Nerve Regeneration through Cryo-treated Xeno-

geneic Nerve Grafts

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Summary. Cryo-treated nerves whose Schwann cells had been killed by repeated freezing and thawing were xenogenically grafted into sciatic nerves from rats (Wistar, as donor) to mice (ddy strain, as recipient) to examine whether Schwann cell basal lamina tubes of cryo-treated xenogeneic grafts were effective conduits for regenerating axons. For comparison and evaluation of the effectiveness of this technique, experiments using grafts without the cryo-treatment were carried out.

Cells in cryo-treated xenografts degraded into cell debris immediately after grafting and then were phagocytized by macrophages. After the cellular components had been removed from the graft, Schwann cell basal laminae remained intact in situ, serving as conduits for the regenerating axons. The process of nerve regeneration was almost the same as that observed in cryo-treated auto- and allografts, except that the regeneration was slightly delayed in the xenogeneic graft. In contrast, an extensive cell infiltration occurred in the non-treated grafts. It appeared that the donor's Schwann cells in the graft deteriorated due to immunological reactions and were finally eliminated by macrophages, leaving their basal laminae undamaged in situ. The initiation of nerve regeneration including perineurial sheath formation in non-treated grafts was, therefore, significantly delayed, but once begun, it proceeded in the same manner as in the cryo-treated grafts.

These findings strongly indicate that Schwann cell basal laminae can serve as effective pathways for regenerating axons even in the xenograft. Moreover, cryo-treated xenogeneic grafts are more desirable than non-treated ones, since dead Schwann cells in the former can be removed in the early period (4-14 days) from the graft without causing any immunological reaction, thus resulting in the facilitation of nerve regeneration.

In our previous studies on nerve regeneration, auto- or allogeneic nerve grafts were used after they had been treated by freezing and thawing (cryo-treated). Schwann cells in the grafts having undergone such treatment degenerated and were eventually removed by macrophages, leaving their basal laminae intact in situ. Such basal lamina scaffolds very probably become conduits in nerve regeneration, since it has been observed that most of the regenerating axons grow out through such basal lamina scaffolds (IDE, 1983; IDE et al., 1983).

Concerning nerve allografts, experimental studies have been made in an attempt to obtain successful results using immunosuppressives (POLLARD et al., 1971; POLLARD and FITZPATRICK, 1973 a, b; ZALEWSKI and GULATI, 1981, 1984), or pretreatments such as denervation (DAS GUPTA, 1967; DUCKER and HAYES, 1970; BUCKO and STEINMULLER, 1974; POLLARD and McLEOD, 1981) or irradiation (MARMOR, 1964;
DUCKER and HAYES, 1970; POLLARD and FITZPATRICK, 1973 a, b; BUCKO and STEINMULLER, 1974; HIRASAWA et al., 1974; SINGH, 1976; SINGH et al., 1977). It has been recognized that these treatments improve to some extent the nerve regeneration in allogeneic grafts. In a previous study using sciatic nerve grafts between two different strains of mice, C57BL/6N and C3H/HeN, we demonstrated that Schwann cell basal lamina tubes serve as effective conduits for regenerating nerves even in the allograft (OSAWA et al., 1986). Judging from the fact that basal laminae and collagen fibrils were not phagocytized by macrophages, but left apparently intact in the allogeneic grafts, it can be assumed that there were no significant differences in the components of these extracellular substances in the above two strains of mice, and that little or no immunological reaction to those substances occurred between these strains.

There have been only a few studies on xenogeneic nerve grafting. AGUAYO et al. (1977) and AGUAYO and PRAY (1980) reported that human Schwann cells in the graft could myelinate on immuno-suppressed host mouse axons, whereas after the discontinuation of the injection of the immuno-suppressive, the human Schwann cells were rejected. ROSENBERG and APPENZELLER (1980) mentioned that xenogeneic Schwann cells could produce myelin sheaths on axons of immuno-suppressed host animals to some extent; however, the myelination was delayed and myelin sheaths were thinner than normal. Eventually, the grafts underwent a rejection after ceasing the administration of immuno-suppressives. DUNCAN et al. (1981) also reported that cultured rat Schwann cells implanted into mouse spinal cord were immunologically rejected when no immuno-suppressive agents were used. TANG and BERNSTEIN (1986) studied the xenogeneic grafting of chick neocortex to the dorsal column of the rat, showing that chicken cells were rapidly rejected. In general, it can be said that xenogeneic nerve grafts do not survive, but are eventually rejected from the host animals due to immunological reactions.

In the present study, we examined whether the xenogeneic grafts which had been treated by freezing and thawing could be as effective a pathway as cryo-treated autologous or allogeneic grafts for the outgrowth of regenerating axons. Since regenerating axons require basal lamina tubes as conduits for their outgrowth, if the basal laminae of Schwann cells could survive immunological rejection in xenogeneic grafts, it would be expected that cryo-treated xenogeneic nerve grafts might be effective for nerve regeneration.

MATERIALS AND METHODS

A total of 10 adult male rats (Wistar) and 50 male mice (ddy strain) were used as the donors and recipients, respectively. Nerve segments about 3 cm long were excised from the left sciatic nerve of the donor rats. These nerve segments were treated five times to repeated freezing (ca. –20°C) and thawing (ca. 5°C) to kill the Schwann cells prior to grafting, and then were cut into small segments approximately 6–7 mm long. In the recipient mice, a nerve segment about 5 mm long was removed from the left sciatic nerve near the outlet from the pelvis, and a nerve segment from the donor rat prepared as described above was transplanted in the gap. Both the proximal and distal ends of the graft were sutured to the host sciatic nerve with one stitch of 9-0 silk thread. Two to six animals were sacrificed at each of the following time points: 4, 7, 14, 20, 50, 70 and 100 days after grafting. They were anesthetized with Nembutal (sodium pentobarbital, 30 mg/kg body weight) and fixed by perfusion through the heart.
with a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4).

The grafts were excised together with parts of the recipient sciatic nerve. In addition, the toe pads and lumbrical muscles of the operated limb were excised to examine whether regenerating axons could reinnervate their targets, i.e., sensory corpuscles and motor endplates. Specimens were stored in the same fixative for 24 hrs at room temperature, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C, dehydrated through a series of graded ethanols, and embedded in Epon 812. Thin sections were cut with a diamond knife on an LKB Ultrrotome and stained with uranyl acetate and lead citrate. They were observed with a Hitachi H-700 transmission electron microscope. Semithin sections 1 μm thick were stained with toluidine blue and observed by light microscopy.

In another experiment, the nerve grafting was performed between rats (Wistar) and mice (ddY) using the same procedure as above, except the grafts were not pretreated by freezing and thawing, i.e., the Schwann cells in these grafts were kept alive. Two to four animals were sacrificed at 4, 7, 14, 20, 50 and 70 days post-grafting. The specimens were observed in the same manner as above.

RESULTS

Cryo-treated grafts

The degeneration and subsequent regeneration of nerve fibers in the grafts could be followed in semithin sections by light microscopy (Fig. 1a-f). Myelin sheaths exhibited the initial changes of degeneration, i.e., the disintegration of myelin sheath lamellae (Fig. 1a). The degeneration of myelin sheaths rapidly advanced until all the myelin sheaths had degraded into “myelin balls” by day 7 after grafting. At the same time, these degenerated myelin sheaths were gradually phagocytized by macrophages (Fig. 1b); they had been completely removed and disappeared from the graft by day 20 post-grafting (Fig. 1d). Some axons which appeared to be regenerating were observed at 7 days after grafting (Fig. 1b). Clearly regenerating axons growing in a bundle could be found by 14 days after grafting (Fig. 1c). These regenerating axons had been mostly myelinated and grouped into small fascicles by days 20 after grafting. Such myelinated nerves gradually increased in number as well as in size; this grouping of nerves fascicles became obscure 50 days after grafting, and remained so even up until day 70 (Fig. 1f).

By electron microscopy it was clear that myelin lamellae of all the myelinated fibers were being disintegrated 4 days post-grafting. All the Schwann cells of nerve fibers were destroyed, and no other living cells were seen in the grafts. Thus the cryo-treatment was effective in killing all the cellular elements in the graft, while retaining noncellular elements such as endoneurial collagen fibrils and apparently normal Schwann cell basal laminae in situ (Fig. 2).

Seven days after grafting, all the degraded myelin sheaths were in the process of being phagocytized by macrophages. Macrophages did not attack Schwann cell basal laminae which were left in the form of tubes in situ. Regenerating axons surrounded by immature Schwann cell processes (Fig. 3) were already found in some of these basal lamina tubes. This stage of nerve regeneration 7 days post-grafting was somewhat delayed as compared with auto- or allografts.
Fig. 1. Cryo-treated grafts. These light micrographs show nerve regeneration 4–70 days after grafting. 

a. Four days after grafting. All the myelin sheaths are being disintegrated.

b. Seven days after grafting. Degenerating myelin sheaths appear as “myelin balls” which are presumed to be phagocytized by macrophages. bv blood vessels.

c. Fourteen days after grafting. “Myelin balls” are further degenerated, and unmyelinated regenerating nerves are found extending in fascicles of various sizes (arrows).

d. Twenty days after grafting. Myelin sheath debris is no longer seen. Regenerating axons are separated by flattened cells into fascicles of almost the same size, and many of such regenerating axons are myelinated. bv blood vessels.

e. Fifty days after grafting. Regenerating axons become thicker and myelinated fibers increase in number. Fasciculation of regenerating nerves become less distinct.

f. Seventy days after grafting. The situation is almost the same as that of 50 days after grafting. ×340
Fourteen to 20 days after grafting, axons and accompanying Schwann cells, which were extending through basal lamina tubes, markedly increased in number as well as in size. Due to the growth of regenerating axons and schwann cells, the original basal lamina tubes expanded and gradually became fragmentized. Concomitant with such changes, regenerating Schwann cells became mature enough to produce their own basal laminas. Fine collagen fibrils which were probably synthesized by Schwann cells were deposited between these maturing Schwann cells. These fine collagen fibrils were regarded as corresponding to endoneurial collagen fibrils. Collagen fibrils located outside nerve fascicles were thicker than these new endoneurial collagen fibrils. Regenerating axons became thicker with time (2-3 \( \mu \)m in diameter), sorted out by Schwann cells, and myelinated to some degree. At the same time, thin cells (presumably developing perineurial cells) surrounded nerve fascicles, segregating the endoneurial space from the surrounding connective tissue (Fig. 4). Concurrent with the maturation of regenerating nerves, perineurial cells divided nerve bundles into smaller fascicles containing up to 10-20 nerve fibers. Fifty to 100 days after grafting these perineurial sheaths consisted of two or three layers of flattened cells, having their own basal laminae on both the inner and outer surfaces. The myelination of regenerating axons had proceeded to its completion by this stage, while small axons remained unmyelinated. Macrophages were no longer found at this stage (Fig. 5).

As to the reinnervation of target organs, Meissner corpuscles of the toe pads had received regenerating axons by day 70 post-grafting. Motor endplates of the lum-
brical muscles also had been reinnervated by this stage (Fig. 6a, b). These findings showed that regenerating axons, after growing through cryo-treated xenogeneic grafts, fully extended up to their target organs.

**Non-treated grafts**

The difference in the rate of nerve regeneration between cryo-treated and non-treated grafts was evident in light microscopic observations of semithin sections, i.e., the nerve regeneration was significantly delayed in the latter. Myelination of regenerating axons was not observed by 20 days post-grafting (vs. 14 days for cryo-treated grafts), and, in contrast to the cryo-treated grafts, macrophages were still present in the graft at this stage (Fig. 7d). In addition, there were many cells, other than macrophages, which had invaded the graft by days 14 or 20 post-grafting, indicating that a vigorous cell infiltration was occurring in the non-treated graft (Fig. 7c, d). Fifty to 70 days after grafting there were many myelinated fibers in the non-treated grafts, exhibiting almost the same features as in the cryo-treated grafts (Fig. 7e, f).

Electron microscopy showed that myelin sheaths had been degraded by macrophages, while some others still appeared normal, retaining their original configuration.

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**Fig. 3.** Cryo-treated graft. Seven days after grafting. Macrophages phagocytizing degenerating myelin sheaths (M) are seen within the basal lamina tubes of Schwann cells. Regenerating axons (A) are seen within a basal lamina tube (BL), which are accompanied by immature Schwann cells (S). ×9,500
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four days after grafting. Many macrophages containing myelin sheath debris were found in the endoneurial space (Fig. 8). It was difficult to definitively determine whether the living cells located within the basal lamina tubes were macrophages or Schwann cells. However, judging from the fact that they contained myelin sheath debris in the cytoplasm, most of these cells were considered to be macrophages (Beuche and Friege, 1984).

Regenerating axons were found 14 days after grafting. Groups of axons were surrounded by single Schwann cells within the basal lamina tubes. Usually there was a narrow gap between the basal laminae and Schwann cells within the tube, indicating that these basal laminae were not produced by these accompanying Schwann cells themselves, but were derived from the donor's Schwann cells in the graft. In fact, Schwann cells at this stage appeared very immature, containing an abundance of ribosomes in their cytoplasm. Therefore it is conceivable that the donor's Schwann cells had been destroyed, and that most Schwann cells seen at this stage were those of the recipient, migrating from the proximal stumps of the sciatic nerve. Myelination of regenerating axons was not yet seen, indicating the delayed regeneration and
maturation of axons as compared to the cryo-treated grafts. A noteworthy finding was a conspicuous cell infiltration in the graft at this stage. These infiltrating cells possessed in most cases no phagosomes of myelin sheath debris, suggesting that they were not macrophages, but were derived from lymphocytes (Fig. 9).

Twenty to 30 days after grafting, regenerating axons increased in number and extended in bundles through basal lamina tubes. Myelination was not yet observed on these axons at this stage, although some flattened cells were seen incompletely surrounding these nerve fascicles (Fig. 10).

Fifty days after grafting, most regenerating axons were myelinated in the grafts. The degree of their myelination was almost the same as in the cryo-treated grafts of the corresponding stage. Usually a single cell layer of perineurial cells completely surrounded the nerve fascicles, though such a fasciculation of nerve fibers was not so distinct by light microscopy (Fig. 7e), exhibiting the same pattern of endoneurial compartmentalization as that in cryo-treated grafts. Signs of cell infiltration were no longer seen in the graft at this stage.

Fig. 5. Cryo-treated graft. Fifty days after grafting. Fascicles of regenerating axons are further divided into smaller ones of various sizes by two to three layers of perineurial cells (P). Nerve fascicles consist of one to more than ten myelinated (mN) and unmyelinated fibers (uN). S Schwann cells. ×5,400

Fig. 6. Cryo-treated graft. Seventy days after grafting. a. Regenerating axons (A) are seen in a digital corpuscle of the toe pad in contact with lamellar cells (L) of the corpuscle. E epidermis. ×15,000. b. Regenerating axons (A) are seen in a motor endplate of the lumbral muscle. S Schwann cell, M muscle cell. ×22,000
Fig. 6. Legend on the opposite page.
Fig. 7. Non-treated graft. These light micrographs show nerve regeneration at 4–70 days post-grafting.  

a. Four days after grafting. Myelin sheaths are being degenerated.

b. Seven days after grafting. Degeneration of myelin sheaths has further advanced. The myelin sheath debris is seemingly phagocytized by macrophages (arrows).

c. Fourteen days after grafting. There is an extensive cell infiltration into the graft at this stage.

d. Twenty days after grafting. The cell infiltration has become less distinct, but there are still many macrophages (arrows) containing myelin sheath debris in the graft. Regenerating axons cannot be clearly identified in this micrograph.

e. Fifty days after grafting. Many myelinated nerve fibers are seen, some of which are as thick as those in Figure 1e. Fasciculation of regenerating axons is not evident in this light micrograph.

f. Seventy days after grafting. Regenerating fibers tend to be compartmentalized into fascicles. There is no difference in nerve regeneration in cryo-treated grafts (cf. Fig. 1f). ×340
Regenerating axons were found in Meissner corpuscles of the toe pads on day 70 in the non-treated grafts which were very similar to those found in the cryo-treated grafts.

**DISCUSSION**

The evidence in the present study indicates that an almost identical process of nerve regeneration occurs in the cryo-treated xenogeneic graft as in similarly treated autografts (IDE, 1983; IDE et al., 1983) and allografts (OSAWA et al., 1986). In short, Schwann cells in the graft are degraded, whereas their basal laminae remain in the form of tubes without being phagocytized by macrophages, and regenerating axons grow out through such basal lamina tubes. Such regenerating axons can extend far enough to reach their targets of the skin and muscles, as in the case of allogeneic grafts (OSAWA et al., 1986).

The process of nerve regeneration through xenogeneic grafts appears slightly delayed in the early period of regeneration (4 and 7 days after grafting). Such a delay in nerve regeneration is especially conspicuous in non-treated grafts. The vigorous cell infiltration seen in non-treated grafts 14-20 days after grafting suggests that
Schwann cells may be damaged by such an immunological cell infiltration, and then eliminated by macrophages. It seems that the basal laminae of Schwann cells are not affected by this immunological reaction, being left intact in situ. Therefore, nerve regeneration, though it is delayed due to such an early immunological reaction, can proceed in the same way in the non-treated graft as in cryo-treated ones. It can be said that dead Schwann cells in the cryo-treated grafts cause much less inflammatory reaction compared with living cells in non-treated grafts, thus enabling the early removal of cell debris by macrophages, which in turn leads to the facilitation of nerve regeneration in cryo-treated grafts.

It is generally thought that ordinary xenografts are sooner or later rejected by immunological reaction, as has been reported for cardiac (Corry and Kelly, 1975; Homan et al., 1981; Sugimoto et al., 1985), renal (Green et al., 1980), and spinal cord grafts (Tang and Bernstein, 1986). However, in the present study, xenogeneic grafts, even if they were non-treated ones, were not eliminated but served as conduits for the regenerating axons. It is probable that the donor's cellular elements including Schwann cells were gradually destroyed by the immunological reaction and finally removed by macrophages. Therefore, as in all ordinary xenogeneic grafts, non-

Fig. 9. Non-treated graft. Fourteen days after grafting. The delay of nerve regeneration in this graft is evident when compared with cryo-treated graft (cf. Fig. 4). The cell infiltration of the graft is evident. Most of the infiltrating (IC) are considered to be lymphocytes. Regenerating axons (A) which are accompanied by immature cells are seen within the basal lamina tubes. No myelination is seen on the regenerating axons. The compartmentalization by developing perineurial cells is not seen yet. ×5,400
treated xenogeneic grafts were made essentially ineffective due to the removal of all the cell constituents from the graft. However in nerve regeneration, the survival of the cells in the graft is not necessary because the Schwann cell basal lamina tubes are sufficiently effective conduits for the elongation of regenerating axons. It is known that the extracellular matrix such as collagen fibrils and basal laminae causes little or no immunoreaction (WATSON et al., 1954; STEFFEN et al., 1970; ELVIS, 1983). In this regard, the basal laminae of Schwann cells can remain without being eliminated, serving as conduites for regenerating axons even in non-treated grafts.

In the present study, the grafts used were short, being only 6-7 mm in length. If longer nerve segments were used as grafts, the nerve regeneration would be less successful in the distal half of the graft, regardless of whether the grafts were treated by freezing or not, because Schwann cell basal laminae of cryo-treated grafts can usually survive only up to 20-30 days after grafting (OSAWA et al., 1986). Thus, it is believed that the maximum effective length of the graft would be about 2-3 cm based on the evidence that axons regenerate approximately 1 mm per day. Repeated surgical procedures, in which a new graft is added to the distal end of the old one every 20 days after the previous grafting, might possibly solve this problem.

It has been suggested that basal laminae of cells other than Schwann cells are also
effective for nerve regeneration. IDE (1984) has found that the muscle basal laminae act as conduits for the regenerating nerves. It is probable that some substances effective for the cell attachment may be located on the basal laminae. It has been reported that laminin is localized mainly on the cellular side of the basal laminae of Schwann cells (TOHYAMA and IDE, 1984), epidermal cells (FOIDART et al., 1980; OSAWA, 1986), and glomerular epithelial cells (MADRI et al., 1980). It has been established that cultured sympathetic neurons and the outgrowth of neurites are stimulated by laminin, and that the heparin binding domain of laminin is responsible for such effects on neurons (EDGAR et al., 1984). In this respect, it can be considered that regenerating axons can attach themselves to basal laminae at least partly through the interaction with laminin.

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