In Vivo Gene Therapy for Pyridoxine-Induced Neuropathy by Herpes Simplex Virus-Mediated Gene Transfer of Neurotrophin-3

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Neurotrophic factors have been demonstrated to prevent the development of peripheral neuropathy in animal models, but the therapeutic use of these factors in human disease has been limited by the short serum half-life and dose-limiting side effects of these potent peptides. We used peripheral subcutaneous inoculation with a replication-incompetent, genomic herpes simplex virus-based vector containing the coding sequence for neurotrophin-3 to transduce sensory neurons of the rat dorsal root ganglion in vivo, and found that expression of neurotrophin-3 from the vector protected peripheral sensory axons from neuropathy induced by intoxication with pyridoxine assessed by electrophysiological (foot sensory response amplitude, and conduction velocity, and H-wave), histological (nerve morphology and morphometry), and behavioral measures of proprioceptive function. In vivo gene transfer using herpes simplex virus vectors provides a unique option for treatment of diseases of the sensory peripheral nervous system.

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Neurotrophic factors were identified originally by the ability of these peptides to prevent the programmed cell death of subpopulations of neurons during development.[1,2] Although a specific role for these factors in the adult peripheral nervous system has not been established, evidence in several different animal models of peripheral neuropathy has shown that systemic administration of pharmacological doses of a trophic factor may be effective in protecting sensory neurons from degeneration characteristic of peripheral neuropathy.[3] Nerve growth factor (NGF) prevents cisplatin neuropathy,[4] and the sensory neuropathy caused by experimental diabetes,[5] insulin-like growth factor-1 can prevent neuropathy caused by administration of vincristine,[6] and neurotrophin-3 (NT-3) can reverse neuropathy caused by administration of cisplatin and prevent the neuropathy caused by pyridoxine overdose.[7] Despite the impressive effects of trophic factor administration in the treatment of rodent models of neuropathy, effective treatment of human disease with these factors has not yet been achieved. The peptides have a very short half-life in serum, measured at 7.2 minutes for NGF and at 1.28 minutes for NT-3 in the rat,[8] and systemic administration is limited by dose-related side effects. In a phase I trial of recombinant human NGF human subjects reported muscle pain at doses above 0.1μg/kg.[9] A multicenter phase III trial, in which 0.1μg/kg recombinant human NGF was administered twice a week, proved ineffective in preventing the progression of diabetic neuropathy in patients,[10] but the effective doses reported in animal studies ranged from 3 to 5mg/kg.[4,5]

Gene transfer offers the possibility to deliver a therapeutic neurotrophin transgene directly to the vulnerable cell population, so that the local synthesis and continuous release of the neurotrophic factor, acting in an autocrine or paracrine fashion may protect those neurons from degeneration, while avoiding the undesirable complications created by systemic delivery of high doses of the peptide. Among the gene transfer vectors, herpes simplex virus (HSV) is ideally suited for the delivery of genes to the peripheral nervous system.[11] The wild-type virus is neurotropic, taken up and transported with high efficiency from peripheral inoculation to sensory neuronal perikarya in the ganglion.[12] Like the parental wild-type virus, which naturally establishes a lifelong latent state as an intranuclear episomal element in peripheral sensory neurons,[13] non-

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replicating genomic HSV vectors created by inactivation of essential immediate early HSV genes establish the same persistent state[14,15] and can be used to transfer and express transgenes in the nervous system. Genomic HSV vectors effectively deliver and express transgenes in neurons of the central nervous system when targeted by stereotactic inoculation[16–18] and, after subcutaneous injection in the skin, can be used to deliver biologically active transgenes to the DRG.[19]

In an effort to determine whether HSV vector-mediated delivery of a neurotrophic factor can protect peripheral sensory neurons from degeneration, we used a model of pyridoxine (PDX) intoxication in the rat. In high doses, PDX causes a selective degeneration of large and small myelinated sensory axons in the peripheral nerve,[20] resulting in numbness and loss of proprioception that manifests clinically as a sensory ataxia without weakness or central nervous system dysfunction.[20,21] In rodent models, intoxication with PDX causes axonal degeneration in the dorsal roots, and the central projections of the same neurons in the dorsal horn of spinal cord, but sparing the ventral roots.[22] The effect is dose related; at lower doses, the predominant damage occurs in the distal axon, while with progressively increasing doses produce pathological abnormalities in the perikarya of neurons in the dorsal root ganglia (DRG).[23,24] A major advantage of PDX neuropathy as an animal model of peripheral neuropathy is the absence systemic toxicity that often confounds analysis of treatment effects in other animal models of neuropathy such as those caused by diabetes or chemotherapeutic agents. Using PDX neuropathy in the rat, Helgren and colleagues[7] demonstrated that systemic delivery of NT-3 (2 to 20 mg/kg/day, subcutaneously) largely attenuates the behavioral, electrophysiological and morphological sequelae of pyridoxine toxicity.

Although NT-3 is protective in the rat model, it is unlikely that patients would tolerate NT-3 in mg/kg doses by daily injection, so the need for alternative means of delivery is acute. In the present study, we report that a nonreplicating recombinant genomic HSV vector expressing human NT-3 delivered to the DRG by subcutaneous inoculation in the foot protects rats from the development of a sensory neuropathy after pyridoxine intoxication, measured by histological and electrophysiological parameters. The protective effect of the vector was demonstrated by prevention of the behavioral effect of the sensory neuropathy, measured by the ability of the rats to walk along a narrow beam.

Materials and Methods

Vector Construction

The control vector QOZHG was created by a genetic cross of the virus TOZ.1 (ICP4$^-$, ICP27$^-$, ICP22$^-$, UL24$^-$::ICP4p-tk, UL41$^-$::ICP0p-lacZ) with the virus d106 (ICP4$^-$, ICP27$^-$::HCMV IEp-GFP, β-ICP22, β-ICP47, a gift of N. DeLuca, Pittsburgh, PA).[25] GFP-positive lacZ$^+$ plaques were purified by three rounds of limiting dilution and the genomic structure confirmed by Southern blot. The rat NT-3 gene was cloned from genomic DNA using nested polymerase chain reaction (PCR) primers (primer set 1, forward primer-TTCTTTCAGATCTTACAGGTGAAC; reverse primer-AATAATTTATATGTGGGGACAGAT) in a standard 20μl reaction. The resultant single band (~840bp) was excised and second amplifications performed using primer set 2 (forward primer - *GGCAATC*-GGATCC-*CCACC*-ATGTCCATCTTGTTTTATGTGATA; reverse primer-*CGGTACC*-CGGCCGC-*ATCCAC*-TCATGTTCT-TCCGATTTTTCTTGA) containing *Bam*HI and *Not*I sites (underlined) bounded by nonsense sequence (italics), respectively. The NT-3 PCR product was ligated with *Xho*I linkers, digested with *Xho*I, and inserted into the *Xho*I site of p41L2HlacZ, a plasmid containing a LAP2HCMVlacZ polyA cassette in which the lacZ sequences are flanked by *Xho*I sites, and the entire cassette flanked by HSV UL41 sequences 90145–91605 and 92193–93857.[26] LAP2[27] included the HSV sequences from 11864 to 119500[27] and the SV40 late polyA included the *Bam* to *Pst* fragment. *Xba*I linearized p41L2HNT3 was cotransfected with QOZHG, clear plaques purified by three rounds of single plaque purification,[28] and the genetic structure confirmed by Southern blot, using NT-3 as a probe.

Cell Culture

DRG from 17-day rat embryos were dissociated with 0.25% trypsin, 1mm EDTA for 30 minutes at 37°C with constant shaking and then plated on poly-D-lysine-coated coverslips at 10^5 cells per well in a 24-well plate in 500μl of defined Neurobasal media containing B27, Glutamax I, Albumax II, and penicillin/streptomycin (Gibco-BRL, Carlsbad, CA), supplemented with 100ng/ml of 7.0S NGF per ml (Sigma, St Louis, MO).

ELISA and Western blot

NT-3 immunoreactivity in vitro and in sera was measured using a commercial NT-3 enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, WI). Total cell extract (20μg protein per lane) were separated by PAGE, transferred to an Immobilon-P membrane (0.45μm, Millipore, Bedford, MA), incubated with the primary antibody (anti-human NT-3 1μg/ml, R and D Systems, Minneapolis, MN), followed by horseradish peroxidase-conjugated anti-mouse IgG (1:15,000, Sigma, St. Louis, MO) and visualized with ECL (Amersham).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated using a commercial kit (MicroRNA, Stratagene, La Jolla, CA) and reverse transcription performed using Sensiscript Reverse Transcriptase kit (Qiagen, Valencia, CA) with primers forward, 5′-TCCGTGGCATCCA-AGGCAAC-3′; reverse 5′-GGGGCTCCAAAGGGGTGC-TG-3′ for 35 cycles (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute), separated on 1.2% agarose gel.

Experimental Protocol

All the experiments were carried out on young adult male Sprague-Dawley rats weighing 200 to 250gm at the start of the experiment, and in compliance with approved institutional animal care and use protocols. Three days after vector inoculation, the animals were intoxicated with PDX (Sigma, St. Louis, MO) prepared at 100mg/ml in distilled water immediately before injection, and administered at 400mg/kg intraperitoneally twice a day for 8 days. On day 7 after intoxication, the animals were evaluated by an experimenter blinded to the treatment received by the individual animals.

Electrophysiological Measurements

All recordings were made using a standard clinical electromyography device (Viking II, Nicolet Biomedical, Madison, WI) and Grass needle electrodes. Rats were anesthetized with chloral hydrate (400mg/kg IP), with hindlimbs secured at an angle of 30 to 45 degrees relative to the long axis of the body, subcutaneous temperature maintained at 36 to 37 degrees, and a ground electrode inserted into the tail. Motor nerve conduction velocity and amplitude in the sciatic nerve was determined with a recording electrode inserted in the gastrocnemius muscle. The stimulating electrode pair was placed proximal to the sciatic notch or the knee and a reference recording electrode inserted subcutaneously into the fifth digit of the hindlimb. Both latencies and amplitudes were determined and conduction velocity was calculated. The H wave was recorded after stimulating at the sciatic notch and recording from a clip electrode placed proximal to the fifth digit. At least eight responses were obtained and the maximal H-wave amplitude determined. For sensory nerve recordings, the electrode placed in the sciatic notch was used as the recording electrode; a stimulating electrode was placed at the ankle and the reference electrode placed at the first digit. The statistical significance of the difference between groups was determined by analysis of variance (ANOVA) (Systat 9), using Bonferroni's correction for the multiple post hoc analyses performed.

Behavioral Evaluation of Neuropathy

In order to assess peripheral nerve sensory function, the rats were trained before intoxication to traverse a 3cm-diameter dowel 185cm in length. A pair of black lines 0.6cm in width were painted along the length of the dowel, 1.05cm lateral to the midline on each side. At 7 days after the conclusion of PDX intoxication, each rat was given five trials to cross the beam. The placement of the paw (metatarsophalangeal joint) in relation to the score line, and the number of slips from the dowel were counted from a videotape recording played at slow speed. The statistical significance of the difference between groups was determined by ANOVA (Systat 9) using Bonferroni's correction for the multiple post hoc analyses performed.

Histological Analysis

At the conclusion of the behavioral analysis, rats were perfused with 4% formaldehyde, 2% glutaraldehyde; the lumbar spinal cord, DRG, and sciatic nerve osmicated and embedded in epoxy resin (EPON 812 kit, Tousimis Research, Rockville, MD); and 0.5μm sections stained with toluidine blue. The size distribution of myelinated fibers was determined using a PC-based image analysis program (MCID, Imaging Research, Brock, ON) with a Zeiss Axiophot microscope by a technician blinded to the treatment group.

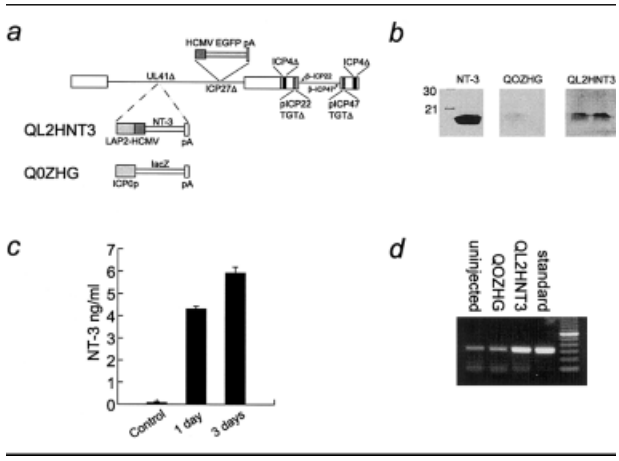

Fig 1. (a) The neurotrophin-3 (NT-3) gene transfer vector expresses NT-3 both in vitro and in vivo. Schematic representation of the herpes simplex virus (HSV) gene transfer vector and control used in this study. Both vectors are defective for the expression of HSV genes ICP4, ICP22, ICP27, and ICP47. The NT-3 expression cassette contains the LAP2-HCMV IEP fusion promoter element in the UL41 locus of the HSV vector. QOZHG has a lacZ sequence under the control of the HSV ICP0 promoter in the UL41 locus. Both vectors contain an EGFP reporter sequence under the control of the HCMV IEp in the ICP27 locus. (b) Western blot detection of NT-3 expression from Vero cells transduced with QOZHG or QL2HNT3. The first lane contains recombinant NT-3 as a control. The middle lanes are from Vero cells infected with QOZHG at MOI of 2 and 4. The last lanes are from Vero cells infected with QL2HNT3 at MOI of 2, and 4. (c) Expression of NT-3 in supernatant of primary DRG neurons in culture transduced with QL2HNT3, measured by enzyme-linked immunosorbent assay. The amount of NT-3 in the medium was elevated at 24 hours (1 day) and 3 days after transduction. The half-life of NT-3 in the medium is approximately 8 hours (data not shown). (d) Detection of NT-3 mRNA by reverse transcription-polymerase chain reaction from lumbar dorsal root ganglion (DRG) of rats after peripheral inoculation with QL2HNT3. A low level of endogenous NT-3 RNA is seen in noninjected and QOZHG-transduced DRG. Transduction with QL2HNT3 resulted in a substantial increase in the expression of NT-3 RNA. The standard shows polymerase chain reaction of QL2HNT3 DNA. A 100bp DNA ladder molecular weight marker is shown on the right.

Results

Expression of NT-3 from Vector QL2HNT3

We constructed the genomic HSV vector QL2HNT3 by insertion of the NT-3 coding sequence into the UL41 locus of an HSV recombinant defective for the HSV immediate early functions ICP4, ICP22, ICP27,

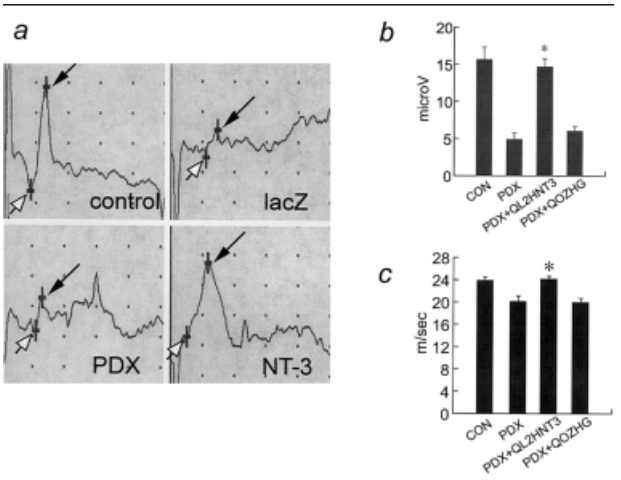

Fig 2. (a) Herpes simplex virus (HSV)-mediated expression of neurotrophin-3 (NT-3) in vivo preserves sensory nerve function after pyroxidine (PDX) intoxication. Examples of individual sensory nerve action potential recorded from rats in the control, PDX-only, QOZHG-treated PDX-intoxicated (lacZ), and QL2HNT3-treated PDX-intoxicated (NT-3) groups, using a Nicolet Viking II. Latency was recorded to the start of the upstroke of the action potential (white arrow) *determined using an algorithm resident in the Viking II. The amplitude was recorded from baseline to the top of the action potential (black arrow), also determined automatically. The amplitude is reduced and the conduction velocity slowed in PDX-intoxicated animals. Both abnormalities are reversed in animals that received QL2HNT3 3 days before intoxication. (b) Mean sensory nerve amplitude (in μV) recorded as shown in (a), for the entire cohort of 8 animals in each group. *$p < 0.005$ compared with either PDX treatment or PDX + QOZHG, analysis of variance (ANOVA). (c) Mean conduction velocity (in m/sec) recorded as shown in (a), for the entire cohort of 8 animals in each group. *$p < 0.005$ compared with either PDX treatment or PDX + QOZHG, ANOVA.*

and ICP47 (Fig 1a). In this vector, the promoter driving NT-3 expression contains both the HSV latency associated promoter (LAP2) sequence[28] and human cytomegalovirus (HCMV) immediate-early promoter (IEp), to provide a combination of immediate and sustained transgene expression. Infection of VERO cells with QL2HNT3 at a multiplicity of infection (MOI) of 2 or 4 resulted in the production of immunoreactive NT-3 detected by Western blot (see Fig 1b) and by ELISA (data not shown). The biological activity of NT-3 was confirmed by the ability of supernatant of infected VERO cells to promote neurite extension in PC-12 cells (data not shown). Transduction of cultures of dissociated primary DRG neurons at an MOI of 1 with QL2HNT3 resulted in the release of 4 to 6ng/ml of NT-3 in supernatant collected at 24 and 72 hours after transfection (see Fig 1c). Expression in vivo was assessed by RT-PCR. Rats were inoculated into each rear footpad with 25μl containing 5×10^6 plaque-forming units of QL2HNT3 or control vector QOZHG. One week after inoculation, NT-3 RNA was detected by RT-PCR of DRG from rats inoculated with QL2HNT3 (see Fig 1d). A low level of expression of endogenous NT-3 was seen in RNA from DRG in uninjected animals, similar to that found in animals injected with the control vector QOZHG (see Fig 1d). No band was seen on PCR of DNA isolated from the same tissue after DNase treatment (data not shown). We also assessed the release of NT-3 into the systemic circulation by ELISA of plasma collected from animals in the experimental groups ($n = 8$ per group) at the time of sacrifice. There was no significant rise in the level of NT-3 in plasma, and no significant difference between QL2HNT3-inoculated (3.9 ± 1.5ng/ml) and QOZHG-inoculated (2.0 ± 0.3ng/ml) animals ($p = 0.59$, t test).

Pyridoxine Neuropathy in the Rat

We examined the biological effect of NT-3 expression in the model of PDX neuropathy. Rats were intoxicated by the injection of PDX, 800mg/kg/day divided into two doses, for 8 days, which produced a characteristic sensory neuropathy, identical to that which has previously been described.[7] Except for the neurological dysfunction, there were no signs of adverse systemic effects of the treatment. The ability of the animals to walk was severely impaired, and treated animals were distinguishable from controls by day 3 of treatment by their abnormal stance and gait, which became progressively worse over the course of PDX treatment. Four groups of 8 animals/group were examined: (1) control, (2) PDX-intoxicated, (3) PDX-intoxicated QOZHG-treated, and (4) PDX-intoxicated QL2HNT3-treated. The animals in the treated groups were injected with vector or control 3 days before PDX intoxication, and formal blinded behavioral analysis and electrophysiological recordings were performed 7 days after the conclusion of the PDX treatment, followed by sacrifice.

Protection Against Neuropathy Measured by Peripheral Sensory Electrophysiological Parameters

Measurement of the evoked sensory nerve action potential revealed a marked decrease in amplitude and slowing of the foot sensory nerve conduction velocity in rats intoxicated with PDX compared with control (Fig 2a). Sensory nerve amplitude was reduced from 15μV in control animals to 5μV in PDX-intoxicated animals (see Fig 2b, $p < 0.005$, ANOVA), and the conduction velocity reduced from 23.5m/sec to 20m/sec (see Fig 2c, $p < 0.005$, ANOVA). In animals transduced with QL2HNT3 3 days before intoxication with PDX the sensory nerve amplitude was 14.8μV (see Fig 2b, $p < 0.005$ compared with PDX alone, ANOVA), while in animals transduced with QOZHG 3 days before intoxication the sensory nerve amplitude (5.5μV)

was no different from the PDX alone treated group (see Fig 2c). Similarly, PDX-intoxicated QL2HNT3-treated animals had a sensory nerve conduction velocity (23.5m/sec) that was identical to control (see Fig 2c, $p < 0.005$ compared with PDX alone, ANOVA), while in PDX-intoxicated animals treated with QOZHG the sensory nerve conduction velocity was 20m/sec, identical to the PDX-treatment group (see Fig 2c). The pure sensory nature of the deficit was confirmed by measurement of the H reflex, which was severely attenuated in rats receiving PDX compared with control (Fig 3a), while the direct M response was unattenuated (see Fig 3a). Animals intoxicated with PDX, or intoxicated with PDX and treated with QOZHG had essentially no detectable H reflex (see Fig 3b), while in animals that had received an injection of QL2HNT3 3 days before intoxication the H wave was 0.6 mV (compared with 1 mV in control animals), demonstrating a substantial though incomplete preservation of the H wave (see Fig 3b, $p < 0.005$ PDX + QL2HNT3 compared with PDX alone or PDX + QOZHG, ANOVA).

Protection Against Neuropathy Measured by Behavioral Performance

In order to test proprioceptive sensory function, the rats were trained before PDX intoxication to walk on a 3.0cm diameter beam, and tested at 7 days after completion of PDX treatment (15 days after the start of treatment and 18 days after vector inoculation) on the same beam. Control animals had no difficulty traversing the beam, indicated by no slips below the score line.[7] Animals intoxicated with PDX experienced substantial difficulty, recording an average of 12 slips from the beam during the test period. Rats transduced with QL2HNT3 3 days before PDX intoxication performed substantially better than PDX-only animals both qualitatively and quantitatively. The performance was videotaped and representative examples are provided as supplementary material on Wiley InterScience (http://www.interscience.wiley.com/jpages/0364-5134/suppmat/index/html). Animals treated with QL2HNT3 recorded an average of three slips (see Fig 3c, $p < 0.005$ compared with PDX-only, ANOVA), while animals that had received the control vector QOZHG recorded an average of 12 slips from the bar during the trial period (see Fig 3c, no different from PDX alone).

Protection against neuropathy measured by histology of nerve After the electrophysiological and behavioral measurements, the animals were sacrificed and cross sections of sciatic nerve reviewed by a blinded examiner for histology and analyzed by quantitative morphometry for fiber size distribution. Sciatic nerve from PDX-intoxicated animals showed substantial fiber loss and the presence of degenerating fibers (Fig 4). The sciatic nerve from animals transduced with QOZHG was indistinguishable from the nerve of PDX-only animals (see Fig 4), while the sciatic nerve of NT-3 treated animals showed preservation of normal nerve morphology. The fiber size distribution (cross-sectional area, including myelin sheath) was determined by measuring all of the fibers in the sciatic nerve from 3 QOZHG-treated PDX-intoxicated and 3 QL2HNT3-treated PDX-intoxicated animals. A total of >14,000 fibers were counted in the three QOZHG-treated nerves, and >17,700 in the three nerves from the QL2HNT3-

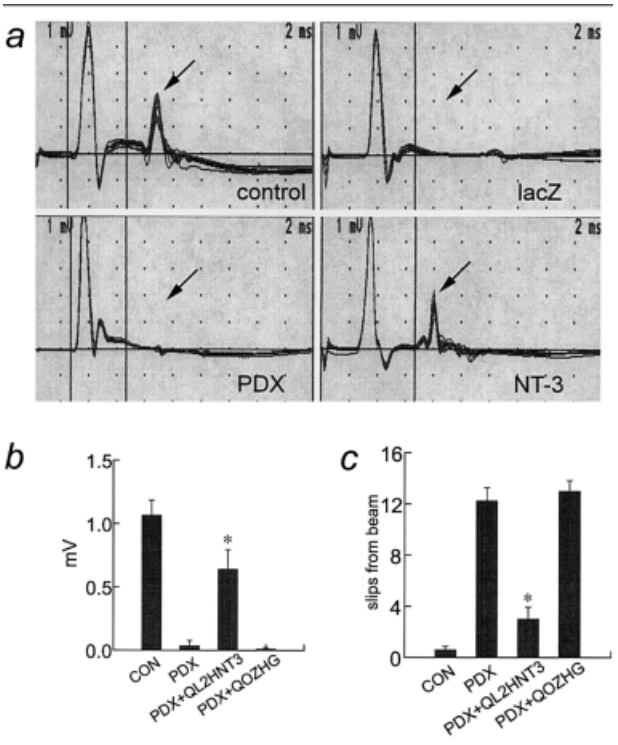

*Fig 3. (a) Herpes simplex virus (HSV)-mediated expression of neurotrophin-3 (NT-3) in vivo preserves H wave after pyroxidine (PDX) intoxication. Examples of individual M wave and H reflex (black arrow) recorded from the gastrocnemius muscle after stimulation at the sciatic notch. At least eight responses were obtained and the maximal H-wave amplitude determined. Representative examples from each of the groups are shown, including control, PDX-only, QOZHG-treated PDX-intoxicated (lacZ) and QL2HNT3-treated PDX-intoxicated (NT-3). (b) Mean H-wave amplitude (mV) recorded as shown in (a), for the cohort of 8 animals in each group. *p < 0.005 compared with PDX alone or PDX + QOZHG, analysis of variance. (c) Behavioral analysis of proprioceptive function. The rats were trained to walk across a 3cm beam, and the number of times there foot slipped below a score line (0.6cm) painted along the length of the beam 1.05cm lateral to the midline during a 3-minute session counted from a videotape recording of the training session. PDX-intoxicated animals have great difficulty traversing the beam because of the deficit in position sensation caused by PDX-intoxication, but this defect is prevented by inoculation with vector QL2HNT3. *p < 0.005 compared with PDX alone or PDX + QOZHG.*

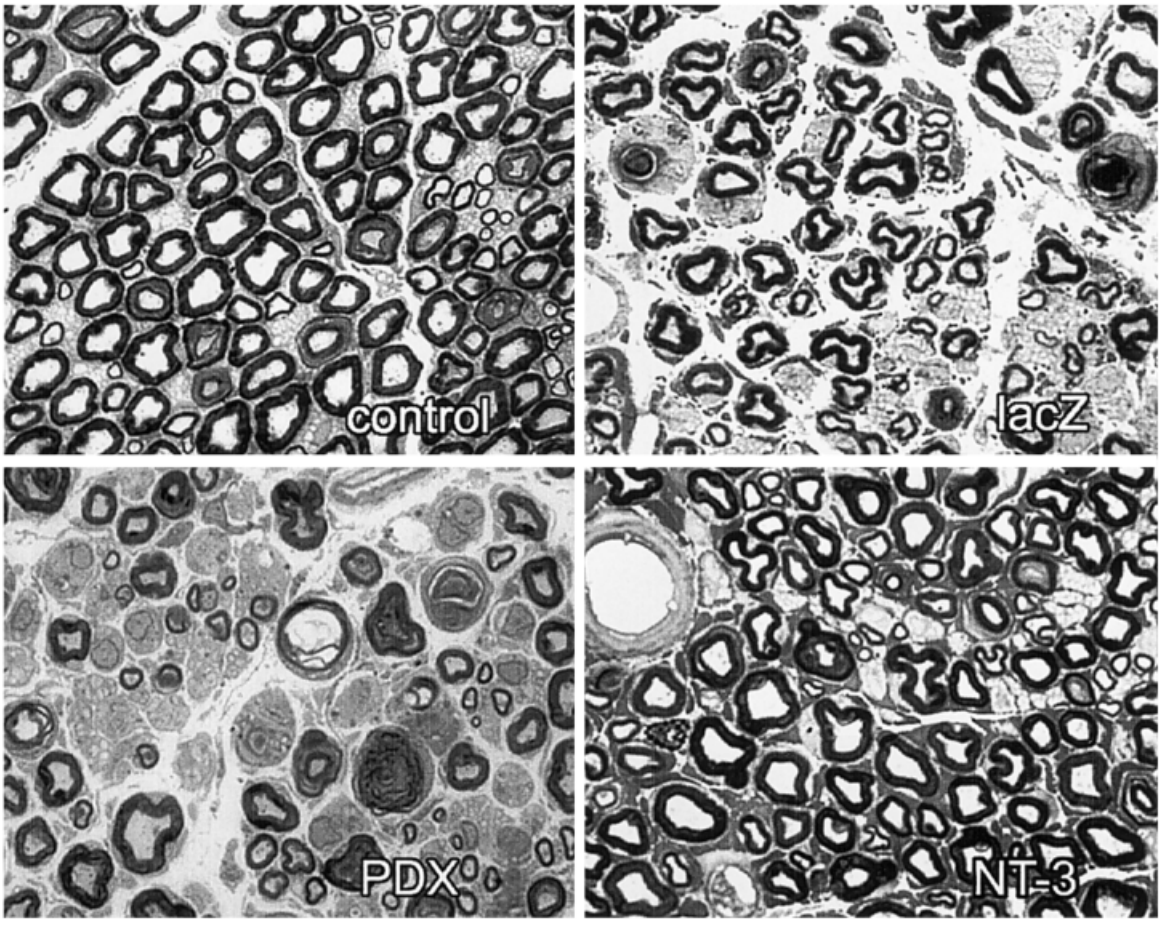

Fig 4. Herpes simplex virus (HSV)-mediated expression of neurotrophin-3 (NT-3) in vivo prevents axonal degeneration caused by PDX intoxication. Cross section of sciatic nerve, 7 days after completion of PDX intoxication. Both the PDX-intoxicated (PDX) and PDX-intoxicated QOZHG-treated (lacZ) nerves show substantial loss of fibers and the presence of degenerating fibers. The PDX-intoxicated QL2HNT3-treated (NT-3) animals have substantial preservation of nerve fiber morphology, in agreement with the preservation of electrophysiological parameters and behavioral measures of nerve function.

treated animals. The loss of approximately 1200 of the 6000 fibers/nerve (approximately 20%) reflects the axonal loss seen in the histology. Quantitative morphometry demonstrated that QL2HNT3-treated PDX-intoxicated animals had preferential preservation of larger myelinated fibers compared with QOZHG-treated PDX-intoxicated nerves (Fig 5), similar to the findings reported after systemic administration of NT-3.[7]

Discussion

We have explored in vivo gene therapy in a rat model of pyridoxine neuropathy, using a recombinant genomic HSV-based vector expressing NT-3 delivered to DRG by subcutaneous inoculation in the foot. This approach resulted in the expression of NT-3 in DRG that prevented the neuropathological changes and preserved sensory function in PDX-intoxicated animals as reflected in electrophysiological measures, and in the ability of the PDX-intoxicated QL2HNT3-treated animals to walk on a beam. Despite substantial evidence in animal models demonstrating that neurotrophic factors are beneficial in the treatment or prevention of peripheral neuropathy, the treatment of human neuropathies has been limited, in part because of the short half-life of these peptide factors and the side effects engendered by systemic administration. The results of the current report demonstrate that gene transfer of the coding sequence for NT-3 to the DRG using an HSV-based vector, to produce expression of NT-3 directly in DRG, may circumvent the delivery problem, and thus allow the use of the therapeutic potential of these trophic factors in the treatment of neuropathy.

There are several reasons why we chose to investigate pyridoxine neuropathy. First, the toxin has a selective neurotoxic action on large DRG neurons without causing systemic morbidity. Second, a relatively short course of intoxication results in the subacute development of neuropathy. Third, there are dramatic and unambiguous histological, electrophysiological, and corre-

lated behavioral manifestations. Fourth, a previous study demonstrated that systemic administration of NT-3 prevents the development of neuropathy in the same model.[7] PDX-induced neuropathy in the rodent mimics precisely the human disease in its clinical, electrophysiological, and histological parameters, and in that way is an excellent model for the human disease. Human pyridoxine-induced neuropathy is not a common condition, but as a pure degeneration of peripheral sensory neurons provides data that may be applied to more common peripheral neuropathies such as those that complicate a systemic disease (eg, diabetes), or resulting from the administration of neurotoxic drugs for other purposes.

PDX intoxication causes a predominant but not exclusive degeneration of large diameter neurons in the DRG and the large sensory fibers derived from those neurons.[7,22,23] The molecular mechanisms responsible for the axonal degeneration in PDX neuropathy are not fully understood[22,23] but can be reversed in animal models by systemic administration of NT-3.[7] During development, NT-3 promotes the survival of proprioceptive afferents in vitro,[29] and NT-3 knockout mice lack proprioceptive afferents and peripheral sense organs.[30–32] Knockout mice lacking the high-affinity NT-3 receptor trkC lack Ia muscle afferents,[33] although cell loss in NT-3 knockout mice is not limited to trkC-expressing neurons.[34] In the adult rat, trkC continues to be expressed in a population of predominantly large sensory neurons,[35] but the role of NT-3 and activation of trkC in the adult remains unknown.

In this study, the vector was administered by subcutaneous inoculation and carried by retrograde axonal transport from that site to the DRG, where expression of transgene-mediated NT-3 could be demonstrated by RT-PCR. Some of the neuroprotective effects, such as the protection measured by sensory nerve amplitude recorded from stimulation of the foot, occurred in neurons whose axons project to the inoculation site and are therefore likely to contain the vector. Other protective effects of the vector, such as preservation of H-wave amplitude, reflect effects on primary spindle afferents whose peripheral projections terminate in the gastrocnemius, distant from the subcutaneous inoculation site. It is possible that transgene-mediated NT-3 released by the central terminals of transfected skin afferents in the spinal cord was taken up by the central terminals of the spindle afferents to provide trophic support from the periphery. Transgene-mediated NT-3 may be released by the neuronal perikaryon of transfected cells in the DRG, binding directly to trkC receptors on other neurons in the ganglion. There is no evidence to support the transneuronal transport of nonreplicating vectors from cell-to-cell in the DRG, although this occurs for replicating virus in sensory ganglia.

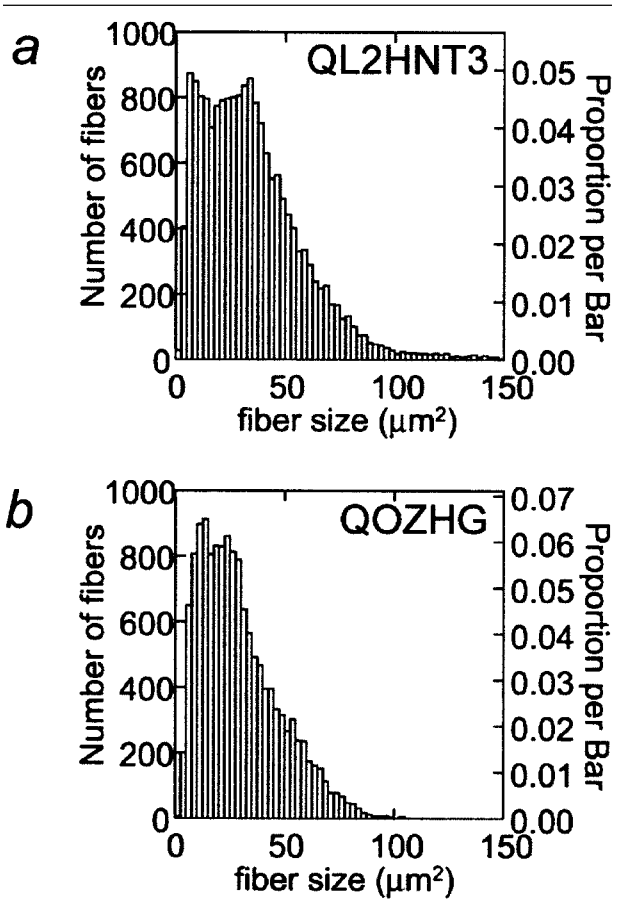

Fig 5. Herpes simplex virus (HSV)-mediated expression of neurotrophin-3 (NT-3) in vivo prevented the loss of large myelinated fibers. Size distribution of fibers in the sciatic nerve was determined by quantitative morphometry. All of the fibers in three nerves from each group were analyzed using a PC-based morphometry program (MCID, Imaging Resources). Histograms representing the number of fibers (left axis) and the proportion of the total fibers (right axis) plotted against the fiber size (in μm^2 are shown for (a) PDX-intoxicated QL2HNT3-treated and (b) PDX-intoxicated QOZHG-treated animals. Treatment with QL2HNT3 results in the substantial protection of fibers in the larger ($\geq 30 \mu m^2$) range compared with treatment with QOZHG.

Systemic administration of trophic factors is effective in preventing the development of neuropathy in a wide array of rodent models. However, difficulties inherent in achieving adequate concentrations of the trophic factor in the specific locations where they may be required, without causing toxicity related to their effects at distant sites, limits their application to the treatment of human disease. Previous demonstrations of the delivery and expression of trophic factor genes after peripheral inoculation have been limited to neonatal mice; an adenoviral vector expressing NT-3 injected into muscle 3 days after birth protects motor neurons from degeneration in *pmn* mice,[36] but no previous

studies have addressed the problems of the adult peripheral nervous system. HSV is uniquely suited as a vector for gene transfer to sensory neurons of the peripheral nervous system. The virus naturally and efficiently targets neurons in peripheral sensory ganglia, directed by high-affinity binding to a receptor (nectin-1),[37,38] which is expressed to high levels on sensory neurons.[39] The wild-type parental virus naturally establishes a lifelong latent state in neurons, and replication-incompetent vectors are forced into the latent state by default, prevented from entering the lytic cycle by the deletion of essential immediate early gene (ICP4 and ICP27 in the vector employed). As demonstrated in the current report, expression of a neurotrophic transgene from latent HSV vector genomes produces a therapeutic effect that protects against the development of neuropathy.

In conclusion, HSV-mediated neurotrophin gene transfer represents an effective strategy to protect neurons from degeneration characteristic of peripheral neuropathy. In the experimental model employed in this study the vector was administered before intoxication with pyridoxine. While the same paradigm may be used to prevent a drug-induced neuropathy (eg, taxol) most acquired and inherited neuropathies are subacute to chronic in their progression and not identified until nerve damage has already begun. Nonetheless, the data in the current report would suggest that, even in the course of neuropathy, neurons that have not yet degenerated might be rescued by gene delivery of the HSV vector-mediated neurotrophin.

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