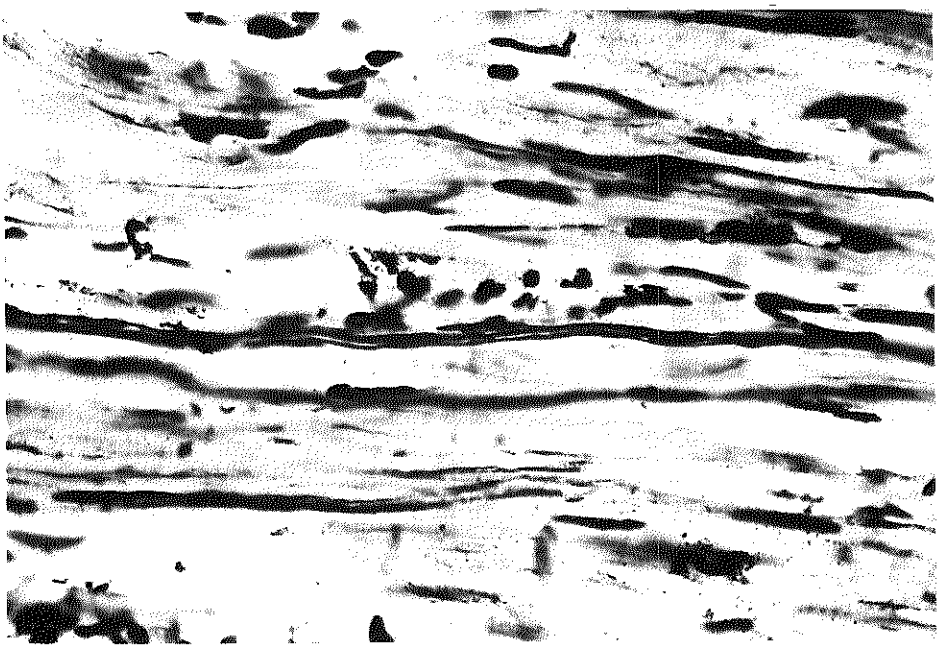


Figure 8c. Distal nerve segment. Uneven thickness of fibres and low density (Bielschowsky, X 280).

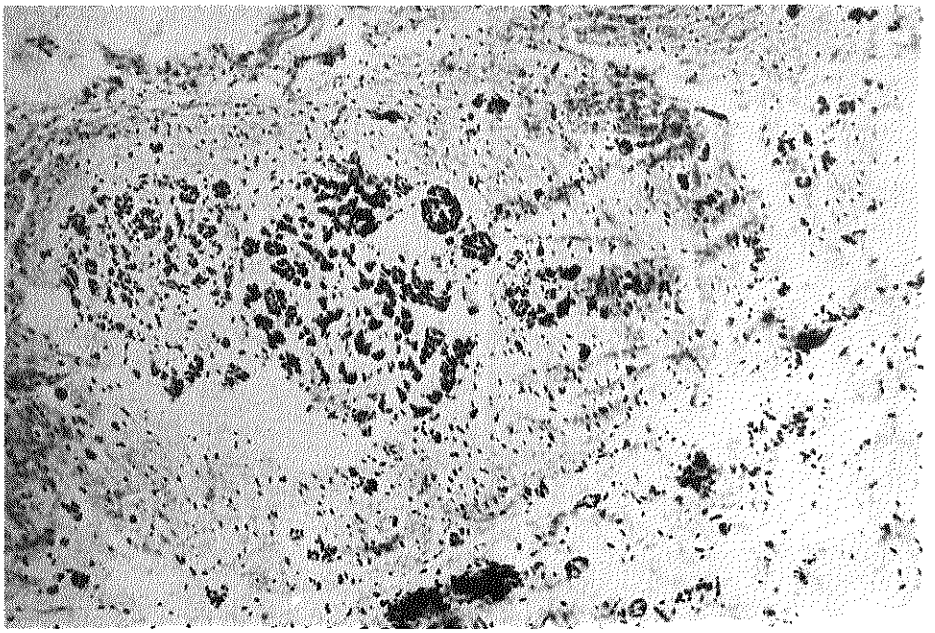


Figure 8d. Distal nerve segment (transverse section). Irregular distribution of axons. Slightly irregular condensation of axons. Many aberrant fibres in the connective tissue of epineurium (Bodian, X 235).

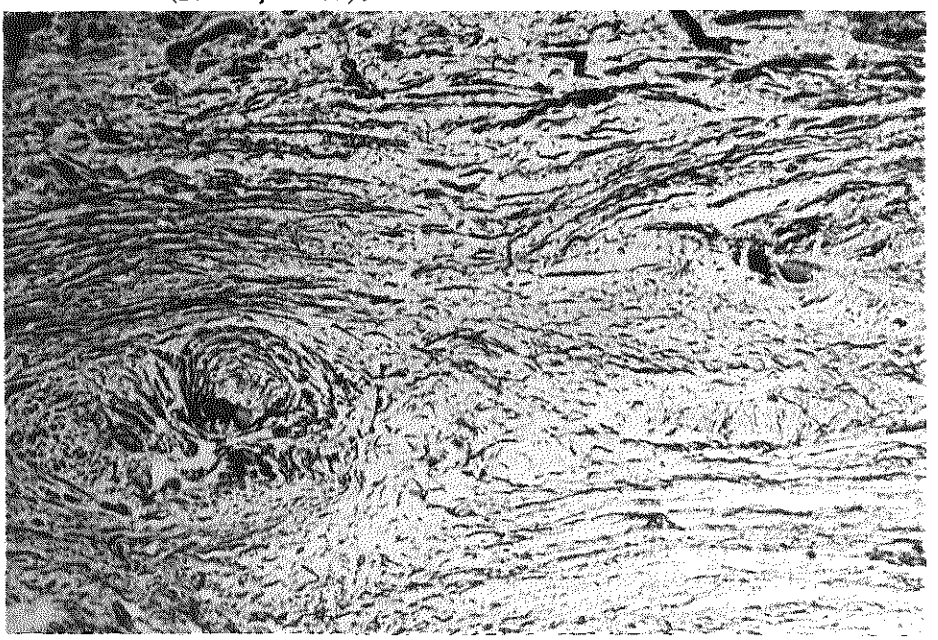


Figure 9a. Site of nerve suture. Marked interlacing of nerve fibres. Considerable proliferation of connective tissue and aberrant fibres (Van Gieson, X 235).

Figure 9b. Distal nerve segment. Diffuse inflammatory reaction in epi- and perineurium (H&E, X 235).

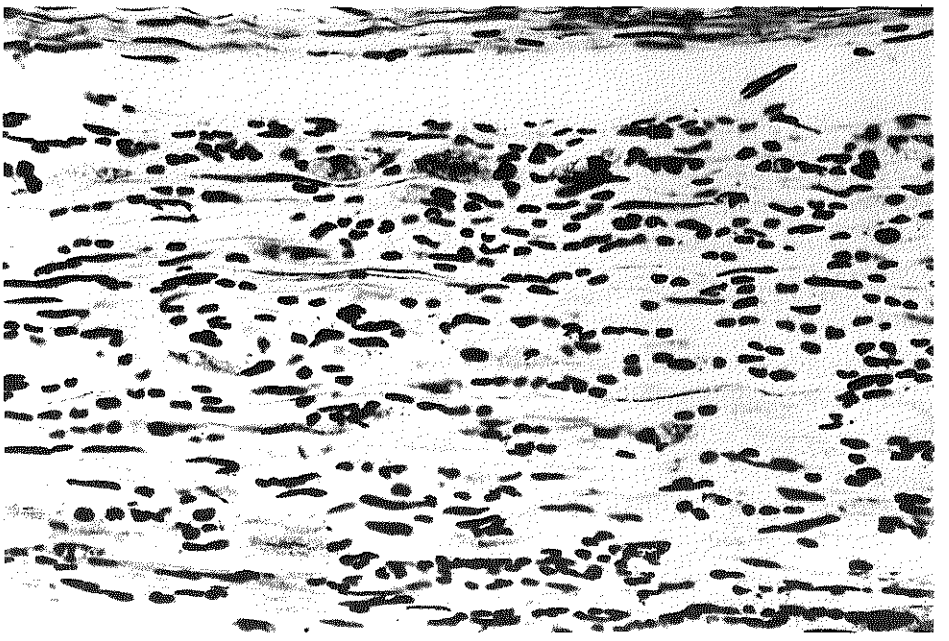


Figure 9c. Distal nerve segment. Poor regeneration (Bielschowsky, X 380).

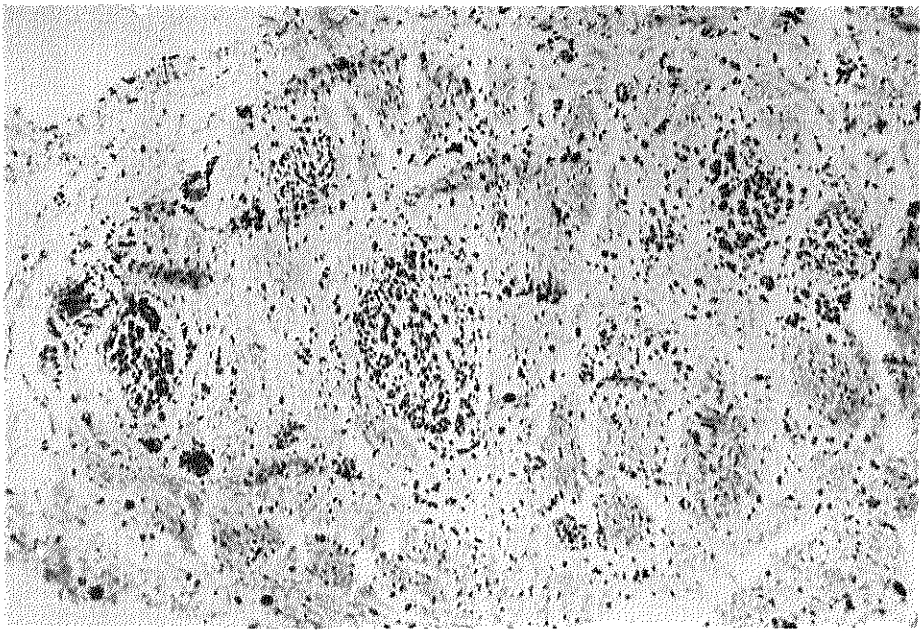


Figure 9d. Distal nerve segment (transverse section). Markedly irregular distribution of axons with many aberrant fibres in the fibrotic epineurium (Bodian, X 235).

Prior to this study, histological preparations were studied following autogenous nerve grafting, carried out under exactly the same conditions. Grade I of this classification resembles the results after autografting and is considered to be the best grade; Grade IV is the worst; Grade II and III are intermediate.

The grading of these histological findings was confirmed by an independent expert in the field of histology, not involved in the research scheme.

CHAPTER 4

RESULTS

4.1. Experiments with 4-cm long cryopreserved allografts

4.1.1. Influence of histocompatibility matching on 4-cm non-irradiated nerve allografts

Introduction

In the early days of such experiments most investigators achieved occasional success with short nerve allografts (2-4 centimeter) (Bentley and Hill, 1936; Sanders and Young, 1942). Sanders (1954) reported that the severity and rapidity of onset of the immune reaction depended upon the amount of tissue transplanted and on the genetic relationship between the donor and the host. Bentley and Hill (1936) concluded that gaps upto 3 centimeter could be bridged satisfactory by nerve allografts in some cases but longer grafts invariably failed, while Ducker and Hayes (1968a, 1968b, 1970) maintained this safe limit was 4 centimeter.

In this experiment cryopreserved nerve allografts of 4 centimeter in length, were tested in DLA-identical littermates and DLA-mismatched unrelated combinations, in order to assess the influence of histocompatibility matching (at two extremes) on the survival and regeneration of these allografts.

Nerve allografts in DLA-identical combinations showed better signs of regeneration on electromyographic studies. In most of these grafts maximum nerve conduction velocity returned to normal or very near normal. On histological examination, there were no obvious signs of tissue

rejection. The pattern of regeneration through the nerve graft was normal in appearance. The DLA-mismatched group was examined electromyographically. The maximum conduction velocity did not reach normal levels in any of these nerve grafts; a delayed conduction velocity was recorded in four of the grafts; one gave no electrical stimulation response, and one a markedly delayed response. On histological examination four of these grafts showed marked inflammatory response activity, thus leading to fibrosis. In two of these grafts, however, a reasonably normal pattern of regeneration was apparent.

Conclusion

Thes results support the views of many other authors (e.g. Ducker and Hayes, 1970), i.e. that some of the grafts not exceeding 4 centimeter in length could show some signs of regeneration even under all adverse circumstances. The difference in the results of these two groups are of statistical significane on histological examination, $P = <0.001$, but not very significant on electromyographic examination, $P = 0.13$ (Wilcoxon's two sample test).

Table II. Results

Group no.	Donor recipient relationship	No. of dogs in electromyographic grade				No. of dogs in histological grade			
		I	II	III	IV	I	II	III	IV
5	DLA-identical littermates	2	4	-	-	4	2	-	-
6	DLA-mismatched unrelated	-	4	1	1	-	2	3	1

4.1.2. Influence of graft irradiation on the survival of DLA-identical and DLA-mismatched cryopreserved grafts 4 cm in length

Introduction

Immunological reactions against the transplanted nerve tissue are responsible for the failures of mismatched nerve allografts. Irradiation has been used to reduce this immunological response in other organ transplants. Marmor (1967) used irradiation in his experimental animals in order to reduce this immunological reaction, and obtained encouraging results. Clinical application, however, remained far beyond expectations (Marmor, 1967). At the same time, Campbell (1971), Ducker and Hayes (1968a, 1968b, 1970) and Pollard et al. (1971) also reported favourable results of irradiation of nerve allografts in their experimental studies. Also in our own clinical experience, some favourable results of irradiated nerve allografts were recorded (Singh and De Lange, 1975).

Material

In this experiment, irradiation of the nerve allografts, with the technique described (Chapter 3), was used in addition to histocompatibility matching. Groups of DLA--identical and DLA-mismatched grafts were used for this study. Results are compared with those of experiment 4.1.1. where no irradiation was applied.
The results of the DLA-identical grafts with or without irradiation are more or less the same. On electromyographic examination, a normal or near normal maximum nerve conduction velocity was recorded in all the grafts. Similary, on histological examination, no marked inflammatory response was noticed in any of these grafts. Results of the DLA-mismatched groups with or without irradiation

did not show any beneficial effect of the irradiation in reducing the immunological response to the foreign tissue. In the irradiated group, the results of both electromyographic and histological examination were poor. Most of the grafts showed massive invasion together with a lymphocytic reaction leading to intense fibrosis. In the non-irradiated group, even better results were recorded on EMG as well as on histological examination.

Table III. Results

Group no.	Donor-reciepient relationship	Irradiated or unirradiated	No. of dogs in electromyographic grade				No. of dogs in histological grade			
			I	II	III	IV	I	II	III	IV
1	DLA-identical littermates	irradiated	3	2	-	-	4	1	-	-
5	DLA-identical littermates	non-irradiated	2	4	-	-	4	2	-	-
4	DLA-mismatched unrelated	irradiated	-	-	3	3	-	-	3	3
6	DLA mismatched unrelated	non-irradiated	-	4	1	1	-	2	3	1

One dog of group 1 was lost shortly after the implantation of the graft.

Conclusion

Results thus show no significant difference between the irradiated and the non-irradiated nerve allografts. Matching for the MHC-antigens remained the predominating factor. On statistical analysis (Wilcoxon's two sample test) the difference in irradiated and non-irradiated

groups in DLA-identical littermates is not significant on electromyographic examination (P = 0.06) as well as on histological examination (P = 0.18). Similar insignificant are the results in DLA-mismatched, irradiated and non-irradiated groups on electromyographic examination (P = 0.06) and on histological examination (P = 0.18).

4.1.3. Influence of DLA-matching on the survival of 4-cm long irradiated cryopreserved nerve allografts

Introduction

Donors of different match grades were available for this study, so that the effect of different grades of histocompatibility could be investigated.

Table IV. Results

Group no.	Donor-recipient relationship	Irradiation	No. of dogs in electromyographic grade				No. of dogs in histological grade			
			I	II	III	IV	I	II	III	IV
1	DLA-identical littermates	irradiated	3	2	-	-	4	1	-	-
2	one DLA-haplotype different littermates	irradiated	1	3	-	1	-	5	-	-
3	two DLA-haplotype different littermates	irradiated	-	1	2	3	-	-	3	3
4	DLA-mismatched unrelated	irradiated	-	-	3	3	-	-	3	3

One dog of group 1 was lost shortly after the implantation of the graft. In the remaining dogs of this group, an almost normal EMG and histological response was observed. Gradual decrease in matching in groups 1, 2, 3, and 4 was negatively correlated with the EMG performance and the histology of the grafts. The partially DLA-mismatched group (group 2) shows better results than the completely mismatched groups 3 and 4. One nerve graft in group 3, in spite of mismatching, showed a good EMG response. This can probably be explained by Sanders' (1954) experience that grafts upto 4 centimeter in length sometimes survive and regenerate against all odds. The marked inflammatory reaction observed on histological examination of this graft suggests a strong immunological response.

Conclusion

These experiments demonstrate the beneficial effect of partial and complete histocompatibility matching on the survival of irradiated nerve allografts. The results in irradiated grafts support the view obtained following the experiment with non-irradiated grafts, that the immunological reaction, responsible for the failure of nerve allografts, is dependent on the genetic relationship between the donor and the host. The occasional results of improvement with irradiation, reported in the past, could be coincidental due to a better match for the major histocompatibility antigens between the host and the donor. On statistical examination, Spearman Rank Correlation Test, the electromyographic results correspond with grades of DLA-matching $P = < 0.001$. The same agreement is also found with histological results $P = < 0.001$.

4.2. *Experiments with 7-cm long cryopreserved nerve allografts*

4.2.1. *Influence of histocompatibility matching on 7-cm nerve allografts*

Introduction

Smaller nerve grafts are known to survive occasionally in spite of adverse circumstances, but longer ones have failed invariably (Bentley and Hill, 1936; Gye et al., 1972; Roberts, 1967). In this experiment, 7 centimeter cryopreserved nerve allografts have been used in DLA-identical and DLA-mismatched combinations. Six dogs in each group were used for this study.

Table V. Results

Group no.	Donor recipient relationship	No. of dogs in electromyographic grade				No. of dogs in histological grade			
		I	II	III	IV	I	II	III	IV
7	DLA-identical littermate	5	-	-	1	4	1	-	1
8	DLA-mismatched unrelated	-	-	3	3	-	-	-	6

In DLA-identical combinations, five of the grafts registered a normal electromyographic response, with maximum nerve conduction velocity within the normal range. One graft suffered suture interruption at the distal nerve end and thus showed no electromyographic response. On histological examination, marked fibrosis was seen but no marked inflammatory response, yet it was provisionally placed in histological grade IV. In the remaining DLA--identical nerve grafts, a normal pattern of regeneration

was noted without any inflammatory response. In the DLA--mismatched group none of the grafts showed any significant signs of nerve regeneration on electromyographic examination. On histological examination, a marked inflammatory response was found leading to extensive fibrosis in all cases. This difference between the two groups is of statistical significance ($P < 0.05$) (Wilcoxon's two sample test) on histological as well as on electromyograhic examination.

Conclusion

The influence of histocompatibility matching is more evident with longer than with shorter allografts. In DLA--mismatched combinations, no significant functional recovery was found in any of the grafts and a marked inflammatory response was predominant. In the technically successful DLA-identical grafts, all showed a normal pattern of nerve regeneration without any evident signs of inflammation. Thus, with optimal matching for Major Histocompatibility System antigens, even longer grafts regenerate like autografts. This difference between the two groups is of statistical significance on electromyographic examination ($P_2 =< 0.05$) as well as on histological examination ($P_2 =< 0.02$) (Wilcoxon's two sample test).

4.2.2. *Result of partial histocompatibility matching on the regeneration and survival of 7-cm long cryopreserved nerve allografts*

Introduction

Optimal matching for MHC antigens leads to a good result, but such MHC identical grafts will be difficult to obtain

when cadaver donors are used. Therefore, it is of interest to study the effect of partial histocompatibility matching. For practical purposes, one haplotype different sibling donors were chosen. In our previous experiment some recovery was recorded of 4 centimeter nerve grafts matched for the antigens of only one MHC-haplotype. This experiment is carried out to find out whether a similar effect can be observed in grafts 7 centimeter in length.

Table VI. Results

Group no.	Donor recipient relationship	No. of dogs in electromyographic grade				No. of dogs in histological grade			
		I	II	III	IV	I	II	III	IV
7	DLA-identical littermate	5	-	-	1	4	1	-	1
11	One haplotype different	2	1	-	3	1	2	-	3
8	DLA-mismatched unrelated	-	-	3	3	-	-	-	6

In this experiment there is also a positive correlation between DLA-matching and the performance of the graft on electromyographic as well as on histological examination. About 50% of the grafts differing by one DLA-haplotype showed good functional recovery without any inflammatory response. Failure of one nerve graft in the DLA-identical group, due to a break in the suture line, has been explained in the previous chapter (4.2.1.).

Conclusion

The results are thus directly proportional to the degree of histocompatibility matching. If donor and recipient differ

with respect to one haplotype in MHC-matching, the graft has a better chance of recovery than a mismatched combination; thus, the importance of matching for MHC is underlined. Similar was the experience of Levinthal et al. (1980) with whole nerve allografts in rats.
In this experiment, there is also a positive correlation between DLA-matching and the performance of the graft on electromyographic as well as on histological examination. On statistical analysis $P < 0.001$ on histological examination and $P < 0.01$ on electromyographic examination (Spearman Rank Correlation test).

4.2.3. *Comparison of results of 7-cm long and 4-cm long cryopreserved nerve allografts of DLA-identical related and DLA-mismatched unrelated combinations*

Introduction

In chapter 4.1.1. and chapter 4.2.1. it was shown that histocompatibility matching was important for the outcome of both 4 and 7 centimeter long nerve allografts. In this chapter the results of matching for smaller or longer nerve allografts are put together for comparison.
Even with grafts of 7 centimeter, a good degree of regeneration and functional survival was found if the donor and recipient relationship was DLA-identical. On the other hand, none of the 7 centimeter DLA-mismatched grafts showed regeneration or functional recovery. In some 4 centimeter DLA-mismatched nerve grafts, however, incomplete recovery was observed.

Table VII. Results

Group no.	Donor-recipient relationship	Length graft in cm.	No. of dogs in electromyographic grade				No. of dogs in histological grade			
			I	II	III	IV	I	II	III	IV
7	DLA-identical littermate	7	5	-	-	1	4	1	-	1
5	DLA-identical littermate	4	2	4	-	-	4	2	-	-
8	DLA-mismatched unrelated	7	-	-	3	3	-	-	-	6

Conclusion

On statistical analysis the results in DLA-identical littermates groups are not significant on electromyographic examination (P = 0.10) as well as on histological examination (P = 0.10). In DLA-mismatched groups, however, the results are significant on histological examination (P = 0.02) and not significant on electromyographic examination (P = 0.06). However, this difference is minimal (Wilcoxon's two sample test). One could conclude, therefore, that matching for the major histocompatibility complex appears to be of even greater importance for the survival of longer nerve allografts.

4.3. *Influence of time of harvesting after death on the functional result of DLA-identical nerve allografts*

Introduction

The major histocompatibility complex is extremely poly-

morphic. It is, therefore, difficult to find compatible unrelated donor-recipient pairs. In order to have an effective selection for nerve allografting, it would be desirable to have a cryobank of many stored nerve grafts from tissue--typed donors of different tissue type. Furthermore, for the various clinical problems that can be solved by nerve transplantation, nerves of different length and neurolemmal pattern are required. Obviously, removal of such nerves from living donors is seldom acceptable and usually impossible. For these reasons, the feasibility of using nerves obtained from cadavers for transplantation has been studied. Nerves (7 centimeter in length) were removed 3 and 6 hours after the death of the donor dogs and were subjected to exactly the same process of preservation and transplantation as other nerve grafts. In order to be able to measure the effect of the circulatory arrest on graft function, only DLA-identical dogs were used for this study. The results are compared with those of DLA-identical nerve grafts removed under living conditions.

Table VIII. Results

Group no.	Donor-recipient relationship	Time of graft removal	No. of dogs in electromyographic grade				No. of dogs in histological grade			
			I	II	III	IV	I	II	III	IV
7	DLA-identical littermates	alive	5	-	-	1	4	1	-	1
9	DLA-identical littermates	3 hours after death	5	-	-	1	5	1	-	-
10	DLA-identical littermates	6 hours after death	5	-	-	1	6	-	-	-

The influence of the time interval between the death of the dog and removal of the graft from DLA-identical donors on nerve regeneration is shown in table VIII.
On electromyographic examination, one graft from each group failed to register any response. This can probably be explained by a technical error at the site of the nerve suture, as on histological examination these grafts did not show any marked inflammatory reaction nor fibrosis, making a plausible explanation of these failures difficult. Hence, in all DLA-identical donor-recipient combinations, cryopreserved nerve grafts 7 centimeter in length show a good degree of regeneration and functional recovery even if removed 6 hours after the death of the dog. A comparison between the cadaveric and fresh grafts show no difference of statistical significance ($P > 0.10$) on histological as well as on electromyographic examination (Wilcoxon's two sample test).

Conclusion

In DLA-identical major histocompatibility complex combinations, even the cadaveric nerve allografts, harvested 3 to 6 hours after circulatory arrest, regenerate as good as non-cadaveric grafts. This is evidently a great practical advantage that enormously enhances the number of potential donors.

4.4. *Influence of long term preservation (56 weeks) on 7-cm DLA-identical matched nerve allografts*

Introduction

Preservation of certain tissues, such as cornea, bone marrow and to certain extent skin is a well recognized and

and accepted procedure. Kaufman et al. (1966) and Mueller et al. (1964) have established a preservation technique for cornea, involving freezing at low temperatures, with good results. Precursors of the haematopoietic system and of the cellular immune system can be stored at cryogenic temperatures for extended periods of time (Lewis, 1974). Long term storage of skin can also be achieved using cryoprotectant, although there are some doubts about its biological behavior following transplantation (Berggren et al., 1966). In order to fulfil the requirements for an ideal nerve graft, the possibilities of using prolonged preserved (56 weeks) nerve allografts were explored. Nerve preservation was carried out as described in Chapter 3. Six DLA-identical littermate donor-recipient combinations were used for this procedure and the other variants kept constant. The results of graft preserved under similar conditions for 6 weeks will be compared.

Table IX. Results

Group no.	Donor-recipient relationship	Duration of preservation	No. of dogs in electromyographic grade				No. of dogs in histological grade			
			I	II	III	IV	I	II	III	IV
12	DLA-identical littermate	56 weeks	5	1	-	-	6	-	-	-
7	DLA-identical littermate	6 weeks	5	-	-	1	4	1	-	1

Histologically these grafts follow a very normal pattern of regeneration. No obvious signs of tissue rejection were seen. All these grafts could be graded histologically as Grade I. On electromyographic examination a normal conduction velocity was recorded in five cases, and reduced conduction velocity in one graft. Thus, even on EMG

examination normal patterns were observed.

Conclusion

Prolonged preservation does not seem to have any detrimental effect on nerve allografts of 7 centimeter. Comparison of these results with those following short preservation (6 weeks) shows no statistically significant difference. $P > 0.10$ on electromyographic as well as on histological examination (Wilcoxon's two sample test).

4.5. Agreement between Histology and EMG

Introduction

The entire study is based on the results obtained by histological and electromyographic studies of the grafts. In order to emphasize the integrity of these two parameters, an attempt has been made to establish a correlation between them.

Table X. Results

Grading	Histology	EMG-grading			
		I	II	III	IV
grade I	32	26	5	-	-
grade II	12	2	8	-	2
grade III	9	-	3	6	-
grade IV	17	-	-	3	14

The total number of grafts is 70. Complete grading agreement exists in 54 cases (77%). In 16 cases a discrepancy exists (23%). In 13 of these cases the differences between

histological and EMG-grading was only one grade, and so the correlation can be still considered to be reasonably good. A far worse EMG than histological grading was observed in three dogs, i.e. the graft was not rejected or only moderated rejected histologically, but it did not transport nerve impulses.
Technically failures possible prevented the growth of nerve sprouts into the distal nerve in these cases, no other explanation can be given. The overall correlation between the two evaluation methods is good. However, the overall agreement between both evaluation methods is thus good, KAPPA = 0.67 with SE = 0.07 (KAPPA-test for measurement of agreement).

CHAPTER 5

GENERAL DISCUSSION

5.1. General problems of nerve grafting

5.1.1. Choice of graft size

In clinical practice nerve grafts are used in cases where end-to-end suturing is not possible without producing tension at the suture site. This is usually the case when the gap exceeds 2-3 centimeter. Small nerve allografts have been reported to survive and function occasionally, in experimental as well as in clinical studies.
Klar (1943), Sanders (1942) and Ducker and Hayes (1970) found that the success rate of the grafts declined when the length of the grafts exceeded 4 centimeter. The experience of Singh et al. (1977) has been similar. Verhoog (1978) also used small nerve grafts of 3-4 centimeter in his study on the immunological aspects of nerve transplantation.
Different constituents of the nerve grafts have been found to constitute principal antigens responsible for the immunological reaction. Marmor (1964) and Dasgupta (1967) suggested myelin, while Pollard and McLeod (1982) the Schwann cells and Levinthal et al. (1978) epineurium and the surrounding connective tissue to constitute the principal antigens. By these experiments one could conclude that the entire nerve structure as a whole is responsible for the immunological reaction and not one constituent from it.
The only correct way to handle this problem seems to treat the nerve graft as an individual organ, whose immunological problems are the same as other vital organ transplants.
In this study 4 centimeter and 7 centimeter nerve allografts have been used in order to study the influence of

matching for MHC on the smaller and longer nerve grafts, most frequently used in clinical practice.
As the dog was the experimental model in this study, 7 centimeter was the maximum permissible length of graft which could be removed from the peroneal nerve, without jeopardizing the function of the sciatic nerve. In our experience, complete lesion of the sciatic nerve in the dog gives rise to traumatic ulcers of the leg which lead to gangrene with severe infection, and this sometimes necessitates amputation.

Thin long nerve grafts survive better compared to thick, lengthy grafts according to many investigators (Levinthal et al., 1978). Revascularization of the graft stems initially from the proximal and distal stumps and somewhat more slowly from the tissues of the nerve bed. In longer grafts the central part of the graft may suffer ischaemic necrosis (Miyamoto, 1979).
This reaction seems to be more evident when the transplanted graft tissue is not accepted by the host. This effect is also shown in our study (Chapter 4.2.1. and 4.2.2.). In histo-identical combinations, 7 centimeter grafts showed excellent recovery while in mismatched combinations, none of the grafts of the same length showed any signs of reasonable recovery. Certain smaller nerve grafts, 4 centimeter in length, showed signs of reasonable recovery even in mismatched histocompatibility combinations (Chapter 4.1.2.). The peroneal nerve in the dog is a relatively thin nerve; it might, therefore, be worthwhile conducting more experiments with thicker nerves. This is, however, hampered by the difficulty of selecting a proper experimental animal.

5.1.2. Choice of preservation method

For prolonged preservation of tissue, many techniques are available but these are often complicated, require highly specialized equipment and procedures, and are available only at well equipped centres.
In order to simplify the procedure and allow it to be carried out at most centres, a simple intermediate cooling procedure was used until the temperature reached a constant level of -70^{o}C, employing widely available tissue culture Medium 96 as a protectant.
Verhoog (1978) studied the effect of different methods of nerve preservation on the host's immunological reaction to the graft in rats. Temperatures varying from 20^{o}C to -192^{o}C with TCM 199, DMSO (15%) as cryoprotectant and Cialit as preservative, were used in his study. The preservation period lasted from 60 minutes to 14 days.
He conclused that preservation in TCM 199 supplemented with DMSO (15%) at -192^{o}C does not reduce the immunogenicity of the graft. He also found that the endoneural tubes were still filled with normal neural tissue, and were thus responsible for the immunological reaction. Preservation in Cialit together with lyophilization produces empty endoneural tubes and according to Verhoog reduces the immunological reaction.

In our study, reduction of the immunological reaction against the nerve allografts was attempted by irradiation and matching for the Major Histocompatibility Complex system antigens, but the effect of preservation on reducing this response has not been the subject of a separate study. Zalewsky and Gulati (1982), however, have reported that freezing even for two weeks destroys the Schwann cell of nerve grafts in rats and, therefore, is not suitable for nerve preservation.

5.1.3. *Viability of the graft*

Nerve grafts are supposed to act as a type of scaffolding for the growing nerve axons, to help them reach their final destination, the end plate. Viability of this transplanted nervous tissue has never been properly studied. Indirect evidence for the viability of nerve grafts has been collected by Verhoog (1978) during his study of different methods for preservation of the nerve grafts. The endoneural tubes of nerve grafts preserved in TCM 199 and DMSO (15%) at -192°C were found to be filled with normal neural tissue which retained its immunological properties. Nerve grafts preserved with other methods for the same period of 2 weeks gave different results: the endoneural tubes were empty. These findings support the hypothesis that viable nerve tissue is transplanted and also acts as a viable tissue when the nerve grafts are preserved for a short time in TCM 199 and DMSO (15%) at -192°C.

In the present work, only the final results of nerve grafting have been studied. Histological studies of the DLA-identical related group reveals a neural structure so near that of normal tissue, that one tends to believe that these allografts are acting as viable tissue. It appears that even during longer periods of preservation under deep-freeze conditions the viability of the nerve is preserved. Further study is necessary to confirm this hypothesis.

5.2. *Immunological problems of nerve grafting*

5.2.1. *Frozen nerve grafts are liable to be rejected*

Nerve allografts have been used for a long time with disappointing results (Davis and Ruge, 1950a, 1950b; and Barnes et al., 1945). The classical work of Sanders and Young (1942) and Gutmann and Sanders (1942) showed that the failure of nerve allografts was caused by the immunological response of the host, provoked by the foreign tissue implanted. Based on their own experiments, Dasgupta (1967) and Verhoog and Van Bekkum (1971) also formed the conclusion that nerve allografts generally follow the same rules as other organ transplants. They also concluded that this rejection response was proportional to the amount of nerve tissue transplanted, hereby explaining the occasional successes with small nerve allografts. Nerve allografts upto 2-4 centimeter in length were reported to have occasional success even under adverse circumstances. Marmor (1964) and Dasgupta (1967) suggested myelin to have major antigens responsible for immunological reaction, while Aguayo (1979) and Pollard and McLeod (1981) believed that major antigens carried on Schwann cells are more important than those in myelin. Lennon et al. (1978) have also suggested that within the central nervous system HLA antigens are not expressed by myelin. Contrary to the above reports Levinthal et al. (1978) have suggested that major transplantation antigens may reside mainly in the epineurium and the surrounding connective tissues.
Patients, however, often require longer nerve grafts, and consequently a solution for the problem of graft rejection had to be sought. We, therefore, initiated a study in dogs to investigate the problem of nerve allograft rejection.

It appeared that random nerve allografts 4 centimeter in length, obtained from unrelated DLA-mismatched donors were reasonably successful in four out of six cases; a severe reaction destroyed the other two grafts (table I). The

longer grafts of 7 centimeter, however, from random unrelated DLA-mismatched donors always failed (6 out of 6). The histological findings were in accordance with immunological rejection of the grafts.
Apparently, the rejection response in our dog model is dependent on the amount of nerve tissue transplanted. This is in accordance with the findings in other experimental models (Sanders, 1954; Gutmann and Sanders, 1943; Roberts, 1967; Ducker and Hayes, 1970).
The observation that 4 centimeter grafts are sometimes still functional after 210 days after transplantation is of interest. The other mismatched organs, transplanted to immuno-suppressed recipients, are almost always rejected. Several explanations can be forwarded for the high incidence of good functional recovery of short mismatched nerve grafts. In the first place, the small amount of nerve tissue transplanted, might contain only a sub--threshold dose of transplantation antigens, insufficient to activate the immune apparatus.
Alternatively, such a small graft might contain the approximate quantity of antigens just necessary to induce enhancement of the graft, i.e. antibodies are produced that protect rather than harm the graft.
Another possibility is that in some instances, in spite of rejection, the graft still provides a skeleton for the growing nerve sprouts to find their way to the distal segment of the nerve. This hypothesis is supported by the observation that the histological grades of groups 3 and 6 are inferior to the EMG grades. The finding that 7 centimeter DLA-mismatched nerve allografts were all rejected confirmed the need for further study, directed at preventing this rejection.

5.2.2. Attempts to prevent allogenic rejection

Theoretically there are the following possibilities:
a.: reduction of the immunogenicity of the graft,
b.: histocompatibility matching,
c.: modification of the immune response of the recipient by immunosuppressive drugs,
d.: modification of the immune response of the recipient by immunological manipulation.

5.2.2.1. Reduction of the immunogenicity of the graft

Irradiation has been said to reduce the antigenicity of the allografts and thus promote the capacity of cryopreserved nerve allografts to transmit regenerating axons. In animal experiments, Bohler (1962, 1967), Campbell et al. (1963a, 1963b, 1970) and Marmor (1964) reported better results following irradiation of the frozen dried nerve allografts. McGuirt and McCabe (1977), however, did not find any adverse effect of autogenous facial nerve cable grafts in cats. Their clinical experience, however, as ours (Singh and De Lange, 1975) is still disappointing. Our attempts to reduce the immunogenicity of the grafts by irradiation were not successful; none of the random mismatched irradiated grafts (6 out of 6) of length 4 centimeter still functioned after 210 days, while in the non-irradiated group 4 out of 6 were moderately successful.
Irradiation was not, however, harmful to well-matched grafts, as a good degree of regeneration was found in both groups of DLA-identical, irradiated and non-irradiated.
It is difficult to explain why irradiation is harmful for mismatched and not for matched grafts. Earlier, the hypothesis was forwarded that short mismatched grafts provided

a scaffolding through which the nerve sprouts could grow, regardless of rejection (Ducker and Hayes, 1970).

The additive damage caused by irradiation and rejection in these grafts, might make regeneration impossible (Stearns, 1982). In matched nerve allografts, rejection does not play a part in destroying this skeleton, and so one may conclude that the damage caused by irradiation alone is insufficient to destroy the graft.

Verhoog and Van Bekkum (1971) concluded from their rat studies that major histocompatibility structures on nerve grafts disappeared after storage at -70°C and irradiation. However, the poor results in DLA-differing by two haplotypes and DLA-mismatched cryopreserved irradiated nerve allografts, compared to the good results in DLA-identical littermates, demonstrates the presence of major histocompatibility antigens on these grafts. This discrepancy between their results and the results of this study might be explained by the following factors:

a.: the 2-week observation period, used by Verhoog and Van Bekkum, may have been too short, and a rejection process occurring at a later stage may have been missed;
b.: the 0.5 centimeter nerve grafts used in rats were too small to show the effects of major histocompatibility structures; and
c.: the histocompatibility difference between the rat strains used may be weaker than the histocompatibility difference in our mismatched dogs, and this in itself may be insufficient to elicit nerve graft rejection. This situation is probably comparable to that in the one haplotype mismatched dogs.

One may conclude that in our experiments, we could not

demonstrate a sufficiently beneficial effect of two techniques, namely irradiation and freezing at -70°C, in reducing the immunogenicity of the grafts.

5.2.2.2. Histocompatibility Matching

The awareness of dog immunogenicity, developed in our laboratory by Vriesendorp et al. (1977a, 1977b) and Westbroek et al. (1971), has made it possible to study whether histocompatibility matching could improve the survival rate of nerve allografts. For this purpose, the grafts from DLA-identical littermates were transplanted, these being the best match donor-recipient combinations obtainable in outbred dogs. It appears that matching greatly improves the results, especially in longer grafts, as these are more susceptible to rejection. In fact, in our experiments, DLA-identical nerve grafts, both 4 and 7 centimeter in length, irradiated or non-irradiated, were never shown to be rejected. This is in contrast to the observation on other vascularized organ transplants in dogs, where a graft rarely survives for more than 200 days without postoperative immunosuppression (Vriesendorp, 1973). This discrepancy could be explained by:

a.: lack of effect of minor histocompatibility structures on nerve allografts;
b.: the disappearance of minor histocompatibility structures on nerve allografts following storage at -70°C; or
c.: the induction of immunological tolerance or enhancement by frozen nerve allografts.

In the present study no argument can be found which favours

one of these possibilities, although Zalewski and Silvers (1980) concluded that minor antigens alone are as potent as major and minor antigens together in evoking an immune response with their experiments with nerve allografts in normal and tolerant rats.
Further studies using fresh nerve grafts would be required to find a solution to the issue. As the use of fresh nerve allografts has been found to provoke more severe tissue rejection (Seddon and Holmes, 1944; Woodhall, 1955; Whitcomb, 1959; and Pollard and McLeod, 1981), their use is no longer recommended.

Moreover, fresh nerve allografts cannot be used in clinical practice; therefore, we did not use fresh nerve allografts in our studies.
Of course, clinically, it will be impossible to obtain nerve grafts from the human correlate of the DLA-identical littermate, namely an HLA-identical sibling. This experiment was in fact, only devised to demonstrate whether matching has any influence at all on improving graft results. This was clearly the case in our experiments.
In addition, for 4 centimeter allografts, partial matching for one haplotype also produces reasonable good results, although Zalewski and Silvers (1980) found minor antigens alone as potent as major and minor antigens together in evoking the immune response, in rats.

Now that the beneficial effect of histocompatibility matching for nerve allograft survival in the related donor--recipient combinations has been shown, it remains a matter for further study to investigate whether matching in unrelated donor-recipient pairs is equally successful.
Presently, histocompatibility matching for other organ transplantation, especially kidney transplantation, is carried out for two classes of antigen (Chapter 2.4.2.)

Matching for the class I antigens, the so-called HLA-A, -B and -C antigens, follows conventional serological methods, using a microlymphocytotoxicity test. The serological matching technique described for dogs (Chapter 3.2.) is similar to this test in humans. For kidney transplantation, matching for the HLA-A, -B and -C antigens (comparable to matching for the DLA-A, -B and -C antigens in dogs) seems to induce improvement of graft survival. The evidence is, however, contradictory to the efficacy on this matching as reports vary only slight (Opelz et al., 1947b) to considerable (Van Rood et al., 1975) improvement in graft survival.

The weak point of matching for these class I antigens is that the polymorphism of the involved antigenic system is so enormous that using cadaveric donors, only a small proportion (10-20%) of the recipients will obtain a kidney that is identical in the whole set of HLA-A, -B and -C antigens. As evidence emerged that correspondence of MLR (or D) antigens might be associated with even better graft survival (man: Van Rood et al., 1977; Rhesus monkeys: Van Es et al., 1977; dogs: Bijnen et al., 1977) an attempt was made to design a rapid, serological test to predict the identity in MLR. This resulted in typing for class II antigens, called "DR" typing, i.e. antigens were being recognized that were somehow related, but not identical to the earlier recognized MLR- or D-antigens.
Currently, the generally held view is that matching for DR-antigens alone improves kidney graft acceptance to a greater extent than matching for class I antigens (Persijn et al., 1981). Furthermore, the DR-antigenic system is less polymorphic, and so one might expect to find a greater number DR-identical donors (in comparison to the rather small percentage of class I identical donors). It is, however, questionable whether this will also hold for nerve

tissue. A few distinct differences between nerve and kidney grafting must be noted.
In the first place, different organs may carry a different antigenic composition - both qualitatively and quantitatively - on the cell surface. Thus it follows, that if typing for HLA-A, -B, -C and DR-antigens improves kidney graft survival, it does not necessarily improve nerve graft survival.
Our study showed, however, that the important antigens for nerve graft survival belong to the major histocompatibility system, as matching for some of the markers of the MHC, the DLA-A, -B antigens improves the results of nerve grafts from related donors.

It is worthwhile to note here again that partial matching for DLA-antigens already proved beneficial for nerve grafting. This may imply that if, by whatever method, a partial matching for "nerve graft antigens" is obtained, a reasonable degree of success can be expected. Studies in various species (man: Van Rood et al., 1977; Rhesus monkeys: Van Es et al., 1977; dogs: Bijnen et al., 1977) has shown the linkage disequilibrium among the different antigenic system of the MHC. Linkage disequilibrium means that pairs of antigens of different genetic systems are found together more often than can be expected from their individual population frequencies. The practical consequence of this phenomenon is that by matching for some antigens which might in themselves be irrelevant for graft survival, one sometimes also matches for the important transplantation antigens.
Thus matching for class I and/or class II antigens will probably result in matching for antigens that are relevant for nerve graft survival. As partial matching for the MHC (as in the one haplotype different related combinations) is already beneficial, matching for markers of the MHC such as

class I and/or class II antigens, may also be beneficial. Secondly, nerve grafts are not as highly vascularized as kidneys are. In other similarly unvascularized grafts, such as skin grafts, MLR-matching improves graft survival only slightly or not at all and DR-matching alone is even less effective.
Lastly, in our model of nerve grafting where frozen nerve grafts are used, it is not certain whether vital tissue is transplanted. It is certainly possible that dead or dying cells are being transplanted which serve merely as the conductive skeleton for the ingrowing nerve sprouts.
The immunizing antigens on the graft may, therefore, have undergone qualitative or quantitative alterations, so that the afferent and/or efferent limb of the rejection mechanism may be quite different from that in a fresh transplant. We have shown that matching can definitely improve the result of nerve grafting in our related dogs. It remains to be demonstrated whether the same will hold true for unrelated humans.
In any case, we still suggest submitting the possibility of transplanting unrelated matched grafts (whether matched for class I or class II antigens or both) to clinical trial, on the following grounds.

In the present study we have shown that nerve grafts preserved for a long time remain in good condition, and can give promising results. It would, therefore, be possible to install a nerve bank containing with a large spectrum of nerves of differing tissue type. Thus, for most recipients a class I and/or class II antigens-matched allograft can be selected. This will most probably result in a partial match for the relevant nerve transplant antigens in a number of cases. If immunosuppression is not desirable, and if the risk of surgery is acceptably low, it would be worthwhile to carry out a clinical trial in cases which are otherwise

desperate, e.g. due to severely debilitating neurological inadequacies.

5.2.2.3. *Modification of the immune response of the recipient by immunosuppressive drugs*

Marmor (1967), Pollard and Fitzpatrick (1972, 1973), Hirasawa and Inoue (1981) and Pollard and McLeod (1981) reported better regeneration on using the immunosuppressant Azathioprine rather than irradiation in their experimental studies.
However, Ikeda (1966) and later Ducker and Hayes (1970) reported that immunosuppressive therapy is not only unnecessary but might also interfere with the regeneration of axons and endanger the host.

As a nerve transplant, in clinical practice, is not a life saving procedure, and since the effect of prolonged therapy with drugs are unpredictable and could even prove dangerous to life, their clinical application would not seem advisable. This sort of drug-therapy was, therefore, not included in our experimental protocol.
Azathioprine alone is not sufficient to maintain other grafts, but its combination with corticosteroids is at present the most generally used regimen for clinical vascularized organ transplantation. This continued regimen might be more successful in stimulating the survival of nerve transplants; this remains to be investigated.

Various immunosuppressive regimens for nerve transplantation in rats have already been described by Verhoog (1978) and Parekh (1982). It is not impossible that a nerve transplant is antigenic only shortly after transplantation, depending on the viability of the graft.

In our experiments, this has as yet not been disproved. It would, therefore, be worthwhile testing the effect of a short course of immunosuppressives in an experimental model e.g. the dog. The relatively new agent, Cyclosporin A, which was found to be successful in renal transplant recipients (Calne et al., 1981; Starzl et al., 1981) might also enjoy future use in nerve transplantation.

5.2.2.4. *Modification of the immune response of the recipient by immunological manipulation, specific or aspecific*

Theoretically, there are two methods of improving graft survival by immunological manipulation, namely the induction of specific unresponsiveness (tolerance or enhancement), or the induction of aspecific unresponsiveness by third party blood transfusions.
Enhancement is defined by Kaliss (1958) as an antibody dependent phenomenon, leading to prolonged allograft survival. However, even graft protection by cellular mechanisms can be included in the definition of enhancement. Tolerance is defined as the absence of an immune response to donor antigens, while other immune responses function normally (Medawar, 1973). At present, both enhancement and tolerance can only be induced in small animals such as rodents; so far they have not been repeatedly elicited in preclinical models nor in man.

Attempts to induce enhancement in kidney transplantation in dogs has so far failed (Jeekel et al., 1975). It is, therefore, unadvisable to attempt to induce specific unresponsiveness to nerve allografts in the preclinical dog model. Third party blood transfusions, given to human recipients prior to transplantation, have a pronounced beneficial effect on subsequent kidney graft survival (Opelz and Terasaki, 1976).

Such a beneficial effect could also be elicited in Rhesus monkeys (Van Es et al., 1977) and in dogs (Obertop et al., 1978), but only when postoperative immunosuppression is applied (Bijnen and Obertop, 1980; and Niessen, 1982). It is of course possible that such blood transfusions are also beneficial to nerve graft survival. However, if post-operative immunosuppressive therapy was also required for nerve grafts, then such a policy would be less desirable for the transplantation of these nerve grafts.

5.2.3. Histology of graft rejection

The present concept concerning cellular rejection of foreign tissues is based on the histological studies carried out in different organs in animal experiments (Wiener et al., 1964; Comtet and Revillard, 1980; and Levinthal et al., 1978). This study is also supported by histological studies on rejected organs, mainly kidneys, in man.
The different forms of rejection, can thus be summarized in four commonly observed pattern:

1.: hyperacute rejection which occurs within a few minutes or a few hours of the organ being revascularized.
2.: delayed hyperacute or accelerated rejection, which develops slowly during the first 3 days after transplantation.
3.: acute or intermediate rejection, which is regarded as the classic primary response of cellular immunity, mediated predominantly by the T-lymphocyte population. The great majority of rejection episodes of this type occur after about 3-5 days and within 2-4 months. With cadaveric kidney transplants in man the danger period may extend up to 2 years (Hume, 1968).
4.: chronic or late rejection changes may be present from

2 months onwards and may progress very slowly and insidiously over a period of years. This reaction is characteristic by extensive interstitial fibrosis, which can be focal and irregular. Lymphoid cells are frequently distributed throughout fibrotic areas (Bohler, 1967). Most infiltrating cells are mature lymphocytes (Lindquist et al., 1968; Bieber et al., 1970) accompanied by a few plasma cells and a variable number of large macrophages. Sometimes these reactions cannot be distinguished from chronic interstitial infection (Thorsby and Lie, 1971; Lagrange, 1972).

In our experiments only the residual effects of the late rejection changes have been studied, and these accord fairly well with the above mentioned rule. Acute and intermediate rejection changes may have passed unnoticed. Massive infiltration with lymphocytes and extensive interstitial fibrosis together with necrosis of the nerve structure were found to be the most predominant features in nerve allografts showing poor or no regeneration. The endoneural tube was replaced by fibrous tissue. Similar had been the experience of Kalopissis et al. (1981) and Comtet and Revillard (1980).
In order to understand the sequence of this cellular rejection of neural tissue, further studies are necessary during the acute and intermediate periods following nerve allograft transplantation in well defined donor-recipient combinations.

5.3. Problems of nerve banking

5.3.1. Harvesting

MHC-identical preserved fresh nerve allografts will survive and regenerate as well as autografts (Singh et al., 1977).

Nerve allografts obtained from deceased identical littermates, in tissue-typed matched combinations, react more or less like fresh allografts, as shown in table VII.
So far, cadaveric nerves of animals dead for more than 6 hours, were not used in transplantation because of the anticipated problems of infection and tissue decomposition. In view of the good results obtained with a 6-hour interval between death and graft removal, longer intervals could be a subject for future study. In general, it will be possible to perform harvesting before this time, e.g. following removal of kidneys for transplantation.

For clinical nerve allografting, the present protocol generally followed in most hospitals for removal of cadaveric kidneys could possibly be adjusted to accomodate the removal of nerves.
The present study has contributed to the definition of an acceptable interval between death and nerve allograft removal. The extreme polymorphism of the Major Histocompatibility Complex creates greater problems in finding suitable recipients for nerve allografts. Studies on the real importance of tissue typing in nerve allografting in the unrelated donor-recipient combinations are still necessary, although knowledge about the successful transplantation results of vascularized organs in partly mismatched animal models and in man, does support an optimistic view on the feasibility of nerve allografting in man in selected cases.

5.3.2. Preservation time

Prolonged cryopreservation of nerve allografts has been tried before. Lyophilizing nerve allografts, with the intention of having easy access to all sorts of nerve

grafts has failed (Kuhlendahl et al., 1972), nor did deep--frozen irradiated nerve grafts produce satisfactory results, especially with longer grafts (Marmor et al., 1969; and Singh and De Lange, 1975).
Our dog experiments (group 7, table V) have shown good regeneration of 7 centimeter nerve allografts in DNA--identical donor-recipient combinations, preserved for about 6 weeks. In another experiment, cryopreserved 7 centimeter nerve allografts (table IX) have shown the capacity to survive and retain functional recovery, even after 56 weeks of preservation. The absence of tissue rejection reaction on histological examination, is probably due to the disappearance of minor histocompatibility structures following this prolonged period of preservation or due to the additive effect of other factors discussed in experiment 4.4.
The tolerance of 3 and 6 hours of complete ischaemia (table VIII) and survival and good functional recovery after 56 weeks of cryopreservation are factors which increase the feasibility of establishing a bank of nerve allografts.

5.3.3. Practical problems

Our experiments have proved that nerve grafts can tolerate vascular ischaemia for 6 hours and be stored for more than 56 weeks without losing their capacity for regeneration in DLA-identical MHC combinations. Such results encourages us to explore these possibilities in man.
The nerves most frequently used in clinical practice would be removed from cadavers. The sites of removal of such grafts should correspond approximately to the sections requiring replacement, e.g. median and ulnar nerve at the wrist and radial nerve at the level of the upper arm in

the radial groove. The length of the graft removed should be quite liberal so that it could be shortened, if necessary. For some nerves, segments could be removed at different places, e.g. median nerve at wrist, elbow and arm. These grafts can be separately labelled and stored. In this way nerve grafts would replace the original neurolemmal system more accurately.

Tissue typing of the donor could be carried out before removal of the graft, and later matched with the recipient whenever necessary. Such administration should be performed at an international level, e.g. by the Eurotransplantation Society.

As such an operation would rarely be an emergency, the recipient's blood typing could be logged into the computer system, so that it was matched immediately and automatically as soon as a suitable donor became available. Thus nerves from one donor could be used for several recipients, provided their blood typing matched sufficiently well. One might also consider removing nerves from all kidney donors, to allow wider flexibility in donor-recipient selection.

Transportation of these cryopreserved nerve allografts from place to place in the same country or from one country to another, would be quite easy in sealed boxes in dry ice, thus maintaining a low constant temperature. If such a programme could be organized, one would have a vast number of different tissue typed nerves available and thus more appropriate selection could be made and better functional recovery expected.

CONCLUSIONS

1. The rejection of nerve transplants more or less follows the rejection laws in other organs. The degree of rejection is probably proportional to the amount of tissue transplanted. Smaller nerve grafts have been shown to survive under all circumstances, and rejection becomes more pronounced as soon as the length of the nerve graft exceeds 4 centimeter.

2. Irradiation does not exert any specific beneficial effect on the survival of cryopreserved nerve allografts and may even be harmful for mismatched grafts.

3. Matching for Major Histocompatibility System antigens greatly improves the survival and functional recovery of nerve allografts. Grafts obtained from DLA-identical littermates behave like autografts. The beneficial influence of matching is even more evident with longer nerve allografts (7 centimeter).

4. The degree of rejection of nervous tissue is directly proportional to the grade of DLA-matching. A positive correlation between the DLA match and the EMG performance and histology of the nerve graft is observed in all groups. In small nerve grafts (4 centimeter) even partially mismatched DLA might show some recovery.

5. In DLA-identical combinations, even 7 centimeter nerve allografts can tolerate complete ischaemia lasting 6 hours, without any adverse effects on the final results.

6. In DLA-identical combinations, cryopreserved nerve allografts, 7 centimeter in length, can tolerate prolonged

preservation for 56 weeks, without their regenerating activities being affected and without showing any evident signs of tissue rejection.

7. The fact that DLA-identical nerve allografts can tolerate ischaemia lasting 6 hours and prolonged cryopreservation for 56 weeks without losing their capacity for regeneration, makes it worthwhile to explore the feasibility of establishing a nerve bank, in order to achieve our ultimate goal of the ideal nerve graft.

RECOMMENDATIONS FOR FURTHER RESEARCH

During the above study, many problems came to light which require further investigation and clarification. It would be desirable to carry out further research into the following problems:

1. In our experiments the maximum preservation period is 56 weeks at a constant temperature of -70°C. It might be worthwhile to try other methods of prolonged preservation, e.g. at a constant temperature of -192°C.

2. The maximum safe limit for prolonged preservation should also be determined. This could aid in discarding the preserved nerve grafts following the expiry of the safe period and thus save space unnecessarily occupied by grafts not likely to be used.

3. Attempts have to be made to assess the viability of the transplanted neural tissue. If deep-freeze conditions are employed for prolonged preservation, the viability of the tissue will probably be maintained and hence a living tissue will be transplanted. If one is sure about the viability of the nerve graft, further measures can then be taken to reduce the immunogenicity, if necessary.

4. Preoperative blood transfusions to the recipient have a beneficial effect on the immunological rejection of kidney transplants. It may be worthwhile finding out whether this also works with nerve allograft recipients. As blood transfusion is a comparatively harmless procedure, its use will not have any adverse effect on the recipient nor on nerve regeneration.

5. Immuno-suppressive drugs, such as Azathioprine, in

combination with corticosteroids have a beneficial effect on kidney transplants, as demonstrated by many studies. Whether a nerve allograft should also be submitted to such treatment, remains debatable. As nerve transplantation is not a life saving procedure, one is not justified in using such measures which could be injurious to the donor for a prolonged period.

The nerve transplant, however, probably only acts as scaffolding for the ingrowing host nerve; hence, it is possible that rejection after some months may not have any deleterious effect on the functional outcome. Thus, if short-term, low-dose immuno-suppression, e.g. combined with preoperative blood transfusions, could be used to allow a good functional end-result of mismatched grafts, it would carry only a slight risk for the recipient, and it would greatly facilitate nerve banking, as histocompatibility matching might become superfluous.
In our opinion such a trial would cause many socio-medical problems. Experimental and clinical studies should be undertaken to determine the relative importance of matching unrelated donor-recipient parts for class I and class II antigens, respectively.

SUMMARY

In chapter 1 the importance of nerve grafting in cases of unsuccessful nerve sutures, has been emphasized. The high percentage of nerve suture failures necessitates the search for an ideal nerve graft.
An ideal nerve graft for the most frequently occurring nerve defects should be the one which is easily available and easily accepted by the host without producing any significant untoward effects.
The gap in a particular nerve should probably be replaced by the same nerve segment, thus restoring the original neurolemmal pattern of the nerve. Thus, the requirements for the ideal nerve graft can only be fulfilled by establishing a nerve bank, the ultimate goal for this study, although many questions still exist.
Many studies have been performed concerning preservation of nerve grafts and different ways of circumventing the immunogenicity of nerve grafts have been studied. Conclusive results in a proper subclinical experimental model, however, are not yet available. These uncertainties have motivated the study presented and influenced the objectives put forward.

Chapter 2 summarizes the literature on nerve anatomy, the healing process of peripheral nerves after trauma and nerve transplantation data presented in the literature.
Neural as well as non-neural tissues have been used to bridge the nerve gaps. Most investigators reached the same conclusion, i.e. non-neural tissue no longer has a place in nerve transplant surgery and should be regarded as being of historical interest only. Neural tissue in the form of xenografting should also be considered to be obsolete.
The use of nerve allografts, together with the additional

beneficial effects of irradiation and use of immuno-suppressive therapy have been discussed. The importance of combating the rejection reaction of nerve allografts by matching for the Major Histocompatibility Complex (MHC) system is explained.

In chapter 3 the experimental set-up is presented. The techniques and animals used are described. Matching for MHC has been used as the sole measure for suppressing the immunological reaction in transplanted nerve grafts. In a few experimental groups, irradiation has been used in addition to matching for the MHC system. As neurological clinical examination of the dog is not a reliable criterion for interpretation of outcome of nerve allograft function, histological and electromyographical means were selected to evaluate the results. These results were interpreted by experts in their own fields who were not involved in this research programme. Finally the peroneal nerves of 70 beagle dogs were used for this study.

The results of the experiments performed are described in chapter 4. Irradiation of 4-cm nerve allografts has no beneficial effects in either DLA-identical or DLA-mismatched combinations. Certain nerve allografts of 4 cm length did show some signs of regeneration even in DLA-mismatched unrelated combinations, thus proving the finding of others that some grafts up to 4 cm in length could show a certain degree of recovery even against all odds. DLA-matching had a positive effect on regeneration of 4-cm grafts. One haplotype mismatched graft fared better than two haplotype mismatched grafts in related combination or mismatched grafts exchanged between non-related animals, thus emphasizing the importance of matching for MHC-system. Similar influence of MHC-matching was noted with 7-cm nerve

grafts. A one haplotype different related nerve allograft was slightly more successful than a DLA-mismatched unrelated graft and worse than a DLA-identical related combination. Seven centimeter nerve allografts showed good regeneration in DLA-identical combinations comparable to the 4-cm grafts. None of the 7-cm grafts in DLA-mismatched unrelated combinations showed any reasonable recovery, in contrast to 4-cm grafts in which some recovery was found under similar circumstances. This suggests that DLA-matching plays an even more important role in long nerve allograft survival. Furthermore, it was found that nerve grafts of 7-cm length in DLA-identical combinations could tolerate warm ischaemia for up to 6 hours before removal from the donors. Regeneration of these grafts was similar to that of nerve grafts removed without warm ischaemia. These grafts further could be preserved for about 56 weeks and showed the same degree of regeneration as that recorded with similar grafts stored for about 6 weeks.

In chapter 5 the importance of matching for MHC for the survival and regeneration of nerve allografts has been discussed in detail and conclusions from the study presented are drawn. Possibilities to improving the results of partly MHC-mismatched donor-recipient combinations either by using immunosuppressive therapy or pre-operative blood transfusion, have been suggested. Recommendations for further studies are put forward. The success of nerve allografts in MHC-matched combinations in dogs with cadaveric nerves, preserved for about one year, have provided the stimulus to look into the possibilities to install a clinical nerve bank as well as clinical trials.

SAMENVATTING

Zenuwtransplantatie is aangewezen wanneer een primaire of secundaire zenuwnaad niet slaagt. Hoofdstuk 1 behandelt de noodzakelijkheid - in verband met het grote aantal mislukkingen op dat gebied - tot onderzoek naar een ideaal zenuwtransplantaat.
Een ideaal zenuwtransplantaat dient aan een aantal eisen te voldoen; het dient gemakkelijk beschikbaar te zijn en goed te worden geaccepteerd door de ontvanger, zonder dat deze behandeld hoeft te worden met medicamenten met nadelige bijwerkingen.
Het defekt in een zenuw moet bij voorkeur overbrugd worden door een zelfde zenuwsegment, teneinde de oorspronkelijke neurolemmale opbouw te kunnen herstellen. Derhalve kan slechts door het oprichten van een zenuwbank aan de vereisten voor een ideaal zenuwtransplantaat worden voldaan. De verwezenlijking van zo'n weefselbank vormt het uiteindelijke doel van deze studie. Voor het begin van deze studie waren er nog veel vragen betreffende de optimale methode van langdurige preservatie en de verschillende wijzen waarop afstoting van zenuw-allografts kon worden voorkomen. Deze onzekerheden vormden de stimulans tot het huidige onderzoek. Daarom wordt in dit proefschrift nagegaan in hoeverre weefseltypering een belangrijke rol speelt bij zenuwtransplantatie. Dit zou dan een extra argument kunnen zijn voor het oprichten van zo'n bank. Bovendien werd gekeken in hoeverre zenuwweefsel enige uren na overlijden van de donor nog bruikbaar was. Hierdoor zou het eventuele donorpotentieel belangrijk uitgebreid kunnen worden. Essentieel is uiteraard ook dat het transplantaat langere tijd kan worden bewaard; ook hier werd onderzoek naar verricht.

In hoofdstuk 2 wordt de literatuur over zenuwtransplantatie

besproken. Voor het overbruggen van zenuwdefekten is zowel zenuwweefsel als ander weefsel gebruikt. De meeste onderzoekers zijn tot de conclusie gekomen dat niet-neuraal weefsel ongeschikt is als transplantaat en thans slechts van historisch belang is. Ook het gebruik van neurale xenotransplantaten is verouderd. Het gebruik van allotransplantaten, al of niet bestraald, en met of zonder postoperatieve immuno-suppressie, en de rol van histocompatibiliteit, wordt beschreven.

In hoofdstuk 3 wordt beschreven hoe in het eigen onderzoek gebruik gemaakt werd van getypeerde beagles, waarbij de nervus peroneus na 6 weken in diep gevroren toestand bewaard te zijn, werd getransplanteerd. Het belang van weefseltypering - verschil en overeenkomst tussen donor en ontvanger - voor de immunologische reaktie op zenuwtransplantaten werd bestudeerd. In enkele groepen werden de transplantaten bestraald. Histologisch en elektromyografisch onderzoek werd verricht, omdat neurologisch onderzoek bij een hond niet op betrouwbare wijze uitvoerbaar is.
De resultaten werden bewerkt door desbetreffende specialisten, die verder niet bij het onderzoek betrokken waren.

Hoofdstuk 4 beschrijft hoe bestraling van 4-cm lange zenuwallotransplantaten, onafhankelijk van de mate van histocompatibiliteit, geen gunstig effekt had. Wel was er een duidelijke relatie tussen de mate van histocompatibiliteit en regeneratie. Echter, enkele niet compatibele allotransplantaten van 4 cm lengte toonden ook enige regeneratie; korte allotransplantaten tot 4 cm lengte kunnen dus toch enig herstel tonen, ondanks dergelijke ongunstige omstandigheden.
De situatie bij 7-cm lange transplantaten was enigszins anders. Compatibele allotransplantaten van die lengte

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CURRICULUM VITAE

R. Singh was born on the 2nd of July, 1930, at Meerut, in India. He passed his M.B.B.S. (arts-diploma) examination in 1955, from the King George's Medical College, Lucknow, India. From 1955 till 1959 he received training in the Department of General Surgery and passed examination for Masters of Surgery degree in 1959, from the same institution. During the training for general surgeon, he also wrote a thesis on "Evaluation of splenic artery-ligation and splenectomy in cirrhosis of liver (clinical study)", which was accepted by the University of Lucknow, India. From 1959 till early 1963 he worked as a general surgeon at the Irwin Hospital, "attached to the Maulana Azad Medical College", New Delhi, India.
He started neurosurgical training in 1963 at the Academisch Ziekenhuis Leiden. After finishing the training he is working as a neurosurgeon since 1968 in the Academisch Ziekenhuis Rotterdam-Dijkzigt and since 1978 also in the St. Franciscus Gasthuis, Rotterdam.