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Regeneration through a long nerve graft used in the correction of facial palsy

A qualitative and quantitative study

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Summary

A surgical technique has been developed for the correction of established unilateral palsy in man. A long (20 cm or more) sural nerve graft is anastomosed to a facial nerve branch on the unaffected side and its distal end left lying free in the cheek of the affected side. After regeneration times of 5.5–14.5 months, the distal end of the graft is joined to a free (pectoralis minor) muscle graft. In due course the muscle graft contracts in unison with the unaffected side giving near normal symmetry to facial movements. In 30 cases (ages 6–52 years) qualitative and quantitative examination was made of the distal end of grafts taken at the time of joining the graft to the muscle. Total axon counts, myelinated plus non-myelinated, confirmed abundant regeneration when compared with total axons in the supplying facial nerve; myelinated fibres remained small (mean diameter 2.5 μm) over the range of regeneration times. Quantitation included non-myelinated axons because they probably have the potential to become myelinated once the nerve is functional. Numbers of regenerating axons were not correlated with age, nor with regeneration time. Lack of a distal connection did not appear to lead to secondary degeneration of the regenerated myelinated fibres. These were maintained in an 'immature' state for many months. This observation is of practical interest since it has been suggested that delayed connection to the distal target may have a deleterious effect on the outcome of the procedure.

Keywords: facial palsy; nerve graft; nerve regeneration; axons; quantification

Introduction

A technique has been developed for reanimation of the face in cases of established unilateral facial palsy, using nerve and muscle grafts in a two-stage operation (Harrison, 1990). In the first operation a long (20 cm or more) sural nerve graft is anastomosed to the buccal branch of the facial nerve on the unaffected side of the face. The graft is tunnelled across the face above the upper lip and its distal end is left lying freely in the preauricular region of the affected side of the face. After confirmation of a Tinel sign at the distal end of the sural nerve graft, a second operation is performed. The pectoralis minor muscle is transferred to the cheek of the affected side of the face, and the facial artery and vein, and the distal end of the sural nerve graft are anastomosed to it. In due course the muscle contracts in unison with the unaffected side giving near normal symmetry to facial movements in a high proportion of cases (Fig. 1).

This two-stage operation provides a unique opportunity to observe nerve regeneration through a long nerve graft in man *in vivo*. Before anastomosis to the muscle, a portion of nerve from the distal end of the cross facial nerve graft was removed for examination. In addition the buccal branch of the facial nerve was examined as the supplying nerve for the graft.

Some specific aspects of this procedure were investigated: in particular whether there is evidence of age differences in the efficiency of regeneration as judged by quantitative assessment of numbers of myelinated and unmyelinated axons; also, since the second operation was often performed some time after the recognition of a positive Tinel sign, whether this delay might affect the graft adversely.

Material and methods

The study was approved by the District Ethical Committee. Thirty patients with established unilateral facial palsy of

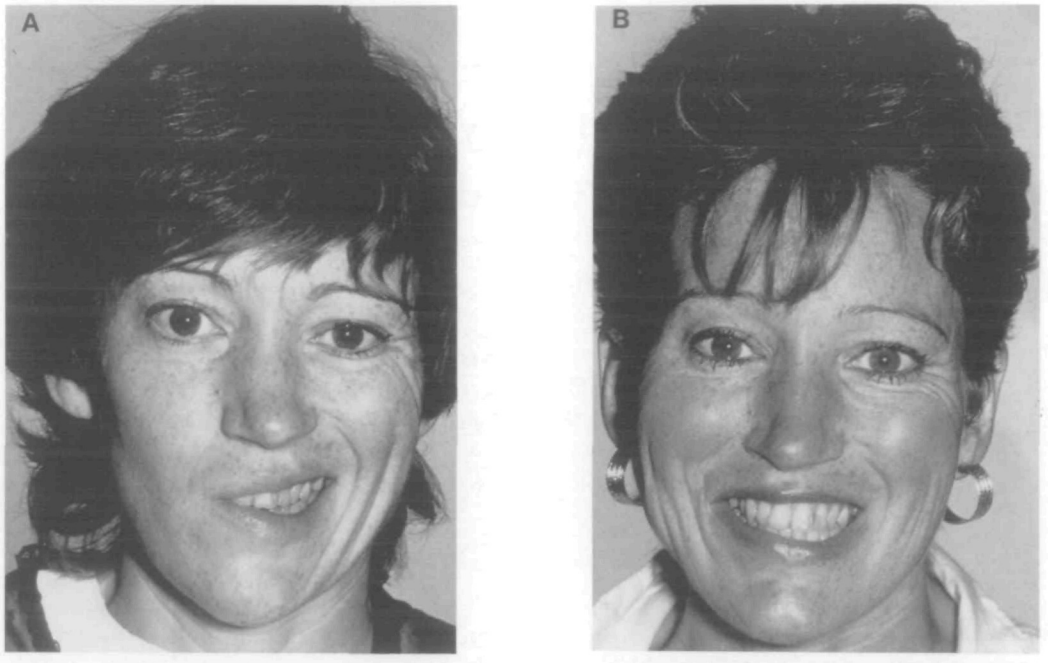

Fig. 1 (**A**) A patient with right-sided facial paralysis following facial nerve section during surgery for an acoustic neuroma. (**B**) The patient 18 months after nerve grafting and right-sided pectoralis minor reconstruction.

various aetiologies (33% congenital, 27% following surgery for acoustic neuroma) were studied. The duration of palsy ranged from 8 months to 42 years (mean 9.1 years: <5 years 30%, 5–10 years 30%, >10 years 40%). Consent was obtained in all cases.

Figure 2 illustrates the procedure diagrammatically. Biopsies from the buccal branch of the facial nerve and the distal end of the graft were treated similarly. In some cases a biopsy was taken of the sural nerve used for the graft.

Immediately on removal, each biopsy was straightened on a piece of card, allowed to adhere to the card and then placed in fixative consisting of 3% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4. The biopsies remained in fixative at 4°C for several days before being cut into thin (~1 mm) segments for post-fixation in osmium tetroxide and processing by routine methods into Araldite resin. The distal end of the graft was often distorted and therefore sections were taken from the cut (proximal) end, representing the approximate level at which the graft was joined to the muscle. Semi-thin and ultra-thin sections were cut for light and electron microscopy, respectively. Semi-thin sections were stained with methylene blue, azure II, basic fuchsin.

The distal ends of some grafts were transferred to formal saline and embedded in paraffin wax. Semi-serial longitudinal sections were cut and stained alternately with haematoxylin and eosin, and with the Glees and Marsland stain for axons in order to observe the manner in which the nerve fibres terminate.

Quantitative methods

These are similar to those described in a previous paper (Jacobs and Love, 1985).

Myelinated fibre counts

These were counted directly from the microscope using ×40 or ×100 oil immersion objectives, a squared eyepiece graticule and a hand counter. All myelinated fibres were counted in grafts and in facial nerves. From previous studies it has been found that an axon with one or two myelin lamellae can be recognized as a myelinated fibre by light microscopy provided that adequate magnification is used.

Fascicular areas

These were measured at a microscope fitted with an extension tube positioned above a bit pad. Using a cursor with a light-emitting diode whose image was visible in the microscope, the inner layer of the perineurial sheath was traced to measure fascicular area. The magnification was calibrated from a slide micrometer.

Unmyelinated axon counts

Representative areas were selected from semi-thin sections of grafts and facial nerves for ultrathin sectioning. Micrographs covering an area of at least 0.02 mm^2 were taken at a final magnification of about ×8000–10 000 (accurately calibrated

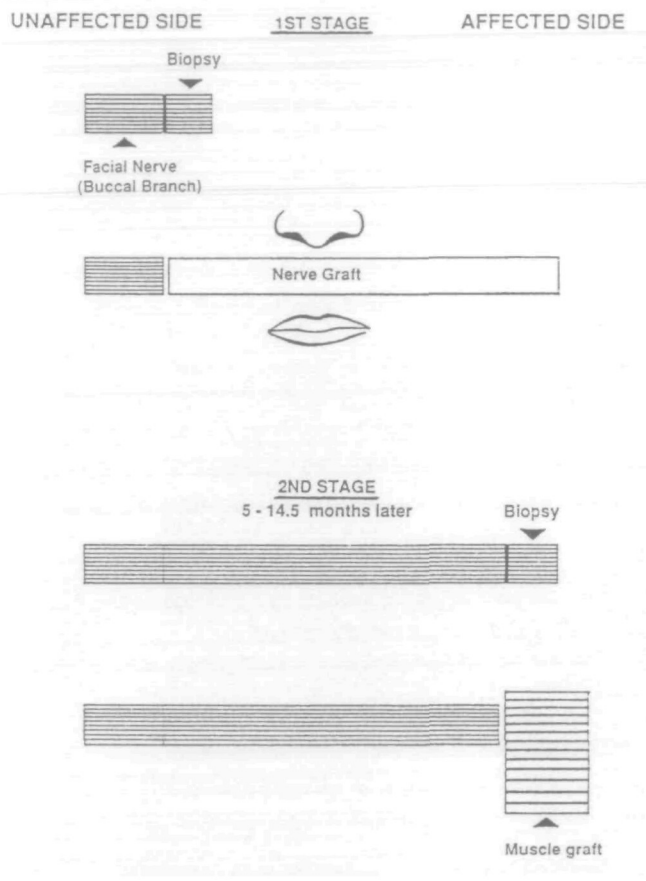

Fig. 2 Diagram of operating procedure. In many cases, biopsies were taken of the normal buccal branch of the facial nerve supplying the graft; sometimes a biopsy was taken of the sural nerve at the first stage operation (not indicated in the diagram). The sural nerve graft was left unattached in the cheek of the affected side of the face. At the second stage operation, a biopsy was taken from the graft just before its anastomosis to the pectoralis minor muscle graft.

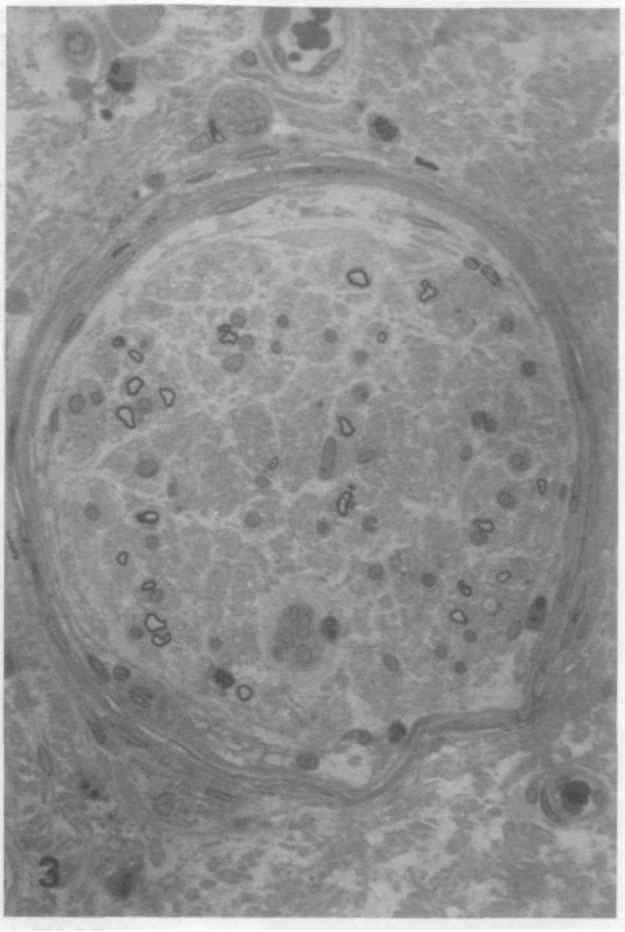

Fig. 3 Semi-thin resin section of graft after 14 months regeneration. A small fascicle is shown, with numerous small myelinated fibres. Endoneurial oedema is seen within the fascicle and subperineurially. ×560.

using a graticule at the time of each microscopy session) and axon densities were calculated. Criteria used to identify unmyelinated axons were: (i) entire or almost complete encirclement by Schwann cell processes; (ii) often clearly rounded profiles; (iii) often with more microtubules than Schwann cell processes; (iv) with no ribosomes or rough endoplasmic reticulum. The main difficulty lay in distinguishing the very smallest processes either as axons or of Schwann cell origin. Where there was any doubt these very small structures were excluded from the counts, therefore there may be some underestimation of numbers in some cases. Total numbers of unmyelinated axons were obtained from measurements of fascicular areas.

Results

Qualitative findings

Buccal branch of the facial nerve (the supplying nerve)

This usually consisted of one or two larger fascicles and one or two much smaller fascicles, although there was some variation in fascicular arrangement. The distribution of fibre size was uneven with most of the small fibres tending to be found in one of the larger fascicles. Electron microscopy showed small collections of unmyelinated axons.

Grafts

The general architecture of the fascicles closely resembled that of the original sural nerve used for the graft (Fig. 3); nerve fibres were only seen within the fascicles.

A first impression of many of the grafts was that the total fascicular area was large, and much of this area was taken up by fluid. In the staining method used, this endoneurial compartment (clearly differentiated from red-staining collagen) varies in hue from almost colourless to a darker blue indicating the presence of proteinaceous material. Only in a few cases was there marked collagenization of the graft. When it occurred it was patchy, affecting perhaps one of a number of fascicles; there appeared to be less regeneration in the more heavily collagenized fascicles.

Blood vessels were numerous and often of considerable size. The perineurium appeared normal, although occasionally

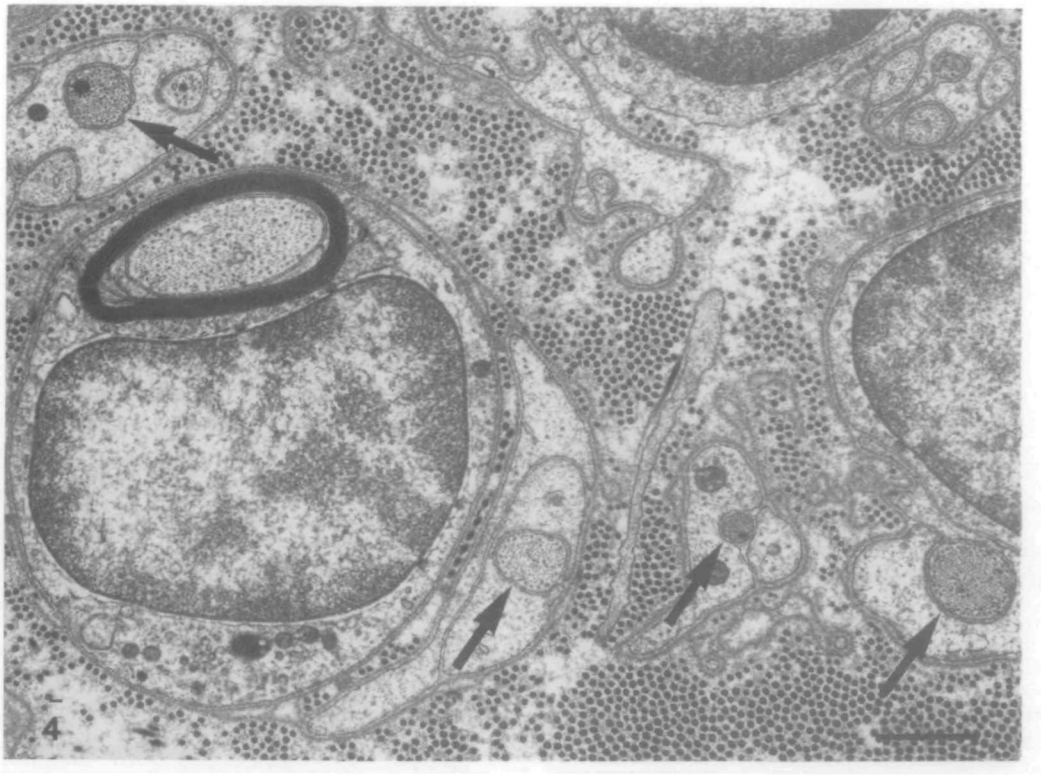

Fig. 4 Electron micrograph of graft after 11 months regeneration showing a small myelinated fibre and several unmyelinated axons (arrows). Non-staining endoneurial fluid, sometimes with a finely granular component, is evident. Bar = 1 μm.

there was increased thickness of this sheath due to the presence of larger amounts of collagen.

Myelinated fibres were small and mostly single (Fig. 4) with only occasional small clusters. Myelin debris was seen only very occasionally.

Electron microscopy

Because of the prominent fluid content of many nerves the structure of the blood vessels and perineurium was examined for evidence of features suggesting a change in blood–nerve barrier function. Endothelial cells sometimes displayed many pinocytotic vesicles, but no fenestrations were seen, nor open junctions between endothelial cells. There was reduplication of basement membranes round some larger vessels perhaps suggesting growth of new vessels within the basement membrane of original vessels. Some 'closed' vessels without a lumen were possibly newly produced. The basement membrane of perineurial cells often appeared thick but in other respects the cells were normal and tight junctions could be identified between the cells. In some grafts granular material was seen between endoneurial collagen fibrils; this has the appearance of protein containing fluid seen in regions without a blood–nerve barrier such as the endoneurium of dorsal root ganglia (Jacobs *et al.*, 1976) and confirms the blue staining material in semi-thin sections.

In addition to small myelinated and unmyelinated axons (Fig. 4), there were occasional promyelin fibres (Fig. 5). This

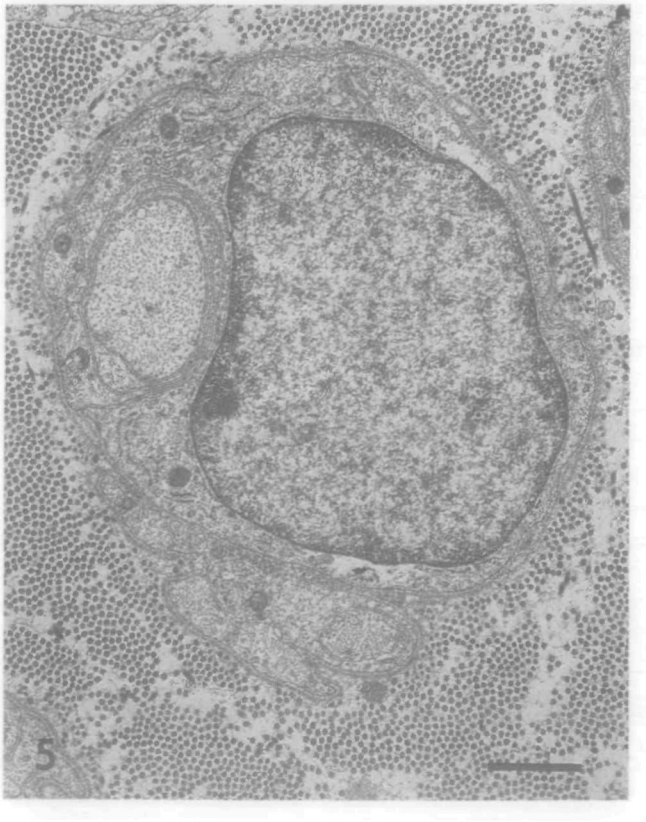

Fig. 5 Electron micrograph of graft after 11 months regeneration. A promyelin fibre is shown with about one and a half 'turns' of mesaxon. Bar = 1 μm.

Table 1 *Quantification of normal buccal branch of facial nerves (means±SD)*

	Total myelinated fibres	Total unmyelinated axons	Total myelinated fibres and unmyelinated axons	Fascicular area (mm^2)
Mean	1736	3853	5589	0.285
±SD	784	2216		0.138
n	11	9		10

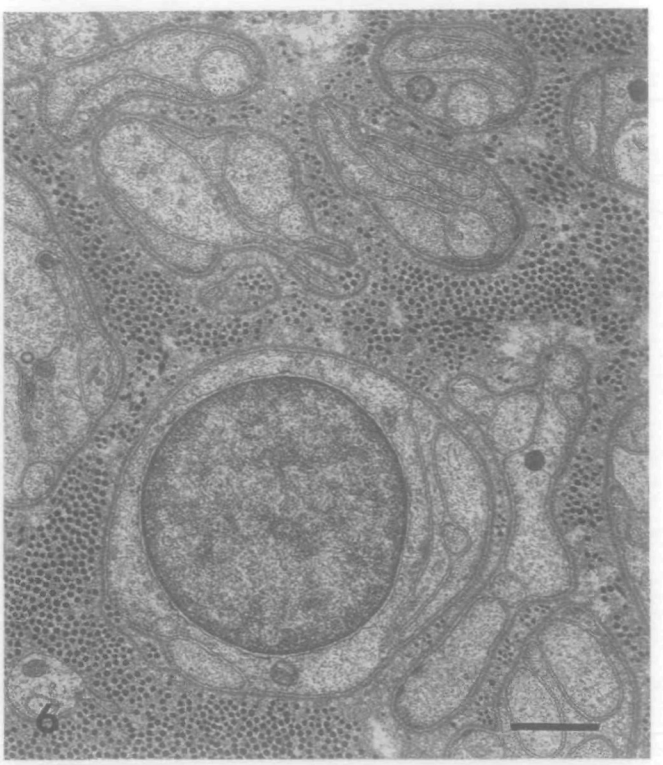

Fig. 6 Electron micrograph of graft after 9 months regeneration showing Schwann cell processes without axons. Finely granular, proteinaceous oedema is seen in the endoneurium, particularly between the collagen fibres. Bar = 1 μm.

suggests that myelination is a slowly continuing process even after long periods of regeneration and emphasizes the necessity of considering both myelinated and unmyelinated axons in a study of this type. Schwann cell processes unassociated with axons were seen quite often (Fig. 6). Very occasionally, only strands of basement membrane remained, indicating some loss of Schwann cells.

Although the interval between the first and second stage operations (the regeneration time) varied from 5.5 to 14.5 months, all biopsies had essentially similar appearances. In grafts with the longest regeneration times (14.5 months) myelinated fibres appeared to be maintained at a similar stage of regeneration to that of the grafts with the shortest regeneration times (5.5 months). There was no evidence of axon degeneration in the longer surviving grafts, and observation of the myelinated fibres showed no evidence of the axonal atrophy that might be expected in fibres surviving for a long time with no distal target.

Quantification

Buccal branch of the facial nerve

Table 1 shows the mean number and SD of myelinated and unmyelinated axons, total axons and fascicular areas in this nerve supplying the graft. Measurements of myelinated fibre size showed the bimodal distribution expected in a motor nerve.

Sural nerve

The mean and standard deviation of total numbers of myelinated fibres in seven sural nerves used for grafting was 6271±1205. These figures are similar to those from a similar age-range (mean = 5901±1080, $n = 7$) in a previous study of normal human sural nerves (Jacobs and Love, 1985). Figures for total unmyelinated axons in the same study ranged from 18 000 to 25 000. The mean fascicular area of 16 sural nerves used for grafts in the present study was 0.64 mm^2±0.14 mm^2.

Grafts

Table 2 shows the results of 30 grafts and Table 3 the mean ±SD of myelinated fibres (426±437), of unmyelinated axons (11 043±7790), and mean total axons 11 469, i.e. about double the total number of axons in the supplying buccal branch of the facial nerve. Fascicular area of the graft (mean = 0.70 mm^2±0.26 mm^2) was seen in many cases to exceed the range of fascicular areas of the sural nerves.

For an indication of the total regenerative response, myelinated and unmyelinated axons have been added together. The numbers are seen to vary considerably. There is no clear evidence that age is a factor in determining the extent of regeneration, although the situation is complicated by the different regeneration times. However, there is no indication that more axons are produced with increasing time of regeneration. Multiple regression analysis showed no correlation between axon number and age, or interval of regeneration.

Mean diameter of myelinated fibres in short- and long-term regeneration grafts was ~2.5 μm, with a maximum of just over 3 μm. It is of interest that the highest count of regenerated axons was found in the nerve graft from a patient with neurofibromatosis.

In one case, a graft which had been functional for 5 years, was infiltrated with lignocaine at about its mid-point during

Table 2 *Quantification of nerve grafts*

Age (years)	Sex	Total myelinated fibres	Total unmyelinated axons	Total myelinated fibres and unmyelinated axons	Area of graft (mm^2)	Interval between graft and biopsy (months)
4	M	70	6192	6262	0.60	8
5	F	166	8549	8715	0.47	9
6	M	1067	13756	14823	0.53	14.5
8	M	128	5498	5626	0.79	8
8	F	157	9604	9761	0.57	8
10	F	873	16794	17667	0.56	10
11	F	290	16463	16753	0.50	9
11	F	658	12955	13613	1.12	9
14	M	65	3778	3843	0.57	9
15	F	379	16670	17049	0.78	8
17	F	21	2164	2185	0.32	9
18	M	445	8238	8683	0.58	10.5
21	M	2143	39105	41248	1.20	14
23	F	699	12266	12965	0.85	13
26	M	398	5877	6275	0.46	12
27	F	265	20185	20450	0.88	5.5
28	F	803	16678	17481	0.80	12
31	M	118	8841	8959	0.91	9
38	F	263	8380	8643	0.44	11
39	M	29	4418	4447	0.88	9
39	F	243	4276	4519	0.72	11
39	F	139	25058	25197	1.07	9
40	M	353	9130	9483	0.84	9.5
43	F	902	12167	13069	1.22	14
47	M	650	10837	11487	0.96	13.5
48	M	33	793	826	0.49	8
48	M	600	11434	12034	0.40	10
50	M	509	13001	13510	0.57	10.5
51	M	148	1524	1672	0.37	13.5
52	F	177	6655	6832	0.51	11

Table 3 *Means±standard deviation of quantitative data from nerve grafts*

	Total myelinated fibres	Total unmyelinated axons	Total myelinated fibres and unmyelinated axons	Fascicular area (mm^2)
Mean	426	11043	11469	0.70
±SD	437	7790		0.26
n	30	30	30	30

an unrelated operation, resulting in degeneration of all the nerve fibres distal to that level. This necessitated a repetition of the entire two-stage operation giving an opportunity to examine the proximal (undamaged) end of what had been a functioning nerve graft. The fascicles were packed with large regeneration clusters of well myelinated fibres with a maximum diameter of 13 μm (Fig. 7).

Examination of semi-serial paraffin-embedded sections through the extreme distal ends (or neuromas) of two grafts showed little evidence of substantial disorganized growth of axons. A few fascicles appeared to open out at their distal ends, with increasing endoneurial spaces. No fibres were seen growing outside the boundary of the fascicles.

Discussion

The present study has shown that large numbers of axons regenerate through a long, unconnected nerve graft. A relatively small proportion are myelinated and these remain of small size. However, very large numbers of unmyelinated axons regenerate, and evidence from a functional graft suggests that many of these have the potential to become myelinated once there is a distal target.

Two-stage cross-facial sural nerve and muscle grafts have been used by other groups to correct long-standing facial palsy. A brief description of the morphology of five grafts was published by Vedung and Olsson (1982). The grafts used were shorter (12–14 cm) than those used in the present study.

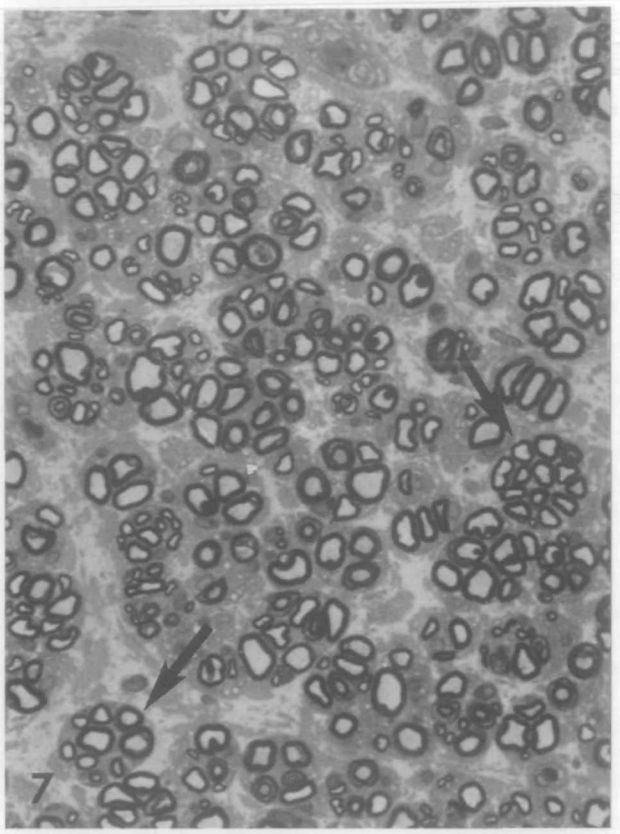

Fig. 7 Semi-thin resin section of graft that had been functional for 5 years. Large regeneration clusters are seen (arrows). The maximum fibre size is >13 μm. ×486.

At the level of the anastomosis to the free muscle graft (palmaris longus or extensor digitorum brevis), after 4–13 months regeneration, the graft contained many unmyelinated axons and some small myelinated fibres. No quantitation was done and there was no clinical follow-up of the cases.

Frey *et al.* (1991) examined nerve grafts from seven cases after 10–14 months regeneration. (The gracilis was used as the free muscle graft.) The distal end of the graft contained 32–251 small myelinated fibres. Unmyelinated axons were not counted. The numbers of myelinated fibres in the graft and sural nerve were smaller than those found in the present study. The morphometric studies were made at a relatively low magnification which may have resulted in underestimation of the numbers.

A few experimental studies have been made on open-ended autologous nerve grafts. Nadim *et al.* (1990) in a study on rats compared 8 weeks regeneration through long (40 mm) acellular (freeze-killed) nerve grafts with fresh grafts. Axon regeneration in acellular grafts only extended for 10–20 mm (attributed to the limited migration of Schwann cells from the proximal nerve stump) but in normal grafts abundant regeneration was found, although no description was given of the distal end of the 40 mm graft. At the midpoint large numbers of well-developed myelinated fibres were seen.

In a study on rabbits, Shibata *et al.* (1991) compared one- and two-stage nerve grafts in order to test the potential benefit of delaying anastomosis of the distal end of the graft and removing a length of 'scar' tissue before coaptation to the distal nerve stump. No description was made of the severed end of the nerve graft, so no proof was provided that the distal region was in fact fibrosed. Experience from the present study suggests that, at least in man, there is relatively little fibrosis at the distal end of the graft. In the Shibata *et al.* (1991) study, no significant differences were found between one- and two-stage grafts when assessed by electrophysiological tests and by myelinated fibre counts.

Frey *et al.* (1992) used long (28–30 cm) unconnected, autologous nerve grafts in a study using sheep. Ipsilateral and contralateral grafts were examined at periods from 3 to 18 months. Numbers of myelinated fibres at the distal end of the graft usually well exceeded that of the supplying motor nerve, and median fibre diameters reached almost 5 μm in some cases. Fibre numbers showed much variability, but generally, and for reasons not understood, contralateral grafts did less well than ipsilateral grafts. Myelinated fibre numbers in the contralateral grafts decreased with increasing regeneration time. Frey *et al.* (1992) concluded from these studies that in man, the second stage operation should be performed without delay once the Tinel sign showed that regenerating fibres had reached the distal end of the graft.

In this, and other experimental studies on nerve regeneration through long unconnected nerve grafts, it appears that a far larger proportion of fibres become myelinated than is the case in the human grafts, and these fibres become larger than those in the human grafts. Therefore extrapolation from animal studies may not always be justified. There are a number of studies of nerve regeneration through long, unconnected nerves following crush injury. This situation differs from that in which nerves are cut and then joined, but it is interesting that even without a distal target, large numbers of myelinated fibres are found which, in rabbits (Aitken *et al.*, 1947; Aitken, 1949), attain a mean diameter of 8 μm and more. The situation regarding non-myelinated axons in the experimental studies is not known.

Some comments should be made on the qualitative examination of the human grafts. These were well vascularized; in experimental studies on the revascularizarion of isolated nerve grafts placed into musculature (Penkert and Samii, 1990), it was found that new vessels entered laterally (through the perineurium) rather than through the cut ends of the nerve, with the implication that the length of the graft is not a limiting factor to successful revascularization. Experimental studies (Kanaya *et al.*, 1992; Mani *et al.*, 1993; Ozcan *et al.*, 1993) indicate that non-vascularized nerve grafts were not markedly inferior to vascularized grafts even when the graft bed was avascular.

Fascicular areas of the grafts were often greater than those of the original sural nerve due mainly to the presence of increased endoneurial fluid or oedema. During nerve regeneration, the blood–nerve barrier becomes permeable (Mellick and Cavanagh, 1968; Sparrow and Kiernan, 1981). It has been postulated that increased permeability is due to

the production of vasoactive substance(s) at the growing axon tips (Sparrow and Kiernan, 1981). Unconnected axons, even if they are not still actively regenerating might continue to produce vasoactive substances leading to increased entry of fluid into the nerve.

Alternatively, or in addition, the 'open-ended' graft might also be a point of entry for fluid from external sources. Whatever the source it seems possible that this fluid may contain 'growth' or 'maintenance' substances that help to sustain the regenerated, but unconnected, axons for a considerable length of time. The importance of Schwann cells in axonal regeneration through long nerve grafts has been demonstrated by Nadim *et al.* (1990), and through muscle grafts by Feneley *et al.* (1991) and Enver and Hall (1994). Whether Schwann cells are involved in the 'maintenance' of the long-term regenerating grafts is not known. Schwann cell processes without axons were seen in all grafts, suggesting that Schwann cells may persist for long periods without axonal contact. In the rat, Schwann cells isolated from axons for 58 weeks (Weinberg and Spencer, 1978) were found to have largely disappeared.

With regard to the quantitative studies, no correlation with age was identified in the assessment of nerve fibre regeneration in the present study, although, in experimental studies (Tanaka *et al.*, 1992; Campbell and Pomeranz, 1993), nerve fibre regeneration was found to be less efficient in older animals.

The length of time between first and second operations ranged from 5.5 to 14.5 months. There is no clear evidence of either an increase or a decrease in numbers of regenerating axons with increasing regeneration time. Shorter term grafts were indistinguishable in appearance from longer surviving grafts. There was no indication of axonal atrophy (in myelinated fibres) nor of degeneration of regenerated fibres that might be expected in a nerve during a prolonged period without a distal connection. For example, in nerves proximal to an amputation, axons become atrophic with eventual centripetal nerve fibre degeneration (Dyck *et al.* 1984).

As stated above, Frey *et al.* (1992) concluded, from their experimental study, that there should be the minimum delay between the presence of a Tinel sign to signal the arrival of regenerated fibres at the distal end of the graft, and performing the second stage operation. Our results suggest that in man there is no obvious deterioration of regenerated axons up to 14.5 months after the first operation; this may be several months after the appearance of a Tinel sign. There appears to be no effect of delay upon the eventual functional outcome of the procedures, which, when assessed for static and dynamic symmetry at follow-up produced 100% functioning muscles with 90% good or excellent dynamic and static position.

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