

SEGMENTS of the sciatic nerve of adult rats were stored in liquid nitrogen for three weeks before being used to repair transected sciatic nerves of four other adult rats of the same strain. Eight months later the animals that had received the cryopreserved segments were sacrificed and compared with two animals that had received fresh autografts. The muscular innervation by the repaired nerves was evaluated by histological methods and electromyographic recordings. No differences between fresh transplanted grafts and cryopreserved grafts were found. This indicates that cryopreserved mature peripheral nerve segments can be used to repair peripheral nerve damage in the rat.

Key words: Nerve injury, Nerve transplantation, Neural grafting, Retrograde tracing, EMG

Cryopreservation of rat peripheral nerve segments later used for transplantation

Steen Jensen^{*ca}, Mark N. Wallace † and Benedicte Dahlerup

Institute of Neurobiology, and * Institute of Forensic Medicine, University of Aarhus, Finsensgade 15, DK-8000 Aarhus C, Denmark
† Dept. of Anatomy, University of California, Irvine CA, USA

^{ca}Corresponding Author

Introduction

The insertion of grafts into peripheral nerves has great clinical importance as a way of re-establishing motor functions after nerve injuries. Unfortunately there are several factors which restrict the use of grafts in repair. At present these problems are overcome by the use of autografts, usually with a large sensory nerve as donor tissue.¹ Another potential solution would be to freeze peripheral nerve segments after tissue typing so that they could be stored or transported for later use.

Cryopreservation has been used for several years in storing various cells and tissues including immature central nervous tissue from rats.²⁻⁷ In this study we froze mature peripheral nerve segments with a method previously used for storage of immature rat brain tissue.⁷ The segments were used later to repair motor nerve damage.

Materials and Methods

All experiments were performed on male Wistar rats (initially weighing 400 g) anesthetized with Nem-butal® (50 mg kg⁻¹ i.p.) administered before any operation or testing was started. Donor animals were killed immediately after the sciatic nerve was removed. The other animals were monitored closely during recovery from surgery and, if necessary, isolated from the other animals until fully recovered from anesthesia. The animals were killed, while anaesthetized, with an additional (lethal) dose of Nem-butal® (approximately 50 mg i.p.).

Donor tissue. Segments of the sciatic nerve (1.0 to 1.5 cm) were removed, cooled at 2°C min⁻¹, stored at -196°C for three weeks, and thawed at 15°C min⁻¹.⁷ In four recipient animals the sciatic nerve was transected and a graft inserted. In another two animals a 1.0-1.5 cm segment of the right sciatic nerve was cut

off, inverted and re-inserted into the nerve. The nerves were sutured in the epineurial sheath, with close approximation and avoidance of traction of the junctions. The covering muscle and skin were sutured. No fixation or immobilization was used. There were no postoperative infections and no antibiotics were given. **Electromyographic recordings (EMG).** Eight months after transplantation, the nerves were exposed (again under Nembutal® anaesthesia), a double-hooked stimulating electrode was placed around the nerve proximal to the graft, approximately 4 cm from the muscle, and a recording needle-electrode was placed in the gastrocnemius muscle. Recordings were obtained with conventional equipment (pulse-stimulator, amplifier, oscilloscope) as described by Hirasawa *et al.*,⁸ photographed and analysed.

Histology. Immediately after EMG-recordings, the animals were killed with an overdose of Nembutal® (see above) and perfused with a fixative (11.4% formaldehyde, 35.6 g sucrose, and 25 g MgSO₄·7H₂O) preceded and followed by a few minutes perfusion with 'rinse' (0.8 g sucrose, 0.8 g NaCl, and 0.4 g glucose in 100 ml H₂O). The spinal cord and the sciatic nerve were isolated, placed in sucrose for 24 h, frozen over gaseous CO₂, and cut in three series at 30 µm in a cryostat. The spinal cord was cut longitudinally and stained with thionine for cells, Nauta⁹ and AChE-staining¹⁰ for fibers, or mounted without staining for fluorescence studies. The graft was cut longitudinally whereas cross-sections of the proximal and distal segments of the host nerve were cut and stained for cells and fibers.

Retrograde tracing. Three weeks prior to sacrifice the animals were injected under light ether anaesthesia at two or three places in the re-innervated gastrocnemius muscle with a total of 12-14 µl Diaceturat True Blue (DTB), using a Hamilton syringe. DTB is a water-soluble derivative of True Blue which is retrogradely

transported in nerves if applied near the nerve endings.¹¹

Results

In the animals which had received a cryopreserved graft and in the controls we found a good recovery of the distal part of the operated limb down to the ankle. Distal to the ankle joint function was severely impaired. The animals were using the operated leg actively when walking although one of the animals with a cryopreserved graft showed some spasticity of the foot.

After the animals had been anesthetized, the operated leg was bimanually palpated; no significant atrophy of the muscles was found in either group.

Two animals, one with a cryopreserved graft and one of the controls had lost the three most lateral toes on the operated foot indicating, at least a temporary, loss of sensation. The control animal which had lost the three toes was the one which had made the best motor recovery of all animals investigated.

Macroscopy. Cryopreserved grafts and controls had the same macroscopic appearance. The grafted part of the nerve had the same colour as the rest of the nerve but was somewhat thickened with adherences to the surroundings. It was not possible to determine the precise junctions between graft and host nerve.

Microscopy. Nauta fiber stain showed fiber-bundles appropriately oriented along the course of the nerve in all grafts (Figs 1—3a, upper sections). The most centrally placed fibers were the best oriented ones, whereas aberrant fibers were seen in the peripheral areas.

AChE-staining confirmed the impression from the Nauta stained sections; cholinergic fibers ran through the graft (Figs 1-3a, lower sections).

Toluidine blue revealed Schwann cells arranged in rows in areas corresponding to the above mentioned fiber-rich areas. In areas without fibers Schwann cells, if present, were reduced in number and diffusely scattered. Several 'islands' of closely packed immunocompetent cells and an increased number of mast cells were found in these areas. Even in grafts judged normal macroscopically, it was found that the central core of nerve fibers was surrounded by a thickened perineurium or by fat tissue. In the autografts accumulations of immunocompetent cells were primarily found surrounding the sutures. In the cryopreserved grafts, the immunocompetent cells were again found primarily surrounding the sutures, but they were also scattered throughout the transplant.

Cross sections were taken from the host nerve within 3 mm of what was judged, macroscopically, to be the host-graft junction.

Both proximal and distal to the graft we found a well organized nerve with a normal sheath (Figs 1-3b,c). The distal cross-section consisted of two or

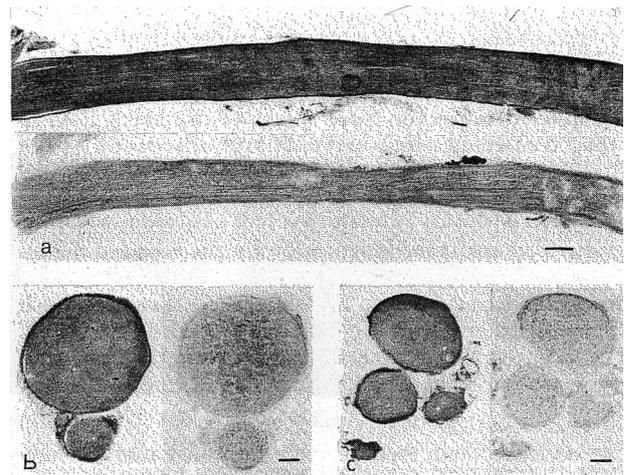


FIG. 1. (a) Nauta- (upper section) and AChE-staining (lower section) of normal sciatic nerve cut longitudinally. Bar 500 μ m. (b) and (c) show cross sections proximal and distal to the longitudinal sectioned part, respectively. Right section of each pair is stained with Nauta silver-stain; left is AChE-stained. Bar 200 μ m.

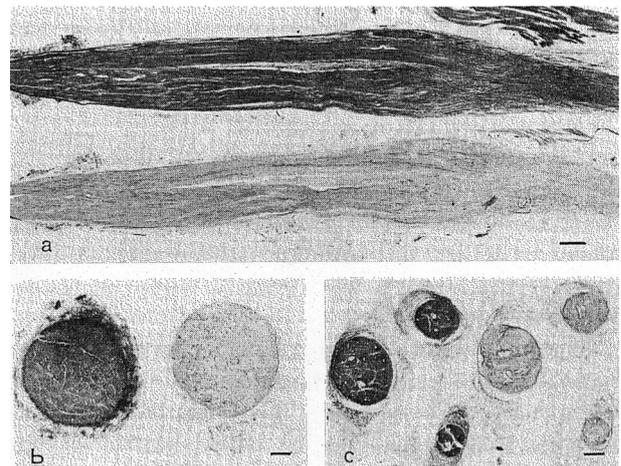


FIG. 2. (a) Nauta- and AChE-staining of autografted sciatic nerve cut longitudinally. The segment sectioned was the part which macroscopically was judged to be the graft. Bar 500 μ m. (b) and (c) show cross sections of the host nerve proximal and distal to the graft, respectively. Bar 200 μ m.

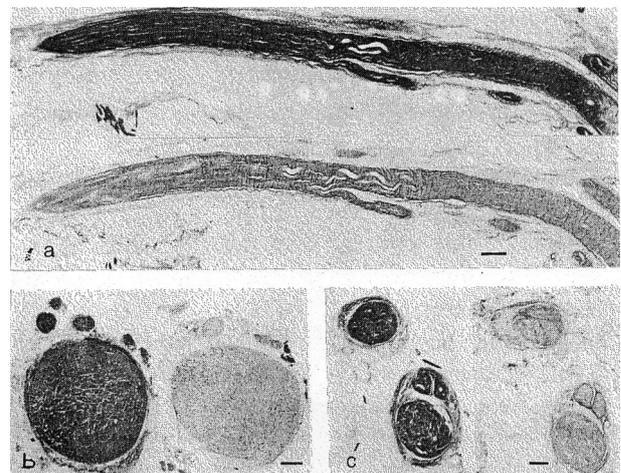


FIG. 3. As Fig. 2 but cryopreserved graft.

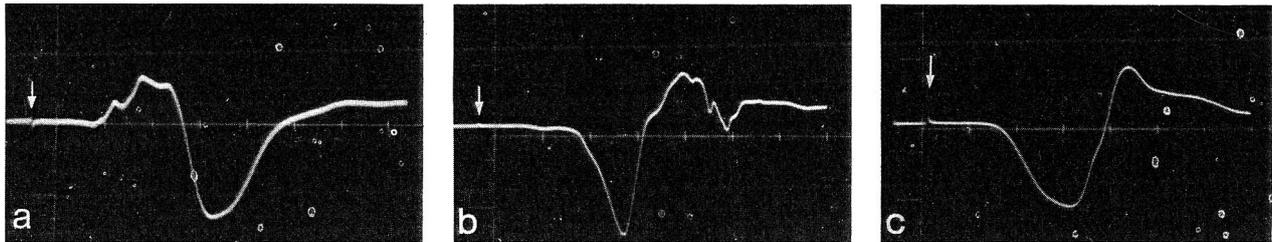


FIG. 4. Electromyograms from (a) normal sciatic nerve, (b) an autografted animal and (c) an animal receiving a cryopreserved graft. Arrow shows stimulus artifact. Total sweep 65 ms, peak to peak voltage in (a) 6 mV; (a), (b) and (c) all at same gain.

three different divisions separated by connective tissue. Each division was well ensheathed and bore a close resemblance to normal nerve. There was no sign of inflammation either in the neural sheath or in the surrounding tissue. There was no cavitation in the nerve. No fibers were found outside the sheath.

EMG. To test the functional recovery of the innervation, EMG recordings were made from the gastrocnemius muscle in both control and experimental animals (Fig. 4).

Similar, complex potentials were recorded from all muscles, indicating the activation of a number of motor units close to the recording electrode. The shape of the electromyogram remained stable for any one position in the muscle but large variations occurred when the electrode was moved between different recording sites in the muscle. As the exact position of the electrode could not be defined, the only stable parameters that could be compared in different animals were the maximum amplitude and the latency between the stimulation artifact and start of the electromyographic response. When these parameters were measured (see Table 1) we found no difference between the different groups of animals.

Retrograde labelling. Injection of DTB into the gastrocnemius muscle of the operated leg resulted in retrograde labelling of motoneurons in all experimental animals. The labelled neurons were only found ipsilateral to the injection site in the ventral horn of the lumbar spinal cord between L4 and L6, which is the area known to give rise to the sciatic nerve.¹² The distribution of labelled neurons corresponded closely to the distribution of AChE-positive neurons seen in adjacent series.

The labelled cells were seen as brightly fluorescent cell bodies lying in a column (Fig. 5). When sections from autografts and cryopreserved grafts were com-

Table 1.

	Latency (ms)	Max. Amplitude (mV)
Control ($n = 1$)	10.6 ± 0.2	7.7 ± 0.6
Fresh grafts ($n = 2$)	9.8 ± 1.9	7.6 ± 1.8
Frozen grafts ($n = 4$)	$*9.4 \pm 1.2$	10.3 ± 2.0

The table shows mean values and standard deviations for each group of animals. The values are calculated from all measurements of all animals in each group.

n = number of animals. There were three measurements taken from each animal except the control in which only two measurements were made.

* In two cases it was not possible to identify the stimulus artifact. This value is, therefore, calculated from a total of ten measurements.

pared, they were found to have a density of labelled cells similar to each other and to normal animals (compare *a*, *b* and *c* in Fig. 5).

Discussion

In peripheral nerves, the perikarya are located in the spinal cord while in the periphery there are fibers and supporting Schwann cells.¹³ If the peripheral part of the nerve suffers minor injury (neurapraxia), the nerve will be able to regenerate and the Schwann cells are essential in this process.¹⁴ If the nerve is severely injured (axotomy or neurotomy) with loss of continuity, the fibers cannot regenerate and continuity must be established operatively. If the gap is minor, the nerve can be sutured¹⁵ but if a longer segment is injured a graft should be inserted.^{15,16} For a transplant to work properly, it is essential that (i) the graft is alive,¹⁷ (ii) there is major histocompatibility between graft and host,^{18,21} and (iii) the donor nerve has approximately the same size as the damaged nerve.¹⁹ If

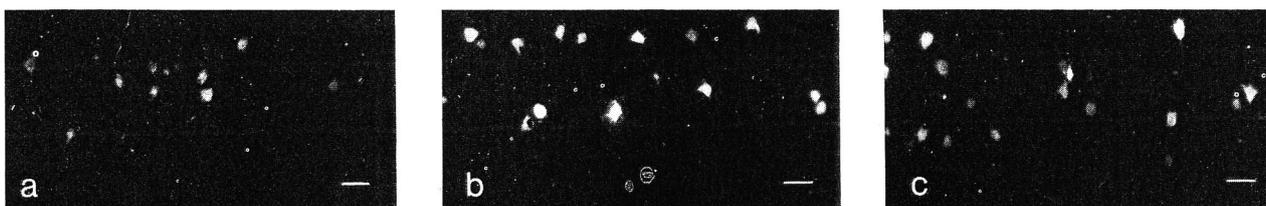


FIG. 5. Labelled motoneurons in the spinal cord in (a) normal animal, (b) autografted animal and (c) animal receiving a cryopreserved graft. Longitudinal sections. Bar 80 μ m.

these criteria are not met, a barrier will form hindering regeneration.

Rats of the Wistar strain are inbred-outbred and thus imitate the clinical situation with major histocompatibility and differences in minor histocompatibility. We wanted to see whether cryopreserved nerves would function under these conditions.

The histological sections (Figs 1-3) showed that the vast majority of fibers traversed the graft and the retrograde labelling showed that the original nerve fibers were re-innervating the target. Finally, electric stimulation and EMG recordings showed that the regenerated nerves had made functional connections.

As this was a preliminary study, no attempt was made to measure functional recovery using objective behavioural criteria. Functional recovery depends not only on the number of regenerating nerves, but rather on their ability to relocate the correct target.²² This study showed that not only did a sufficient number of fibers traverse the cryopreserved graft, but also, that the regenerating fibers were able to relocate and make connections with the correct target.

Conclusion

The results from animals with cryopreserved grafts were compared with results from autografted animals and one normal animal. Although this small number of animals does not allow statistical analysis, the

results are sufficiently consistent to conclude that, in the rat model, cryopreserved peripheral nerve segments can be used in repairing nerve injuries.

References

- Osgaard O, Husby J. *J. Neurosurg.* **47**, 751-754 (1977).
- Playfair HJL, Davies AJS. *Transplantation* **2**, 271-273 (1964).
- Kemp JA, Mazur P, Mullen Y, et al. *Transpl. Proc.* **9**, 325-328 (1977).
- Prümmer O, Raghavachar A, Werner C, et al. *Transplantation* **39**, 349-355 (1985).
- Redmond Jr DE, Naftolin F, Collier TJ, et al. *Science* **242**, 768-771 (1988).
- Houle JD, Das GD. *Brain Res.* **192**, 570-574 (1980).
- Jensen S, Sørensen T, Møller AG, et al. *J. Comp. Neurol.* **227**, 558-568 (1984).
- Hirasawa Y, Marmor L, Junge D. *Arch. Neurol.* **18**, 445-448 (1968).
- Nauta WJH. *Schweiz. Arch. Neurol. Psychiatr.* **66**, 353-376 (1950).
- Geneser-Jensen FA, Blackstad TW. *Z. Zellforsch.* **114**, 460-481 (1971).
- Bentivoglio M, Kuypers HGJM, Catsman-Berrevoets CE, et al. *Neurosci. Lett.* **12**, 235-240 (1979).
- Schrøder, HD. *J. Comp. Neurol.* **192**, 567-587 (1980).
- Geneser F. Chapter 14. Nervous Tissue. In: *Textbook of Histology*. Copenhagen: Munksgaard, 1986: 285-332.
- Angevine JB. The Nervous Tissue. In: Bloom W, Fawcett DW, eds. *A textbook of Histology 10th Ed.* Philadelphia-London-Toronto: W.B. Saunders Company, 1975: 382-383.
- Birch RJ. *Bone and Joint Surg.* **68**, 2-8 (1986).
- Jenq CB, Coggeshall RE. *Brain Res.* **364**, 45-56 (1986).
- Sanders FK, Young JZ. *J. Anat.* **76**, 143-166 (1942).
- Bucko CD, Steinmuller D. *Plast. Reconstr. Surg.* **53**, 326-331 (1974).
- Hirasawa Y, Tamai K, Katsumu Y, et al. *Transpl. Proc.* **16**, 1694-1699 (1984).
- Mackinnon SE, Hudson AR, Falk RE, et al. *Ann. Plast. Surg.* **14**, 334-339 (1985).
- Zalewski AA, Silvers WK. *J. Neurosurg.* **52**, 557-563 (1980).
- Swett JE, Wikholm RP, Torigoe Y, et al. *Anat. Rec.* **218**(1), A133 (1987).

ACKNOWLEDGEMENTS. The skilful technical, photographic and secretarial help of Aa. Lyhr, A. Meier, Th. Nielsen, M. Sørensen, and K. Wiedemann, is gratefully acknowledged. We are grateful to Prof. O. Dann, Friedrich Alexander Universität, Erlangen, West Germany who donated the Diacetatrat True Blue. Special thanks are due to Dr. J. Zimmer for his scientific help and to Dr. M. J. West for his criticism during the preparation of this paper. The study was supported by Aarhus University Research Foundation. We also wish to thank the Wellcome Trust for awarding Mark N. Wallace a Travelling Fellowship.

Resubmitted 16 October 1990;
accepted 18 October 1990