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Leukemia Inhibitory Factor Prevents the Death of Axotomised Sensory Neurons in the Dorsal Root Ganglia of the Neonatal Rat

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Leukemia inhibitory factor (LIF) has several characteristics of a neurotrophic factor for sensory neurons. Here we have investigated whether LIF also supports the survival of axotomised sensory neurons in vivo. Newborn rat pups received a unilateral sciatic nerve transection and the injury site was treated with gelfoam soaked in phosphate buffered saline (PBS), nerve growth factor (NGF), or LIF. Neuronal nucleoli in the L5 dorsal root ganglia were counted, appropriate corrections applied, and the resultant neuronal loss expressed as a percentage of the contralateral intact side. In animals where LIF was administered neuronal loss was significantly reduced: 2 days after LIF treatment neuronal loss was 19.5% compared to 43% in PBS-treated animals; 3 days after LIF treatment neuronal loss was 20.4% compared to 40.2% in PBS-treated animals; however, 7 days after LIF treatment there was no significant reduction in the number of neurons lost. The degree of rescue of sensory neurons in vivo by LIF was found to be similar to NGF, which was not surprising as both factors supported the survival of a similar population of sensory neurons in vitro. Rescue was not observed when LIF-containing gelfoam was placed away from the axotomised nerve, suggesting that LIF's action may be associated with its retrograde transport or direct signalling at the site of nerve injury.

Key words: sensory neuron survival, leukemia inhibitory factor, axotomy, neurotrophic factors, dorsal root ganglion, axonal injury

INTRODUCTION

Neuronal death occurs normally during embryonic development, and has been shown to be lessened by the administration of exogenous growth factors or increased by the depletion of growth factors by antibodies (Barde, 1988, 1989; Johnson et al., 1989; Kromer, 1987; Murphy et al., 1993; Otto et al., 1989). The demonstration that these factors, in particular nerve growth factor (NGF), are also expressed in target tissues at the time of innervation (Davies et al., 1987), which coincides with naturally occurring cell death, further supports the concept that neurotrophic factors are regulators of neuronal survival. Recent evidence suggests that the mechanism of neuronal death which follows injury or degenerative diseases may be similar to the embryonic situation and involve programmed cell death or apoptosis (Franklin and Johnson, 1992). These observations raised the possibility that deprivation of target derived factors causes neuronal loss (Rich, 1992). It follows that the exogenous application of growth factors to damaged neurons may prevent their death (Barde, 1989)

NGF was shown to act in this manner by supporting the survival of axotomised sensory (Otto et al., 1987; Rich et al., 1987; Yip et al., 1984), sympathetic (Hendry and Campbell, 1976), and septo-hippocampal neurons (Hefti, 1986; Kromer, 1987; Otto et al., 1989; Williams et al., 1986). In the latter system, however, it is possible that the results were due to the maintenance of acetyl choline levels rather than the promotion of neuronal survival (Hagg et al., 1988). Ciliary neurotrophic factor also prevents the death of axotomised facial neurons in the neonatal rat (Sendtner et al., 1990), axotomised septo-hippocampal neurons in the adult rat (Hagg et al., 1992), and facial neurons in the mutant progressive motoneuronopathy mouse (Sendtner et al., 1992a), which undergoes a spontaneous motor axonopathy. There is some evidence that brain-derived neurotrophic factor rescues axotomised motoneurons from cell death in the spinal cord (Yan et al., 1992). Likewise, basic fibroblast growth factor prevents the loss of axotomised medial septal (Anderson et al., 1988) and retinal ganglion cells (Sievers et al., 1987).

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Another cytokine with multiple activities, leukemia inhibitory factor (LIF), which is identical to cholinergic differentiation factor, has been shown to support the survival and differentiation of sensory neurons and motoneurons in vitro and to promote cholinergic differentiation in the sympathetic lineage (Martinou et al., 1992; Murphy et al., 1991; Yamamori et al., 1989). Because LIF is also retrogradely transported by sensory neurons in vivo (Hendry et al., 1992), we have investigated the ability of this factor to prevent the loss of sensory neurons in the spinal ganglia following axotomy.

MATERIALS AND METHODS

In Vitro Assays

Dorsal root ganglia (DRG) sensory neurons were isolated from newborn Wistar rat pups which were less than 24 hr old. The ganglia were dissected free of surrounding connective tissue, placed in HEPES buffered Eagles medium (HEM), finely chopped, then incubated in 0.025% trypsin, 0.001% DNAase at 37°C for 15 min. After the addition of fetal bovine serum (FBS), the cells were centrifuged at 300*g* for 5 min, washed twice in HEM, 0.01% DNAase, and triturated through 18–25 gauge needles to obtain a single cell suspension. DRG cells were added to fibronectin-coated wells of HL-A plates (Nunc, Illianois) at 100 cells per well in Monomed medium (Commonwealth Serum Laboratories, Melbourne Australia) containing 10% FBS and growth factors where specified. LIF (*E. coli* derived, murine recombinant, specific activity of 0.9×10^8 units/mg, Amrad Biotechnology Australia) was used at 10 ng/ml and NGF 7S (Boehringer Mannheim) was also used at 10 ng/ml. After 2 days, the wells were scored for live neurons (large, phase bright, spherical cells with fine processes).

Sciatic Nerve Axotomy and Counts of Sensory Neuron Loss In Vivo

Experiments were carried out on newborn (P1) Wistar rat pups of either sex. The pups were operated on within 24 hr of birth under ice-induced anesthesia. The left sciatic nerve of each pup was exposed at the level of the obturator internus muscle and axotomised using a pair of iridectomy scissors. The proximal stump of the sciatic nerve was wrapped with a 1-mm^3 piece of gelfoam (Upjohn) containing either 20 μl phosphate-buffered saline (PBS), 20 μl of LIF (total of 18 μg), or 20 μl of NGF (total of 18 μg). The same LIF and NGF as described above for the in vitro assays were used for the in vivo experiments. The contralateral sciatic nerve and DRG served as the intact control side. A 5-0 Ethicon silk suture was used to close the skin incision. The pups were then warmed until they were fully conscious and they were then reunited with their mothers. The effectiveness of the sciatic nerve axotomy was apparent by the postsurgical ataxia of the hindlimb and the completeness of the nerve transection verified by postmortem dissection.

After survival periods ranging from 12 hr to 36 days, the neonatal animals were deeply anesthetised on ice or, in the case of older animals, killed by asphyxiation in a CO_2 chamber. The animals were then perfused transcardially with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.3. Immediately after perfusion the vertebrae overlying the lumbar DRGs were removed and the whole animal was immersed in the same fixative overnight. The following day, the left (axotomised) and right (intact) L5 DRGs were removed and postfixed for a further 24 hr. These DRGs were then dehydrated and embedded in paraffin. Serial sections 8 μm thick were cut and mounted on gelatinised slides and stained in 0.1% cresyl violet.

Neurons displaying a prominent nucleolus were counted at a final magnification of 400× through a graticule placed in the eye-piece of a Leitz microscope. Counts were performed on every fifth or tenth section. The raw counts were corrected for multiple nucleoli and then for split nucleoli using Abercrombie's formula. Mean nucleolar diameter measurements showed that axotomised neurons did not have shrunken nucleoli. The proportion of neurons lost was calculated as a percentage as follows:

$$\frac{\text{neurons in the intact L5 DRG} - \text{neurons in the axotomised L5 DRG}}{\text{neurons in the intact L5 DRG}} \times 100$$

To ensure an accurate estimate of neuronal loss, two steps were taken. First, the section thickness exceeded the greatest nucleolar height by a factor of about 4. This factor is well above the critical factor of 1.5 which is needed to ensure that the Abercrombie correction factor is reliable and not seriously biased (Clarke, 1992). Secondly, contralateral controls were used throughout and all comparisons between animals were based on *proportional* rather than absolute values. Where proportional values are used (Pollin et al., 1991), Abercrombie's correction factor is not necessary, since any biases introduced in the counting should be common to both sides and cancel out when the ratio is calculated.

The diameters of the neuronal perikarya and nucleoli were determined using a PC-based image-analysis system (MD 30-plus, Leading Edge, Adelaide, South Australia). The means and standard errors of means (SE) were calculated for each group and statistical differences between groups were determined by using the Student's *t* test.

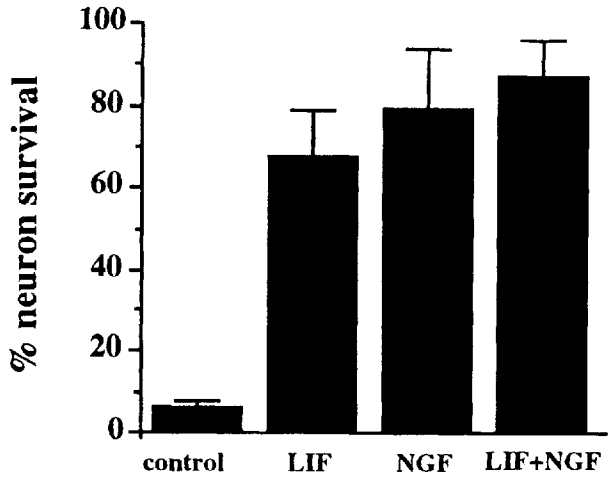

Fig. 1. Survival of dissociated sensory neurons obtained from newborn (P1) rat DRGs in the presence or absence of LIF, NGF, or NGF plus LIF.

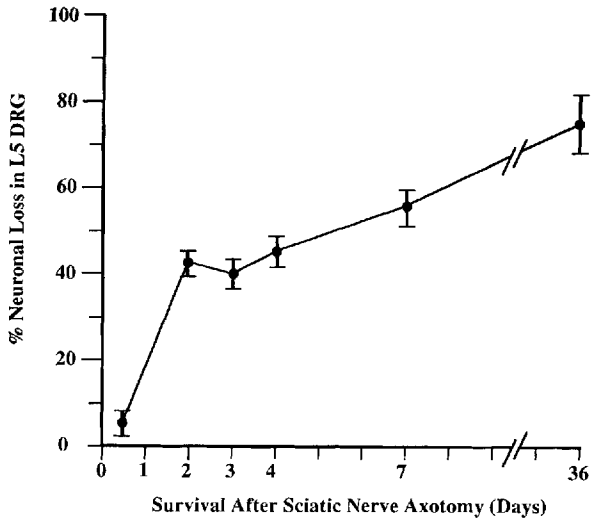

Fig. 2. The loss of sensory neurons in the L5 DRG at several survival times after sciatic nerve transection at P1. Each data point represents the mean value, obtained from three to four animals, with bars indicating the SE.

RESULTS

The sciatic nerve-L5 DRG system was chosen to test the ability of LIF to rescue sensory neurons from lesion-induced cell death in vivo because a significant number of neurons die in these ganglia following neonatal axotomy (Yip et al., 1984). Initial experiments revealed that neonatal rats were more suitable for sciatic nerve axotomy than neonatal mice because they provided easier access and allowed a more accurate positioning of the gelfoam.

In vitro results demonstrated that both recombinant murine LIF and purified NGF supported the survival of the majority of neonatal rat sensory neurons (Fig. 1). The simultaneous addition of LIF plus NGF to the cultures did not significantly increase the percentage of sensory neurons surviving beyond that observed for LIF or NGF alone ($P > 0.05$). This supports the previous findings in mice (Murphy et al., 1991) that LIF and NGF support a similar population of sensory neurons in the postnatal DRG.

Having established that LIF acts as a survival agent for rat sensory neurons in vitro, we next examined its effect in vivo on axotomised sensory neurons. Prior to using LIF we determined the rate and extent of sensory neuronal loss in the ipsilateral L5 spinal ganglion following axotomy and treatment of the axotomised site with PBS in gelfoam. As early as 12 hr following axotomy, a small neuronal loss of approximately 5% was observed; this rose to a mean value of 43% by day 2; 40.0% by day 3; 56% by day 7; and up to 75% by day 36, which was the longest time tested (Fig. 2).

From the time course of neuronal death observed in axotomised animals, we decided to assay the effects of LIF treatment 2, 3, and 7 days after axotomy. Two days following axotomy, the LIF-treated group showed a significant reduction in neuronal loss in L5 DRG sensory neurons compared to controls; LIF prevented the death of about half of the neurons (Fig. 3). A similar reduction was also seen in the group exposed to LIF over a 3-day period (Fig. 3). While the mean rescue was approximately 50% in these two LIF-treated groups, levels as high as 95% were obtained in some LIF-treated animals, which may reflect the varying efficacy of the gelfoam as a method of delivering LIF to the lesion site. After 7 days, there was no significant reduction in the loss of sensory neurons in the LIF-treated DRG. As NGF had been previously shown to rescue DRG neurons (Yip et al., 1984), we compared the degree of rescue with LIF to NGF. NGF treatment for 3 days resulted in a neuronal loss which was not significantly different from that observed for LIF at the same time (Fig. 3).

To determine if the effect of LIF was due to systemic rather than local action, the LIF-soaked gelfoam was placed in the subcutaneous tissue of the shoulder region. In these animals, there was no significant reduction in the percentage of neurons lost at 2 days following axotomy compared to controls (Fig. 3, LIF ectopic).

Neuron diameter measurements were made to determine if neurons of a particular size were lost after axotomy and to see if LIF rescued neurons of a particular size. In two intact DRGs examined, the neuronal diameters had a unimodal distribution with a mean diameter of 33 μm (Fig. 4). Following axotomy and exposure to PBS, the mean as well as the total distribution of neu-

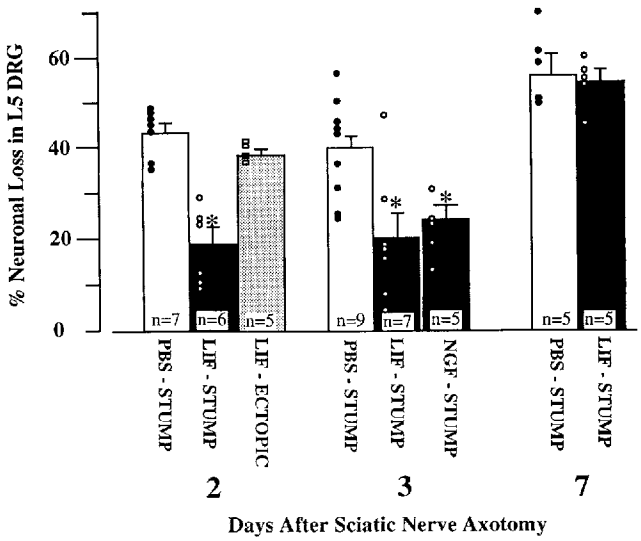

Fig. 3. Neuronal loss in the L5 DRG after axotomy and the effect of PBS, LIF, or NGF treatments. Also shown is the response when LIF was not placed around the proximal nerve stump (LIF-ectopic). The histograms represent mean values with bars indicating the SE. Individual responses and the number of animals (n) in each group are also shown. Statistically significant differences are denoted by asterisks: $P < 0.001$ and $P < 0.05$ after 2 and 3 days following LIF treatment respectively and $P < 0.001$ after 3 days following NGF treatment.

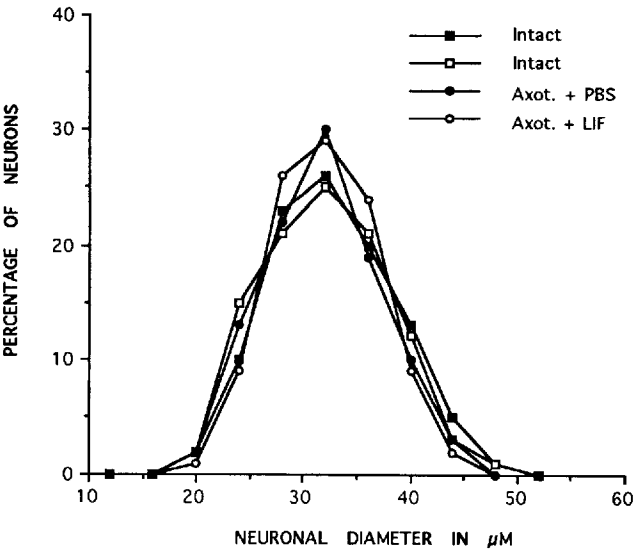

Fig. 4. Size spectrum of neuronal perikarya in L5 DRG from intact controls (n = 423 and 413) or from animals 3 days after sciatic nerve axotomy at P1 and the nerve stump treated with gelfoam containing either PBS (n = 236) or LIF (n = 421).

ronal diameters within the DRG population were not significantly different from the values observed for the intact side (Fig. 4) and showed that neurons of all sizes were lost. Similarly, the mean neuronal diameter in LIF-treated ganglia was also unchanged, suggesting no selectivity of rescue (Fig. 4).

DISCUSSION

This study demonstrates that LIF can rescue approximately 50% of the sensory neurons, at least in the short term, which normally die in the DRG after transection of the sciatic nerve at birth. The degree of rescue was almost identical to that observed with the prototypic neurotrophic factor, NGF. These results also demonstrate that both LIF and NGF can act when placed at the site of nerve lesion and are not required to be given systemically, although previous studies by Yip et al. (1984) showed that the daily subcutaneous application of NGF also promotes the survival of axotomised DRG sensory neurons.

The similarity of action of NGF and LIF in supporting sensory neuron survival in vivo is not surprising given the overlap of biological properties. The in vitro data presented in this study in rats, plus our earlier studies in mice (Murphy et al., 1991, 1993), demonstrate that LIF and NGF support a similar population of postnatal DRG neurons. In addition, both factors are retrogradely transported (Hendry et al., 1992; Raivich et al., 1991) and both are expressed in tissues at the time of peripheral sensory innervation (Murphy et al., 1993). Thus, like NGF, LIF appears to have all the properties of a classical neurotrophic factor.

Our findings reinforce the concept that factors which act during development may also play a role in neuronal survival in diseased or damaged nerves (Bartlett et al., 1991). Of course other factors which appear to be poorly transported retrogradely, such as ciliary neurotrophic factor (CNTF) (Curtis et al., 1992; DiStefano et al., 1992; Smet and Rush, 1992) can also act as in vivo neuronal survival factors. CNTF supports the survival of facial motoneurons after axotomy when placed at the proximal end of the axotomised nerve in gelfoam (Sendtner et al., 1990). In this case, CNTF's ability to promote motoneuron survival correlates with its in vitro activity (Oppenheim et al., 1991). Conversely, it has been shown that molecules, such as brain-derived neurotrophic factor (BDNF), not known to have motoneuron survival activity in vitro, support motoneuron survival in vivo (Yan et al., 1992). Thus, it would seem that any factor possessing one or more of the features of a prototypic neurotrophic factor like NGF is a potential candidate to support the survival of damaged neurons in vivo.

The notion that LIF is an important regulatory molecule in the development of the sensory nervous system is strongly supported by its expression pattern in situ and its promotion of the differentiation of early neuronal pre-

cursors from the neural crest in vitro (Murphy et al., 1993). In addition, LIF also promotes the survival of the majority of sensory neurons in vitro from a period just prior to birth into neonatal life, suggesting it is capable of acting as a maintenance factor for sensory neurons once they have passed through the phase of target innervation and natural neuron death (Davies et al., 1987; Murphy et al., 1993). These in vitro findings are supported by the in vivo data presented here. Although LIF is expressed in target tissue and in the nervous system during development, it cannot be detected in adult target tissue (Murphy et al., 1993), implying that it is unlikely to maintain neuronal integrity in the adult. After injury, however, expression of LIF mRNA can be detected in adult muscle (M. Murphy, M.A Brown, and L. Austin, unpublished observations), demonstrating that LIF can be produced by target tissue following injury or denervation and may play a role as a trauma factor equal to CNTF (Sendtner et al., 1992b). Furthermore, recent experiments have shown that CNTF, which can act via the LIF receptor (Ip et al., 1992), promotes axonal sprouting at, or near, the muscle surface in vivo (Gurney et al., 1992), indicating that LIF-like molecules may, in addition to their survival activity, promote the re-innervation of targets.

The precise nature and mode of transmission of the survival signal generated by LIF is unknown. Evidence from NGF studies (Raivich et al., 1991) implicates the retrograde transport of factors attached to their receptor as part of the signalling mechanism. If retrograde transport is intimately involved in the process of rescuing neurons from cell death, then it would be anticipated that local administration of factors to the site of nerve lesion would be an efficient method of preventing neuronal death. Indeed, this approach has now been used for the rescue of motoneurons using BDNF (Yan et al., 1992) and CNTF (Sendtner et al., 1990), of central cholinergic neurons using NGF and fibroblast growth factor (Otto et al., 1989), and of sensory neurons using LIF and NGF as shown here. Local application of NGF has also been shown to promote the survival of axotomised sensory neurons in the mature rat spinal ganglia (Rich et al., 1987). However, many studies have shown that factors administered subcutaneously or intraventricularly are also effective in supporting the survival of axotomised neurons. Furthermore, combined systemic and local administration have also been employed, presumably—although the relative efficacy of each procedure was not discussed—to maximise survival (Yan et al., 1992). Whilst in this present study we have shown that LIF is ineffective when placed at a distant site from the lesion, indicating that its effect is not mediated via the bloodstream, this does not negate the possibility that growth factors may rescue neurons by binding to the cell soma directly. Although toxicity problems (Metcalf and Gearing, 1989) exclude the use of high levels of LIF in the blood, receptor binding studies (Hendry et al., 1992) clearly indicate that LIF receptors are expressed at high levels on the cell soma, suggesting that if given access to the cell soma, LIF may be effective via this route. Whether the reported effectiveness of subcutaneously administered NGF (Yip et al., 1984) is due to direct action in the ganglia or high levels at the site of injury is as yet unknown.

The temporally restricted survival of sensory neurons after local LIF application found in this study and the finding that neuronal death occurs following cessation of subcutaneous injections of NGF (Yip et al., 1984) suggest that axotomised neurons require a constant source of factor for continued survival. This is unlike the situation in vitro where factor replenishment for isolated sensory neurons is often not required. This may be due to the longer half-life of the factor in vitro or to the neuron losing its dependence for the factor after a critical period in vitro. A similar phenomenon may occur in vivo during neuronal development whereby neurons become more independent as they mature. As neuronal death in most cases is mediated by a positive apoptotic-like phenomenon requiring protein synthesis (Franklin and Johnson, 1992), which can be prevented by either inhibitors of protein synthesis or neurotrophic factors (Oppenheim, 1989), it is possible that axotomised neurons rescued in vivo remain in a pre-apoptotic state as long as they receive an exogenous source of the appropriate trophic factor. The nature and duration of such a pre-apoptotic state, and the event(s) which herald a change of this state, constitute two of the most important questions to be answered if growth factors are to be employed in the treatment of neuronal death in neurological or traumatic diseases.

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