Evaluation of histocompatibility as a factor in the repair of nerve with a frozen nerve allograft

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Host axons in dogs can regenerate through a long nerve allograft provided that the allograft bears only minor transplantation antigens, and is frozen and thawed before transplantation. The authors have tried to confirm this important observation in rats. Host rats received a 4-cm fresh or frozen nerve isograft (that is, a non-antigenic nerve), or a fresh or frozen nerve allograft with cells containing only minor transplantation antigens. The results showed that after 2 and 9 months only a fresh isograft permitted many host axons to traverse its entire length. Only a few host axons grew into the proximal 1 to 2 cm of a frozen isograft or into an allograft (fresh or frozen). Because frozen grafts failed, the authors examined some specimens after 2 weeks and found that freezing killed most of the Schwann cells. On the other hand, many proliferating Schwann cells were found in 2-week fresh isografts. In addition, hosts that received a frozen nerve allograft underwent regrafting after 9 months with an isograft and allograft (of the same genotype as the original nerve allograft) of nodose ganglion. These rats accepted the isograft but rejected the allograft of ganglion. It is concluded that axonal regeneration through a long frozen nerve graft fails in rats because freezing destroys Schwann cells. Moreover, a frozen nerve allograft does not induce a state of immunological tolerance, as has been suggested, because these recipients reject a second allograft. Since the present data failed to confirm findings obtained in dogs, the clinical use of a frozen nerve allograft is not recommended.

Key Words: nerve allograft · transplantation antigen · histocompatibility · nerve regeneration · immunological tolerance · Cyclosporin A · graft

HOST axons can regenerate through a short (2- to 3-cm) but not a long nerve allograft. This result occurs because certain proteins on cells in an allograft (transplantation antigens) elicit an immune response that destroys the graft before many recipient nerve fibers can grow through it. Moreover, the above finding holds true regardless of whether the nerve allograft contains major and minor or only minor transplantation antigens. It seems clear, then, that either the antigenicity of the allograft, or the immune response by the host (alone or in combination) must be altered if a long nerve allograft is to prove successful.

Several investigators have tried to reduce the antigenicity of a nerve allograft by freezing it prior to grafting. However, despite this pretreatment, no significant regeneration occurs. Singh, et al., noted that in previous studies the nerves were not tissue-typed and that in all probability the frozen nerves contained major and minor antigens. Accordingly, they used frozen nerves that lacked major antigens (verified by tissue-typing), and reported that in dogs many host axons regenerated through a 7-cm frozen nerve allograft. Unfortunately, those authors did not present data as to whether fresh (normal) nerve allografts were rejected in these tissue-typed dogs. This point is important, since in that study allografts were exchanged between littermate dogs which could have possessed relatively few antigenic differences. Because the finding by Singh, et al., is important, we tried to confirm it. We avoided the littermate problem by using genetically uniform rat strains which differ only in multiple minor transplantation antigens. In addition, we compared fresh and frozen nerve allografts.

Materials and Methods

Animals and Nerve Graft Procedure

Isogenic strains of Fisher (FR) and Lewis (LE) rats were used. These rat strains differ only with respect
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to minor transplantation antigens. Fisher rats served as hosts, and each received a single 4-cm fresh or frozen, LE or FR nerve graft. The nerve grafts were taken from 350- to 400-gm male donors that were anesthetized with chloral hydrate (400 mg/100 gm body weight, intraperitoneally). In order to obtain a 4-cm graft, the tibial nerve was found in the thigh and traced into the leg where, near the ankle, it divided into medial and lateral plantar branches. The plantar nerves were cut and separated, and one of them was excised and discarded. The remaining tibial-plantar nerve segment was removed and served as the 4-cm nerve graft. The LE or FR nerve grafts were placed into separate sterile tubes which contained culture medium that consisted of 87.5% Dulbecco's modified Eagle's medium, 10% heat-inactivated horse serum, and 2.5% chicken embryo extract. Some LE and FR nerves were transplanted into FR hosts after 5 to 10 minutes in the culture medium; these constituted our fresh-nerve experimental series. Other tubes containing LE and FR nerves were placed into a freezer set at −70°C and frozen for a period of 3 days to 6 weeks; these constitute our frozen-nerve experimental series. Prior to grafting, the frozen nerves were thawed for 5 minutes in water at 35°C, and transferred to fresh tissue-culture medium at room temperature.

The frozen or fresh nerves were transplanted into hosts in the following manner. An FR rat was anesthetized and its peroneal nerve exposed in the mid-thigh and transected. The distal host peroneal nerve was excised down to the point where it crossed the gastrocnemius muscle. The plantar end of the nerve graft was joined to the proximal cut end of the host peroneal nerve. Since a plantar nerve is about the same diameter as a peroneal nerve, this nerve union provided ample nerve stump into which peroneal axons might grow. Nerve union was achieved by tucking about 2 mm of host and donor nerve ends into a sleeve of FR carotid artery graft and holding them there with a clot formed by the topical application of fibrinogen. Only a proximal nerve anastomosis was performed since this procedure was sufficient to determine whether host axons could traverse a long nerve graft. Because a rat's thigh cannot accommodate a 4-cm linear graft, we had to loop the graft up and then back down the thigh.

Evaluation of Nerve Grafts

Fresh and frozen nerve grafts were histologically examined 2 and 9 months after transplantation. At these times, longitudinal and cross-sectional pieces of each nerve graft (or what was left of it) were placed between slabs of skeletal muscle which were frozen in liquid nitrogen. The muscle blocks were transferred to a cryostat and cut to the point where the nerve graft appeared. Frozen sections of the nerve grafts were prepared and stained by a periodic acid-Schiff-hematoxylin (PAS-hematoxylin) or a silver technique. The histological features of successful and unsuccessful nerve grafts are presented in Fig. 1, where they are compared to a normal nerve. A normal nerve contained many thickly and tightly packed myelinated nerve fibers (Fig. 1A). A successful fresh FR nerve graft also had many myelinated nerve fibers, but they were thinner and less tightly packed (Fig. 1B) than normal. The presence of myelin in a successful nerve graft indicated that Schwann cells were present and functioning. Fresh (Fig. 1C) and frozen (Fig. 1D) LE nerve grafts were virtually acellular and lacked myelinated nerve fibers. A frozen FR nerve graft was similar to a frozen LE graft. The silver stain revealed that many host axons had regenerated through a fresh FR graft (Fig. 1E), whereas only a few axons were seen in the proximal region of fresh and frozen LE (Fig. 1F), or a frozen FR graft.

Because the frozen nerve grafts failed, we decided to investigate their fate 2 weeks after transplantation. We also studied 2-week fresh FR grafts and 2-week in situ degenerating host nerve. Four animals in each group were observed. After 2 weeks, Schwann cells had proliferated in in situ degenerating nerve and only scattered remnants of myelin persisted (Fig. 2A). A fresh FR graft resembled an in situ nerve, but it had a lesser degree of Schwann cell proliferation, and more degenerating myelin was present (Fig. 2B). By contrast, frozen nerves lacked any significant numbers of Schwann cells and massive amounts of degenerating myelin were observed (Fig. 2C). Mononuclear cells were found in frozen LE nerve grafts (Fig. 2C), but were absent from frozen FR grafts. An additional observation was that the perineurium survived in fresh FR (Fig. 2D) but not in frozen LE or FR (Fig. 2E) grafts. A large number of host axons were observed growing into the proximal portion of fresh FR grafts, whereas only a few were seen penetrating frozen

Results

The findings were similar in all 10 animals within each experimental group. The results demonstrated that many host axons could regenerate through only a fresh 4-cm FR nerve graft. Host nerve fiber growth into frozen FR, or frozen and fresh LE nerve grafts was confined to the initial 1 to 2 cm of these nerves. The observations on frozen nerve grafts pertained regardless of whether they were stored frozen for 3 days or 6 weeks prior to transplantation. The only difference noted among unsuccessful grafts (that is, grafts in which a few regenerated axons were confined to the proximal 1 to 2 cm) was that at 2 months these grafts were visible throughout their entire length, whereas after 9 months most of their distal segment had disintegrated and was absorbed. On the other hand, a successful nerve graft (that is, a graft in which many regenerated axons were present throughout) was visible throughout its 4-cm length after 2 and 9 months, and it appeared white, indicating the probable presence of myelin.

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FIG. 1. Appearance of longitudinal sections of a normal nerve (A), and nerves grafted for 2 months (B–F). A normal nerve (A) contained many tightly packed and thickly myelinated nerve fibers (PAS stains myelin a red-purple color which in the photographs appears black). A fresh isograft (B) had many myelinated nerve fibers throughout its 4-cm length, but they were thinner and less tightly packed than normal. A fresh (C) or frozen (D) allograft lacked myelinated fibers throughout most of its distal portion; these distal segments were virtually acellular. Frozen isografts were similar to that shown in (D). Many axons were present throughout a fresh isograft (E, one axon at tip of arrow), whereas only a few axons were found in the proximal 1 to 2 cm of fresh and frozen allografts (F), or frozen isografts. A–D: PAS-hematoxylin, × 80; E, F: silver stain, × 330.

Discussion

Our experimental results demonstrated that FR axons regenerated only through a 4-cm fresh FR and not a frozen FR, or a fresh and frozen LE nerve graft. A successful fresh isograft had many myelinated axons throughout, whereas unsuccessful nerve grafts had some axons but only in their proximal 1 to 2 cm portion. Our data in rats fail to confirm the observation that in dogs host axons can regenerate through a long frozen nerve allograft that bears only minor transplantation antigens. We froze our nerve graft in culture medium at the same temperature and for the same duration as did Singh, et al., and cannot readily explain our different experimental results unless a species factor is involved. In our study, freezing clearly killed Schwann and perineurial cells in nerve. Axons were absent in the distal portions of all 2-week grafted nerves.

In a final study, we regrafted five FR hosts that had received a frozen LE nerve graft 9 months earlier. Each of these rats was grafted with an LE and FR nodose ganglion that was implanted into the neck into opposite sternocleidomastoid muscles. After 1 month, all five FR ganglia contained neurons and Schwann cells, whereas these cells were absent in all LE ganglia which, in addition, were infiltrated by mononuclear cells. This regrafting experiment was performed because Singh, et al., suggested that in dogs a frozen nerve allograft might induce a state of immunological tolerance in the host. Our data in regrafted rats showed that their immune system was capable of identifying and rejecting an allograft.
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The appearance of longitudinal (A-C) and cross (D and E) sections of 2-week in situ degenerating host nerve (A), 2-week fresh isograft (B and D), and 2-week frozen allograft (C), and isograft (E). Schwann cells proliferated, and all but a few remnants of myelin (arrow, A) remained in an in situ degenerating nerve (A). A lesser degree of Schwann cell proliferation and more myelin debris (arrow, B) were found in a fresh isograft. The perineurial cells also survived in a fresh isograft (D, arrow points to perineurium). Massive amounts of degenerating myelin and few Schwann cells were observed in a frozen allograft (C) or frozen isograft (E). A frozen allograft but not a frozen isograft was usually surrounded (arrow, C) and infiltrated by mononuclear cells. The perineurial cells were absent from all frozen grafts (E). The arrow in E indicates where the perineurium should have been found in this frozen isograft (compare E to D). PAS-hematoxylin, \( \times 80 \) (A-C) and \( \times 330 \) (D, E).

The graft. In addition, vascularization was probably hindered, because after 2 weeks frozen nerve grafts were not swollen like grafts degenerating in situ, or fresh nerve grafts. From these observations it can be concluded that viable Schwann cells and probably good vascularization are required to promote the success of a nerve graft.

Singh, et al., implied that the allogenic cells in their grafts survived freezing because in their discussion they suggested that the host dog might have become tolerant to the transplantation antigens present in the frozen nerve graft. These investigators did not regraft their dogs to determine this important point. We regrafted our rats that received a frozen nerve allograft and found they were still immunologically competent (that is, they rejected a ganglion of the same genotype as the original nerve allograft).

It would appear that some form of immunosuppression offers the best hope of promoting host axonal growth through a long nerve allograft. Pollard and Fitzpatrick\(^3\) used azathioprine, steroids, and antilymphocyte serum in rats to achieve some regeneration through long allografts. These drugs in our hands, however, have proved to be toxic in rats after prolonged use (unpublished data). Recently, we used the new immunosuppressive agent Cyclosporin A to obtain regeneration through highly incompatible fresh, long nerve allografts in rats.\(^4\) In that study we also found that if Cyclosporin A was abruptly stopped an immune response developed, the nerve allograft was rejected, and the host axons in it degenerated. We are currently trying to determine the minimum dose of Cyclosporin A that is immunosuppressive for a nerve allograft. Once that is ascertained, Cyclosporin A will be gradually withdrawn from the host with the hope that any immune response which develops will be mild and produce no damage to the host axons in the nerve allograft.
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