Peripheral neuropathy is a common and debilitating complication of diabetes. In animal models, neurotrophic factors can prevent progression of the neuropathy, but adverse effects prevent systemic administration in adequate doses to treat human disease. We examined whether gene transfer with replication-defective genomic herpes simplex virus (HSV) vectors modified to express nerve growth factor (NGF) could be used to prevent progression of neuropathy in mice. Diabetes induced by streptozotocin (STZ) resulted in a sensory neuropathy manifest by a decrease in the foot sensory nerve amplitude (FSA; control = 20 ± 0.1 μV, treated = 14 ± 0.1 μV). Transduction of dorsal root ganglia in vivo with an HSV-based vector expressing NGF under the control of the human cytomegalovirus immediate early promoter (vector SHN) or the HSV latency active promoter 2 (vector SLN) by footpad inoculation 2 weeks after STZ administration protected against the decrease in FSA (22 ± 1.4 μV and 21 ± 1.7 μV, respectively) measured 4 weeks later. Injection of SHN into inguinal adipose tissue 2 weeks after onset of diabetes also prevented the decrease in FSA (20 ± 3.3 μV). These results suggest that gene transfer with an NGF-producing herpes-based vector may prove useful in the treatment of diabetic neuropathy. Diabetes 51:2227–2232, 2002

Neuropathy is a common and debilitating complication of diabetes. The most prevalent form of neuropathy is predominantly sensory, with symptoms beginning distally in the lower extremities and ascending as the disease progresses. Motor nerve dysfunction usually occurs some time after sensory and autonomic nerve abnormalities (1,2). Abnormalities of protein glycation, sorbitol accumulation, and microvascular hypoxia have been described. In addition, retrograde axonal transport of both nerve growth factor (NGF) and neurotrophin-3 (NT-3) is decreased in experimentally induced diabetes (3–6), and there seems to be a decrease in target tissue concentrations of both of these trophic factors (5,7,8). A primary role for any of these abnormalities in the cause of neuropathy has not been established (rev. in 2).

Despite uncertainty regarding the role of trophic factor deficiency in the cause of diabetic neuropathy, several studies have demonstrated that systemic administration of various trophic factors can be used effectively to reverse the signs of diabetic neuropathy in animal models. Systemic administration of recombinant human NGF (rhNGF) inhibits diabetes-related changes in nerve fiber morphology and reductions in the sensory nerve peptides substance P (SP) and calcitonin gene-related peptide (CGRP) (4,9) as well as diabetes-related electrophysiologic abnormalities (1,10). Treatment with NT-3 reverses nerve conduction deficits in diabetic rats (4). Insulin-like growth factor halts the progression of hyperalgesia and ameliorates the impairment in sensory nerve regeneration in diabetic rats (11,12). Intrathecal administration of glial cell line–derived neurotrophic factor reverses deficits in nonpeptidergic spinal cord afferents in diabetic rats (13). Systemic injections of brain-derived neurotrophic factor reduce blood glucose in the obese, non–insulin-dependent db/db mouse regardless of food intake (14,15).

The impressive effects of trophic factor therapy in animal models resulted in a human trial of rhNGF to treat diabetic neuropathy. In phase 1, non–life-threatening but bothersome adverse effects occurred at doses of 1 μg/kg (16), so the subsequent phase 2 and phase 3 studies used rhNGF at 0.1 or 0.3 μg/kg administered three times a week. In the phase 2 study, a mild improvement in one measure of sensory function (cooling detection threshold) in patients with diabetes was reported (17). However, in the multicenter phase 3 trial, no effect of rhNGF treatment (administered at 0.1 μg/kg, three times a week) could be detected in the primary outcome measurement (Neuropathy Impairment Score) or in most of the secondary outcome measurements (18). There are several possible explanations for the difference between preclinical animal studies and the human trial. The preclinical animal studies used NGF in doses of 3–5 mg/kg, compared with the dose...
of 0.1 µg/kg used in the human trial. Given the short serum half-life of NGF (7.2 min as measured in the rat [19]), it is not surprising that intermittent low-dose bolus injections failed to achieve a therapeutic effect.

Gene transfer represents a novel means to express identified transgenes in targeted locations in the nervous system, and herpes simplex virus (HSV)-based vectors have a special utility for primary sensory neurons. Wild-type HSV is a neurotropic double-stranded DNA virus that is carried naturally by retrograde axonal transport from the epithelial surfaces to dorsal root ganglia (DRG), where the viral genome may establish a life-long latent state (20–22). Because HSV genes involved in viral replication are expressed in a rigid temporal cascade, deletion of essential immediate early genes from the HSV genome allows the creation of HSV-based vectors that are incapable of replicating in normal tissue in vivo (23) but that nonetheless efficiently establish a quiescent state similar to natural viral latency without the potential for reactivation (24,25).

We have demonstrated that genomic HSV-based vectors that contain the coding sequence of β-NF express biologically active NGF in peripheral sensory neurons in vitro and in vivo (26). We have shown that an HSV-based vector that contains the coding sequence for NT-3 delivered locally to DRG by subcutaneous inoculation into the foot prevents the subacute neuropathy caused by intoxication with pyridoxine (PDX; vitamin B6) in rats in vivo (27). In the current study, we tested the hypothesis that delivery and continuous expression of NGF in peripheral sensory neurons by an HSV vector could prevent the development of neuropathy in STZ-induced diabetic mice. We report that vector-mediated NGF delivered locally to DRG via footpad inoculation or systemically via intraginal adipose tissue injection protected against STZ-induced peripheral neuropathy in the mouse.

**RESEARCH DESIGN AND METHODS**

**Vectors.** Vectors SHN and SLN (Fig. 1) were created as previously described (26). A lox-containing plasmid (pNGF-lox) with the murine β-NF cDNA fused to the SV40 late polyA was inserted into the thymidine kinase (tk) locus of the ICP4-deleted HSV vector d120::tk-lox, a modified version of d120 (23) that had been engineered to contain a loxP site at the tk locus by cre-lox recombination (28). The pNGF-lox plasmid also contained the HSV-1 glyco-converting enzyme (E), which had been engineered to contain a loxP site at the tk locus by cre-lox recombination (26). A lox-containing plasmid (pNGF-lox) with the murine β-NF gene carrying the coding sequence for NT-3 delivered to peripheral sensory neurons in vitro (20,21) was inserted into the viral tk locus. All three vectors are deleted for the immediate early gene ICP4 (indicated by the solid bar).

**Quantitative RT-PCR.** Two weeks after electrophysiological measurements, the L4, L5, and L6 DRGs were removed, immediately frozen on dry ice, and stored at −80°C until used for peptide RNA content. Quantitative RT-PCR was performed using an Applied Biosystems GeneAmp 5700 Sequence Detection System and Real-time TaqMan PCR (Applied BioSystems, Foster City, CA). RNA was extracted from pooled DRGs from each animal using a Stratagene MicroRNA isolation kit as per the manufacturer’s instructions (Stratagene, La Jolla, CA). RNA samples were treated with DNase I (Amersham, Piscataway, NJ), and 5 µl was reverse-transcribed using the Senscript Reverse Transcriptase kit (Qiagen, Valencia, CA) in a 20-µl reaction volume. Real-time RT-reactions were set up as follows: 1 µl reverse-transcribed RNA, 0.225 µM each of 200 µM oligo(dT) forward and reverse primers, 0.125 µl of nucleotide-free water. Forty cycles of amplification were performed: 95°C for 15 s melting, 60°C for 1 min annealing/extension. ABI PRISM Primer Express software was used to design primer/probe sets for CGRP (forward primer 5'-GGCTAGGAGGCT CTAGTGTCACT-3'; reverse primer 5'-CCGCCGATGAGGTCA-3'; probe 5'- FAM-CAGAAGATCTGGAACACGTGCAC-TAMRA-3') and SP (forward primer

**FIG. 1. Schematic representation of genomic HSV vectors.** Expression cassettes for NGF (vectors SHN and SLN) or β-galactosidase (vector SHZ) were inserted into the viral tk locus. All three vectors are deleted for the immediate early gene ICP4 (indicated by the solid bar).
Mice that received an injection of STZ had elevated blood glucose concentrations that did not differ between vector-treated groups. At the time of testing, 6 weeks after STZ administration, the diabetic animals also had a decrease in body weight that reached statistical significance only in the group that received an injection of the SHZ vector. **P < 0.01 ANOVA with Bonferroni/Dunn.

NGF measurements. Tail vein blood was collected and centrifuged immediately, and the plasma was stored at −80°C until assayed. NGF concentrations were measured from 1 μl of plasma using a commercially available enzyme-linked immunosorbent assay (Promega, Madison, WI) according to the manufacturer’s directions. Statistical analysis. The electrophysiological measurements and quantitative RT-PCR results for all treatment groups were analyzed by ANOVA (Statview; SAS Institute, Cary, NC) using the Bonferroni/Dunn or Fisher’s protected least significant difference (PLSD) correction. All values are presented as means ± SE.

RESULTS

Mice that received an injection of STZ had elevated blood glucose concentrations within 1 week of the injection. At the time of testing, 6 weeks after STZ administration, the blood glucose was 22.9 ± 1.3 mmol/l for the diabetic animals and 7.0 ± 0.5 mmol/l for the controls (Fig. 2A). The diabetic animals also had a decrease in body weight (31.0 ± 0.6 g diabetes; 35.1 ± 1.0 g control), a weight loss representing ∼15% (Fig. 2B). There were no significant differences in blood glucose concentrations between the different vector treatment groups. The average weights for all of the diabetic groups were less than that of the normal control mice, but only in the animals that received SHZ was the difference statistically significant (29 ± 1.5 g STZ; 35.1 ± 1.0 g; P < 0.05).

Diabetic animals developed a neuropathy manifest by a reduction in the FSA, which was substantial and significant (diabetic 14.0 ± 0.7 μV; control 19.9 ± 0.7 μV; P < 0.001; Figs. 3 and 4A). Animals that were transduced with SHN showed no decrement in FSA (22.3 ± 1.4 μV), as did animals that were transduced with SLN (20.8 ± 1.8 μV). Transduction of DRG of diabetic mice with the lacZ-expressing control vector SHZ had no effect on FSA, which was reduced to the level of untreated diabetic mice (14.7 ± 1.3 μV; Fig. 4A). There was no correlation between FSA and weight (Fig. 4B). There was no difference in the foot sensory conduction velocity between normal control and diabetic mice or diabetes-associated changes in muscle or tail nerve responses (data not shown).

A consistent finding in animal models of diabetes is reduced expression of CGRP and SP, an abnormality that can be reversed by administration of NGF (1,4,30). Both CGRP and SP RNA amounts determined by quantitative real-time RT-PCR were reduced in the STZ-diabetic mice (P < 0.05 by paired t test). Transduction of the right lumbar DRG by subcutaneous inoculation of SHN into the
right foot resulted in a 1,000-fold increase in CGRP RNA \((P < 0.001)\) and a 400-fold increase in SP RNA \((P < 0.001)\) in diabetic mice (Fig. 5). Transduction of the DRG by subcutaneous inoculation of SLN resulted in a 270-fold increase in CGRP \((P < 0.05)\) and a 70-fold increase in SP RNA (NS). Levels of both CGRP and SP RNA were increased in the DRG on the side opposite footpad inoculation (CGRP 550-fold increase, SP 240-fold increase; \(P < 0.005\)). Although this increase was statistically significant compared with the SHZ control animals, it was significantly less than the increase in CGRP and SP in the ipsilateral DRG (left compared with right, CGRP \(P < 0.0001\), SP \(P < 0.005\)).

Because vector transduction of remote sites in rabbits and primates has been reported to increase circulating NGF (31), we examined whether constitutive release from a site remote from the peripheral nerve might produce elevated concentrations of circulating NGF in the mouse that could be protective against neuropathy. Mice received an injection of 5 \(\mu\)L containing 5 \(\times 10^6\) pfu SHN or SHZ into inguinal adipose tissue bilaterally. Injection with SHN resulted in an increase in plasma NGF that reached statistical significance by 5 weeks (SHN 1,180 \(\pm\) 480 ng/ml; SHZ 290 \(\pm\) 90 ng/ml; \(t\) test \(P < 0.05\)). At 6 weeks, the mice were reinoculated with the same vectors into the same sites. Reinoculation with SHN resulted in a 10-fold increase in plasma NGF (SHN 3,200 \(\pm\) 740 ng/ml; SHZ 340 \(\pm\) 100 ng/ml; \(t\) test \(P < 0.05\)), an increase that persisted for 11 months (Fig. 6). Two weeks after the induction of diabetes by STZ, mice were inoculated with 5 \(\mu\)L containing 5 \(\times 10^6\) pfu of SHN or SHZ into inguinal adipose tissue bilaterally. Animals that were transduced with SHN demonstrated preservation of FSA (20.0 \(\pm\) 3.3 \(\mu\)V) similar to control animals, whereas animals that received an injection of SHZ had a reduction in FSA (SHZ 12.6 \(\pm\) 1.3 \(\mu\)V; control 22.2 \(\pm\) 1 \(\mu\)V; \(P < 0.05\)) similar to the untreated diabetic animals (Fig. 7).

**DISCUSSION**

This is the first report of viral vector-mediated gene transfer in vivo to treat diabetic neuropathy. In our model, the principal difference between the STZ-diabetic and normal mice was a decrease in the FSA, consistent with the most common pattern of nerve involvement in human diabetic neuropathy. Expression of NGF achieved by transduction with either vector SHN or SLN was effective in preserving sensory nerve function in the diabetic mice. Transduction with SHN preserved nerve physiology measured at 6 weeks after the onset of diabetes and 4 weeks after vector inoculation and caused an increase in DRG content of CGRP and SP RNA 8 weeks after the onset of diabetes and 6 weeks after vector inoculation.
Vector inoculation of diabetic animals increased CGRP and SP RNA in the transduced DRG. This is in agreement with other studies that have demonstrated an increase in CGRP and SP protein in NGF-treated diabetic animals (1,4,30) and demonstrates a transgene-mediated effect at the level of the cell perikaryon in addition to the protection of nerve function measured electrophysiologically. We did not examine these animals for evidence of TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling) staining or apoptotic cells in the DRG (32). The smaller increase in CGRP and SP RNA in SLN-treated diabetic animals is consistent with the smaller amount of NGF expressed by the LAP2 promoter element that we have found in other model systems using similar HSV-based vectors (data not shown). Nevertheless, the amount of NGF produced by SLN was sufficient to prevent the reduction in FSA caused by diabetes. Long-term studies will be required to determine whether there is a difference in protection between HCMV IEp– and LAP2-driven transgene expression at 3 or 6 months of diabetes. We observed an effect of transgene-mediated NGF expression on neuropeptide content in the DRG contralateral to the transduced DRG in animals that were inoculated in one footpad. The increase in the contralateral DRG, which was significantly smaller than the increase found in the ipsilateral DRG, suggests that there is both a local effect on transduced DRG and a remote effect that likely is mediated by release of NGF from transduced sensory neurons. Whether this effect occurs through release into the cerebrospinal fluid at the central terminal or release from the peripheral terminal into the systemic circulation has not been determined.

Systemic release of NGF achieved by transduction of adipose tissue with vector SHN also protected against the loss of FSA. Constitutive, continuous production of NGF from vector-injected adipose tissue could provide an attractive pharmacologic alternative to intermittent high-dose bolus injections of short-lived trophic factors (33). Similar results have recently been reported after injection of an adeno viral vector coding for NT-3 into muscle (34). However, in comparison to transduction of DRG, achieving effective systemic levels of NGF by remote inoculation in larger animals may be difficult. Adenoviral injection into muscle of the rat required 5–10 separate injections into four different muscles (20–40 inoculations) of 1010 pfu of the adenoviral vector (34). The volume of SHN injected into fat in our experiments (5 μl into two sites) represents ~0.03% body wt of the mouse; if peptide pharmakinetics were similar and scale-up were linear, then a 70-kg man would require inoculation with 20–25 ml of vector. In contrast, delivery from peripheral inoculation to DRG would not require a commensurate increase in volume.

We have recently demonstrated that an HSV-based vector expressing NT-3 (QL2HNT3) can prevent peripheral nerve degeneration caused by an overdose of PDX (27). An 8-day course of high-dose PDX is toxic to large sensory fibers, causing a marked decrease in the amplitude and slowing of the conduction velocity in sensory nerves, a deficit in proprioceptive sensory function, and a decrease in the number of large fibers in the sciatic nerve. All of these changes were prevented by inoculation with QL2HNT3. The current report is based on the same principles and extends those observations in several important aspects. In STZ diabetes, the neuropathy develops gradually over the course of several weeks; in comparison, the PDX intoxication model is subacute. In the current report, animals were rendered diabetic by injection of STZ and the vector inoculation 2 weeks later; in comparison, in the PDX intoxication model, we injected the vector before PDX treatment. In addition, diabetic neuropathy is a frequent complication of a common human disease, whereas PDX intoxication is an exceedingly rare cause of human neuropathy.

Because SHN and SLN were constructed using the murine NGF gene, we cannot distinguish between vector-produced NGF and endogenous NGF, and we were unable to establish unequivocally vector-mediated NGF expres-
sion in transduced DRGs directly. Two lines of evidence, however, suggest that we have directly transduced DRG neurons in this model. In our study of PDX neuropathy, the QL2HNT3 vector was delivered to rat DRG by footpad inoculation and protected against PDX toxicity without an increase in circulating NT-3 (27). In a study using inoculation of SHN into the bladder wall of rats, NGF immuno-reactive neurons were found in the L6 DRG (35).

The current report demonstrates benefits of the local production and release of transgene-mediated NGF to act in an autocrine or paracrine manner to prevent nerve degeneration in a model of diabetic neuropathy. NGF is not the only factor that may be protective in diabetic neuropathy. There is evidence to suggest that vascular endothelial growth factor (36), IGF-1 (12), or NT-3 (34) may be useful, but all are likely to require an effective means of delivery (33). The current study suggests that HSV-mediated gene transfer to the DRG may have some utility in this regard.

ACKNOWLEDGMENTS

This work was supported by grants from the Department of Veterans Affairs (D.J.F. and M.M.), the National Institutes of Health (D.J.F. and J.C.G.), and the Juvenile Diabetes Foundation International (D.J.F.).

We acknowledge the excellent technical assistance of Mark O’Malley and Veljko Puskovic.

REFERENCES

5. Fernyhough P, Diemel LT, Tomlinson DR: Target tissue production and axonal transport of neurotrophin-3 are reduced in streptozotocin diabetic mice. Exp Neurol 161, 2001