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Cytokine production in motor neurons by poliovirus replicon vector gene delivery

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Poliovirus replicon vectors transiently express foreign proteins selectively in motor neurons of the anterior horn of the spinal cord. Here we intraspinally inoculated mice transgenic for the poliovirus receptor (PVR) with replicons encoding murine tumor necrosis factor alpha (mTNF-α). We detected high-level expression of mTNF-α in the spinal cords of these animals at 8–12 h post inoculation; this returned to background by 72 h. The mice exhibited ataxia and tail atony, whereas animals given a replicon encoding green fluorescent protein (GFP) exhibited no neurological symptoms. Histology of spinal cords from mice given the replicon encoding mTNF-α revealed neuronal chromatolysis, reactive astrogliosis, decreased expression of myelin basic protein, and demyelination. These animals recovered with only slight residual damage. This study shows that replicon vectors have potential for targeted delivery of therapeutic proteins to the central nervous system and provide a new approach for treatment of spinal cord trauma and neurological disease.

The use of neurotrophic viruses as vectors for targeted gene delivery to the central nervous system (CNS) has many applications for the development of new therapies for neurological diseases and spinal cord trauma. Poliovirus is attractive for the development of such a gene delivery vector because it has been established in humans that once poliovirus invades the CNS, infection is restricted to the motor neurons of the hindbrain and spinal cord[1]. To exploit the unique features of poliovirus tropism, poliovirus genomes (referred to as replicons) were constructed to encode foreign proteins in place of the capsid proteins[2–5]. Because replicons do not encode capsid proteins, they undergo only a single round of infection, without spreading to neighboring cells[3,6,7]. Replicons are encapsidated by providing the capsid proteins in trans using a recombinant vaccinia virus (VV-PI). Because no infectious poliovirus is generated during production of replicons, the use of replicons for gene delivery purposes following worldwide poliovirus eradication will not be a concern. Replicons maintain the tropism of poliovirus in the CNS and exclusively infect spinal cord and brainstem motor neurons[8]. However, replicons can mediate gene delivery in animals that have been previously immunized with poliovirus, indicating that preexisting immunity in humans from vaccination will also not be a limitation for use of replicons[9].

Cytokines have the potential to modulate gene expression in many different cell types of the CNS[10,11]. Due to their potent biological activities, untargeted and uncontrolled expression of cytokines can result in severe pathogenesis. For example, a transgenic mouse line with continuous CNS-specific expression of tumor necrosis factor alpha (TNF-α) developed a demyelinating disease, marked by seizures, ataxia, and paresis leading to early death[12]. Thus, a vector system to deliver biologically active cytokines to the CNS should mediate transient but high levels of expression to alter the function of different cell types of the CNS without the pathogenic effects of sustained expression.

To test poliovirus-based replicons for this explicit purpose, mice transgenic for the human poliovirus receptor (PVR) were inoculated intraspinally with the replicon encoding biologically active TNF-α, because this cytokine is known to affect many cell types in the CNS (refs 10, 11). Production of TNF-α in the spinal cord was detected for up to 72 h post inoculation. Histological analysis revealed neuronal chromatolysis, demyelination, astrogliosis, and microgliosis. However, animal death associated with TNF-α did not occur, and histological analysis revealed that the animals partially recovered one month post inoculation. The results of these studies provide the foundation for the further development of replicons to deliver biologically active molecules to the CNS microenvironment.

Results

Replicon encoding murine TNF-α. The replicon encoding mTNF-α was based on the replicon used for expression of biologically active interleukin 2 (IL-2) (ref. 13). The 467 bp gene encoding wild-type, soluble mTNF-α (nucleotides 117–484 encoding a protein of predicted molecular mass of 17 kDa) was subcloned into the replicon cDNA. The resulting construct contained the complete coding sequence for mTNF-α positioned between the VP0 and 2A genes of poliovirus; amino acids corresponding to the cleavage sites for 2A were positioned at the N and C termini of mTNF-α (Fig. 1A). A replicon encoding green fluorescent protein (GFP) (Clontech, Palo Alto, CA) was also constructed; the details will be published elsewhere (Jackson, C.A. et al., in preparation). To confirm the expression of the foreign protein from the replicon, HeLa H1 cells were infected for 6 h with replicons encoding either mTNF-α or GFP (to serve as a control). The cultures were metabolically labeled followed by immunoprecipitation with an anti-mTNF-α antibody. A 17 kDa protein was immunoprecipitated from the lysates of cells infected with the replicon encoding mTNF-α, but not lysates from the replicon encoding GFP (data not shown).

To determine the kinetics of mTNF-α expression, HeLa HI cells were infected with the replicons encoding either mTNF-α or GFP. At
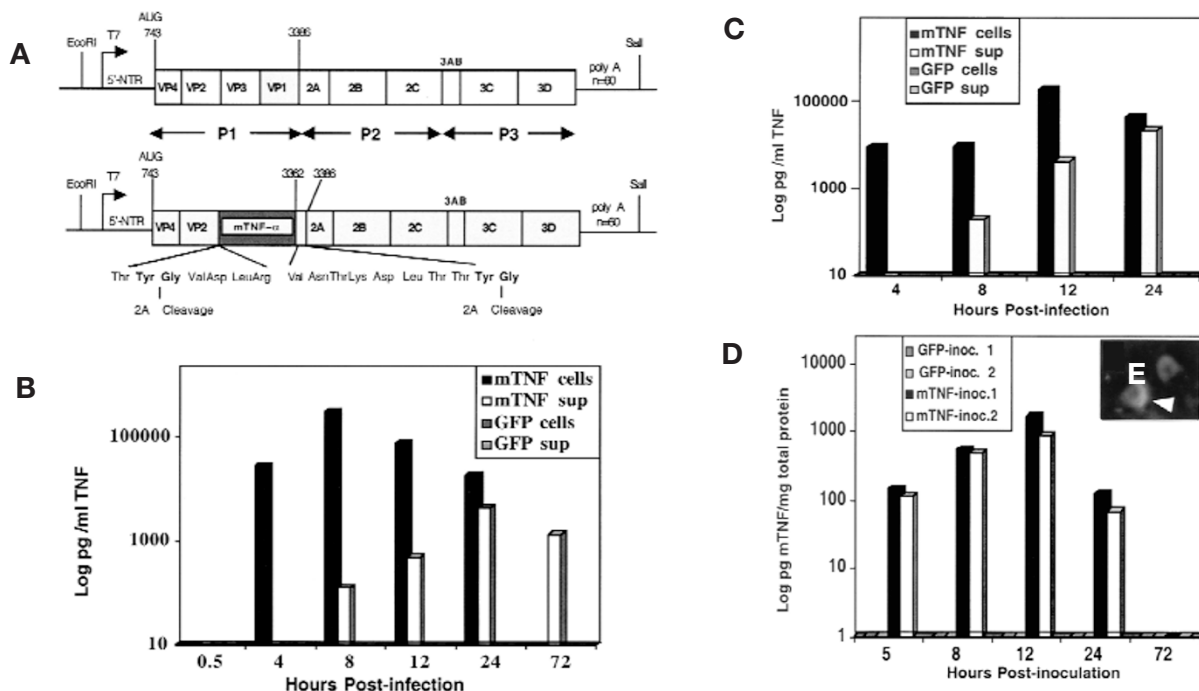
Figure 1. Construction and characterization of the replicon encoding mTNF-α. (A) Replicon encoding mTNF-α. The mTNF-α cDNA was subcloned into the poliovirus infectious cDNA clone in place of the VP2, VP3, and VP1 genes[13]. The resulting plasmid, pT7VP4 VP2 mTNF-α, contains the complete gene encoding soluble mTNF- α, positioned between nucleotides 1766 and 3359 and flanked by cleavage sites for the 2A[pro] of poliovirus to release the mTNF-α protein from the poliovirus polyprotein[13]. **(B)** In vitro expression of mTNF-α. HeLa HI cells were infected with the replicon encoding mTNF-α or, as a control, the replicon encoding GFP at an approximate multiplicity of infection of 10 for 6 h, as described in the Experimental Protocol. Cell lysates and supernatants were analyzed for TNF-α, expression using an ELISA (R&D Systems, Inc). The results shown are representative of three independent experiments. **(C)** Biological activity of mTNF-α expressed from the replicon. HeLa H1 cells were infected with replicons encoding either mTNF-α or GFP as described in the Experimental Protocol for experiments shown in (B). The supernatants and cell lysates were assayed using WEHI cells. To correlate activity with amounts of mTNF-α, a standard curve with known amounts of recombinant mTNF-α (R & D Systems, Inc.) was used. The results shown are representative of two independent experiments. **(D)** In vivo expression of mTNF-α from the replicon. Mice were inoculated intraspinally with the replicons encoding either mTNF-α or GFP. At various times post inoculation, mice were killed, and the spinal cords were extracted, homogenized, and assayed for TNF-α expression by ELISA. Each bar represents the value obtained from the spinal cord of a single mouse at that designated time point. The values presented have been normalized for total protein (1 mg). **(E)** Expression of mTNF-α in neurons. Mice were inoculated with the replicon encoding mTNF-α and killed 8 h later. Spinal cord sections were immunostained with antibodies to mTNF-α and a rhodamine-conjugated secondary antibody; the neuronal marker NeuN and a fluorescein isothiocyanate isomer conjugated secondary antibody was used to identify neurons. The yellow-stained cell (white arrowhead) shows a neuron labeled with antibodies to both TNF-α and NeuN. A neuron not infected by the replicon is also present (green cell). Lumbar enlargement of the spinal cord, ×400.

specified times post infection, the amount of TNF-α produced was determined using an enzyme-linked immunosorbent assay (ELISA) (Fig. 1B). Intracellular mTNF-α was detected at 4 h post infection and peaked between 8 and 12 h. By 72 h post infection, no mTNF-α was detected from cell lysates. At this time, most of the cells in the culture were lysed as a result of a cytopathic effect from the replicons. Starting at 8 h post infection and peaking at 24 h post infection, TNF-α was also detected in the cell supernatant. To test the biological activity of TNF-α, supernatants and cell lysates from cells infected with replicons were assayed for cytotoxicity on WEHI cells[14]. Supernatants and lysates from cells infected with the replicon encoding GFP showed no cytotoxic effect, whereas both cell lysates and supernatants from the replicon encoding mTNF-α exhibited cytotoxic activity on the WEHI cells; ~95% of the amount of mTNF-α detected by ELISA was biologically active in vitro (Fig. 1C).

To determine if the replicon encoding mTNF-α could increase the amounts of mTNF-α in the CNS, PVR transgenic mice were inoculated intraspinally. Poliovirus infection in PVR transgenic mice mimics the CNS pathogenesis seen in humans[15,16]. TNF-α expression was detected in extracts from the spinal cords by 4 h post inoculation, with peak activity between 8 and 12 h; the mTNF-α concentrations returned to background concentrations by 72 h (Fig. 1D). No mTNF-α expression was detected in the lysates from spinal cords inoculated with the replicon encoding GFP. Consistent with previous studies

using replicons encoding luciferase[8] and with wild-type poliovirus[17,18], mTNF-α expression from replicons was confined to neurons (Fig. 1E). Collectively, these results demonstrate that a replicon encoding mTNF-α expresses biologically active TNF-α in vitro and can be used to transiently increase the concentrations of TNF-α within the CNS.

Consequences of mTNF-α expressed from replicons in spinal cord. Previous studies have indicated that TNF-α has a variety of effects on cells of the CNS including neuronal degeneration, apoptosis, and demyelination[10–12, 19–21]. To determine whether mTNF-α expressed from a replicon could modulate the CNS in vivo, PVR mice were inoculated intraspinally with either the replicon encoding mTNF-α or GFP. Most the mice inoculated with the replicon encoding mTNF-α exhibited tail atony and hindlimb ataxia between 8 and 24 h post inoculation (Table 1). In contrast, the mice inoculated with the replicon expressing GFP remained neurologically normal.

Histological and immunocytochemical analysis of spinal cords from transgenic mice sacrificed at various times post inoculation revealed a range of cytological changes. Hematoxylin and eosin (H&E) staining of samples taken between 8 and 72 h exhibited substantial degeneration of motor neurons in the cervical and lumbar enlargements of the spinal cord of animals inoculated with the replicon encoding mTNF-α, even in animals showing no neurological symptoms (Fig. 2B, D–F). The extent of the neuronal damage and inflammation seen in mice given the replicon encoding mTNF-α,

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Table 1. Summary of clinical symptoms following intraspinal inoculation of replicons into PVR mice

	Acute (8–72 h)[a]				Long-term (17–30 days)			
Scale[b]	0	1	2	3	0	1	2	3
Replicon encoding								
GFP	10/10[c]	–	–	–	4/4[d]	-	-	-
mTNF-α	4/12	1/12	3/12	4/12	2/9	0/9	5/9	2/9

[a]Time post inoculation
[b]Scale summarizing clinical symptoms adapted from Taupin et al. (ref. 30). 0, No disease symptoms; 1, tail atony; 2, mild to moderate hindlimb weakness; 3, severe hindlimb weakness, characterized by ataxia and the inability to bear weight.
[c]Numbers of mice exhibiting symptoms/numbers of mice inoculated.
[d]Replicons encoding several other proteins served as additional controls, with similar results.

though always greater than that for mice given the replicon encoding GFP, varied slightly among individual mice. Chromatolysis of the motor neurons was evident, characterized by nuclear irregularities and migration of the Nissl substance to the periphery of the cytoplasm. The chromatolysis was often accompanied by substantial neuronophagia, primarily by microglia, heterophils (the equivalent to neutrophils in the mouse), and lymphocytes (Table 2; Fig. 2F). In contrast, motor neurons in the spinal cords from animals inoculated with the replicon encoding GFP did not exhibit these cytological changes (Fig. 2A, C). To determine if demyelination occurred, adjacent sections of the spinal cords examined by H&E were stained with luxol fast blue. As early as 8 h post inoculation, gaps in the white matter of spinal cords inoculated with the replicon encoding mTNF-α were seen, in addition to localized areas of demyelination, likely resulting from retraction of the axons of degenerating neurons (data not shown). Other histological changes indicative of axonal damage, such as axonal spheroids seen in spinal cords and brains of multiple sclerosis (MS) patients[22,23], were often observed in the white matter of tissues of animals inoculated with the replicon encoding mTNF-α, but not in tissues inoculated with the replicon encoding GFP (data not shown).

To determine if mTNF-α expressed from the replicon affected astrocytes, oligodendrocytes, and microglia, sections of spinal cords were immunostained with antibodies specific for glial fibrillary acidic protein (GFAP) or for myelin basic protein (MBP), or stained with the lectin from *Bandeiraea simplicifolia* (BS-1) (microglia/monocytes[24]). Enhanced immunostaining for GFAP in astrocytes was evident in tissue sections from mice inoculated with replicons encoding mTNF-α (Fig. 3A and B). In contrast, low diffuse concentrations of GFAP were seen in sections from mice inoculated with the replicon encoding GFP. At 8 and 24 h post inoculation, MBP was undetectable in spinal cords from animals inoculated with the replicon encoding mTNF-α, whereas abundant MBP was detected in the spinal cords of mice inoculated with the replicon expressing GFP. Autofluorescence, due to the expression of GFP, was not detected because the tissue was paraffin embedded (Fig. 3C, D).

Increased numbers of microglia were seen in spinal cord sections from mice inoculated with replicons encoding mTNF-α compared with replicons encoding GFP (Fig. 3 E, F). Some staining of microglia was observed in the GFP tissue, because BS-I labels both resting and activated microglia. Increased numbers of microglia (shown as green staining) were consistently detected in the sections obtained from animals given replicons encoding mTNF-α compared with animals inoculated with the replicon encoding GFP. In some instances, the

microglia identified in tissues from animals given replicons encoding mTNF-α were found surrounding neurons, indicative of neuronophagia. Thus, the histological analysis of the spinal cords from mice inoculated with replicons encoding mTNF-α revealed effects consistent with reactive astrogliosis, loss of MBP and microgliosis.

Long-term effects of transient mTNF-α production from replicons. To investigate the long-term effect of mTNF-α expressed from the replicon, PVR mice inoculated with the replicon encoding mTNF-α or GFP were observed for ~30 days. The mice inoculated with either phosphate-buffered saline (PBS) or replicons encoding GFP exhibited no neurological deficits for the entire observation period. Most of the mice that received the replicon expressing mTNF-α developed distinctive neurological deficits (ataxia and tail atony), which began to decrease between 10 and 25 days post inoculation (d.p.i.); the animals with less severe deficits exhibited earlier recovery. Histological analysis of the spinal cords at ~30 d.p.i. revealed less chromatolysis and fewer inflammatory cells (Fig. 4 A, B), with most of the motor neurons in the spinal cords from mice that received either replicon seeming healthy.. Although the neurons seemed near normal at N30 d.p.i., axonal tracts in the white matter of the spinal cord contained gaps in the white matter and the tissue still appeared locally demyelinated (Fig. 4C, D). At 17 d.p.i., the enhanced expression of GFAP as detected by immunostaining was no longer evident in spinal cords from animals inoculated with the replicon encoding mTNF-α (Fig. 4E, F). Immunostaining for MBP in spinal cords at ~30 d.p.i. revealed that fewer cells were stained from the tissues of animals inoculated with the replicon encoding mTNF-α than GFP (data not shown). Thus, some recovery occurred following transient expression of mTNF-α from replicons, although some damage to the spinal cord remained evident even 30 d.p.i.

Discussion

A gene delivery system based on poliovirus can take advantage of many of the unique features of poliovirus cellular tropism in the CNS. Previous studies have established that poliovirus infection in the CNS primarily localizes to motor neurons in the anterior horns of the spinal cord and brainstem[1,15,25]. Replicons based on poliovirus retain the features of poliovirions for the targeted infection of motor neurons[8]. In contrast to poliovirus, in vivo infection of neurons by replicons does not result in observable cellular destruction or disruption of the CNS microenvironment. Several features of the replicon gene delivery system may account for this difference. First, poliovirus has the capacity to spread from the site of inoculation, ultimately resulting in the involvement of numerous motor neurons

Table 2. Summary of histological analysis of CNS following intraspinal inoculation of replicons into PVR mice

	Acute (8–72 h)[a]			Long-term (17–30 days)		
Scale[b]	0	1	2	0	1	2
Replicon encoding						
GFP	10/10[c]	–	–	4/4[d]	-	-
mTNF-α	0/12	5/12	7/12	2/9	4/9	3/9

[a]Time post inoculation.
[b]The scale was derived from histological analysis of spinal cords of replicon-treated mice. The numbers used for the scale correspond to the illustrations presented in Figure 2.
[c]Numbers of spinal cords exhibiting damage as described in the legend for Figure 2/ numbers of mice inoculated.
[d]Replicons encoding several other proteins served as additional controls, with similar results.
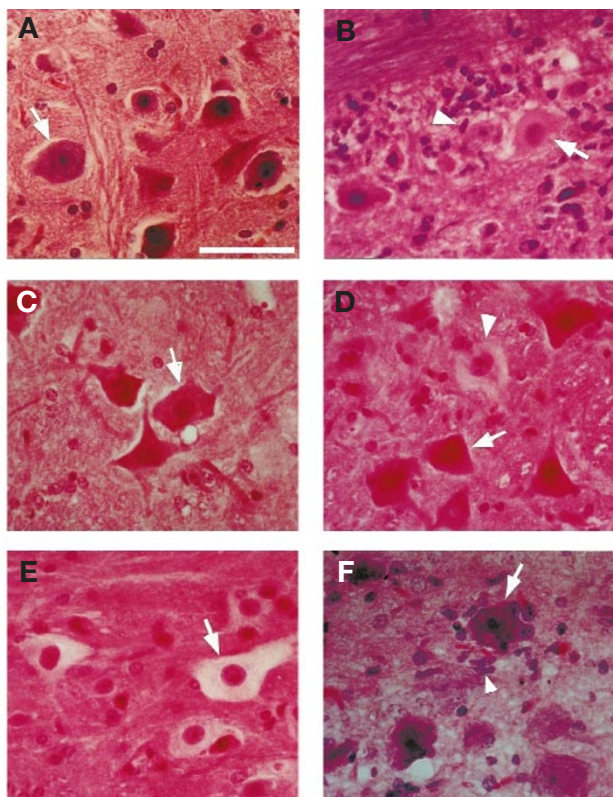
Figure 2. Histological analysis of spinal cord tissue following inoculation of replicons. Mice inoculated with replicons encoding either GFP or mTNF-α were euthanized 24 h later and the spinal cords removed, paraffin embedded, and processed for H&E staining. Representative sections are shown. The total numbers of animals used, along with a summary of the histological findings, are presented in Table 2. All photographs were taken at the lumbar enlargement of the spinal cord and at the same magnification. Scale bar, 500 μm. (A) GFP replicon-inoculated animal. The arrow indicates a healthy appearing neuron, with a well-defined nucleus and Nissl substance distributed throughout the cytoplasm. Few inflammatory cells were seen. The tissue shown in this panel was scored a 0 (see C–F below). (B) mTNF-α replicon-inoculated animal. The arrow points to a neuron undergoing chromatolysis and neuronophagia. The arrowhead indicates inflammatory cells. The tissue shown in this panel was scored a 2. (C) Representative histological analysis of tissue showing no neuronal abnormalities. A representative neuron is indicated by arrow. The tissue shown in this panel is from an animal inoculated with PBS and was given the score of 0. (D) Neuronal abnormalities as a result of the intraspinal inoculation procedure. In rare instances a neuron showing swelling of the nucleus, slightly dispersed chromatin (indicated by arrowhead) and a few inflammatory cells were detected in tissues of mice inoculated with either PBS or the replicon encoding GFP. The arrow points to a healthy neuron, which was characteristic of these tissues. These tissues were scored as a 0. (E) Neuronal abnormalities following inoculation with the replicon encoding mTNF-α. Extensive swelling of the nucleus with dispersion of the Nissl substance to the rim of the cytoplasm and an eccentric nucleus, characteristic of chromatolysis. In addition, moderate inflammation was observed. Tissues in which these abnormalities seen (shown by arrows) were scored as 1. (F) Extensive alterations in the CNS microenvironment as a result of inoculation with the replicon encoding mTNF-α. Similar alterations in cellular architecture as in (E), but with clear neuronophagia, as shown by arrow, as well as extensive inflammation (arrowhead). Tissues in which these abnormalities were seen were scored as 2.

Figure 3. Effect of replicon encoding mTNF-α on astrocytes, oligodendrocytes, and microglia. Mice were inoculated with replicons encoding either mTNF-α or GFP. Spinal cords of the animals were prepared at designated times post inoculation and processed as described earlier. Photographs (A, B, E, and F) were taken at the same magnification. Photographs (C and D) were taken at a lower magnification. All photographs were taken of the lumbar enlargement of the spinal cord. Scale bars, 500 μm. (A and B) Analysis of GFAP expression. Spinal cord sections from mice inoculated with the replicon encoding GFP (A) or the replicon encoding mTNF-α (B) and sacrificed 8 h post inoculation were immunostained using an antibody to GFAP (Pharmingen, San Diego, CA) and a rhodamine-conjugated secondary antibody. The increased expression of GFAP, characteristic of reactive astrocytes, is shown for animals given the replicon encoding mTNF-α, (B). (C, D) Analysis of myelin basic protein expression. Spinal cord sections from mice inoculated with (C) the replicon encoding GFP or (D) the replicon encoding mTNF-α and sacrificed 24 h post inoculation, were immunostained using an antibody to MBP (Biogenesis, Brentwood, NH) and a rhodamine-conjugated secondary antibody. The stained cells (yellow) in (C) were identified as oligodendrocytes. No staining of oligodendrocytes with MBP was seen in the tissues shown in (D). (E, F) Analysis of microglia. Serial sections from the spinal cords shown in panels C and D, of mice inoculated with the replicons encoding either (E) GFP or (F) mTNF-α were stained with a FITC-conjugated lectin from *Bandeiraea simplicifolia* (BS-1; Sigma), which has been reported to stain microglia and monocytes[24]. Arrows point to neurons, either surrounded by microglia indicating possible neuronophagia (as in F) or with no staining (as in E). Arrowheads indicate microglia that are not surrounding neurons.

within the CNS in infected animals. In contrast, replicons remain localized within the CNS, as a result of the single round of infection. Second, the pathogenesis observed for poliovirus infection may be exacerbated by the recruitment of inflammatory cells to the site of infection. As a consequence of the transient protein expression from replicons, the recruitment of inflammatory cells to the CNS is
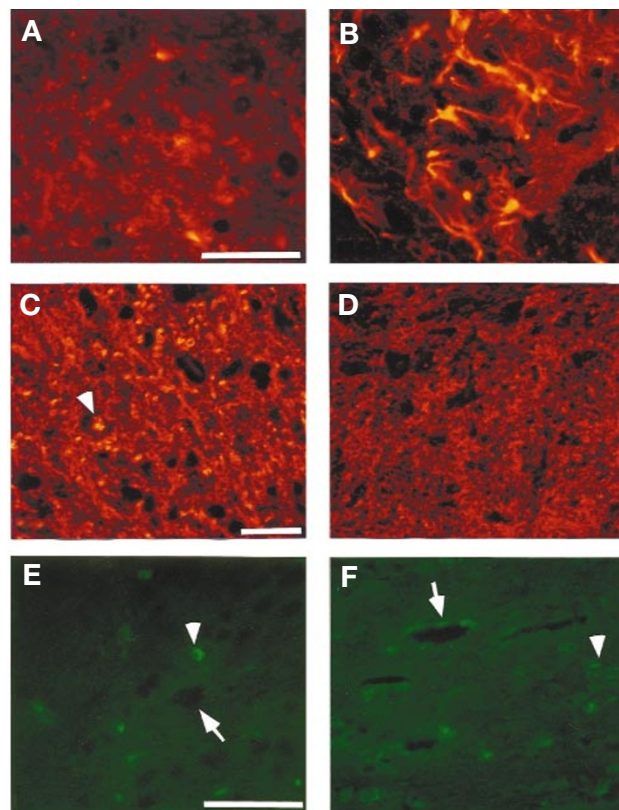
reduced. Finally, during a poliovirus infection, large amounts of virus capsid are produced, which may be toxic to neurons and other cells of the CNS. In this regard poliovirus infection has been shown to induce apoptosis of neurons[26]. Replicons do not encode capsid proteins and the results from a terminal deoxinucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay found that little, if any, apoptosis occurred following inoculation of replicons (data not shown).

If replicons are to have use as a gene delivery system, it is essential to demonstrate that protein expressed from replicons can elicit a
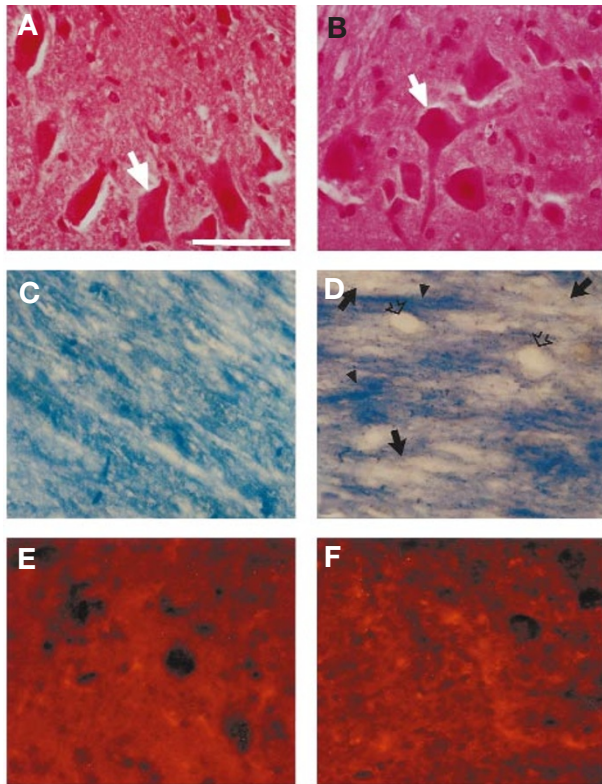
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Figure 4. Analysis of the long-term effect on the spinal cords from animals inoculated with the replicon encoding mTNF-α. All of the mice given replicons encoding either GFP or mTNF- α survived. Spinal cord tissues from mice inoculated with replicons 17 (E, F) or 30 days (A–D) previously were collected and processed for histological analysis. All photographs were taken at the lumbar enlargement and at the same magnification. Scale bar, 500 μm. (A, B) Histological analysis of spinal cord tissues from animals inoculated with replicons encoding either (A) GFP or (B) mTNF-α. Motor neurons in the spinal cords from mice that received either replicon stained with H&E (indicated by arrows). (C, D) Long-term effect on myelin in the spinal cords of animals previously given replicons: encoding GFP or mTNF-α. Serial sections of the tissues of mice inoculated with replicons encoding either (C) GFP or (D) mTNF-α were stained with luxol fast blue/cresyl violet using a commercially available kit (American Master Tech., Lodi, CA). Black arrow points to gaps in white matter fibers and to other demyelinated areas. Arrowheads indicate normally myelinated areas. Open arrows indicate blood vessels. (E, F) Analysis of GFAP expression in the spinal cords 17 days after administration of replicons. Spinal cord sections from mice inoculated with replicons encoding either (E) GFP or (F) mTNF-α were immunostained using an antibody to GFAP and a rhodamine-conjugated secondary antibody. The low, diffuse fluorescence in astrocytes is characteristic of normal spinal cord tissue.

biological response in the CNS in vivo. Previous studies have shown that TNF-α exhibits a multitude of effects on the CNS, some reportedly protective[27,28], whereas others are neurodestructive[12,19,28,29]. Transgenic expression of soluble TNF-α specific to either neurons or astrocytes caused a degenerative disease characterized by random seizures ataxia and early death (by eight months)[12,21]. Histological analysis of these transgenic mice revealed demyelination, astrogliosis, and microgliosis. The striking differences in the extent of the neuropathogenesis between TNF-α gene expression from replicons and the mice transgenic for TNF-α highlight the potential use of this vector system. Extensive demyelination throughout the spinal cord was seen in the mice transgenic for mTNF-α (refs. 12, 20, 30), reflecting severe neuronal damage, as well as damage to oligodendrocytes. In contrast, only local areas of demyelination were observed in mice inoculated with the replicon encoding mTNF-α. The key features of the replicon system, localized, high-level but

transient foreign gene expression, undoubtedly contributed to the fact that expression of TNF-α from replicons in the CNS was not lethal. Furthermore, the animals inoculated with replicons encoding mTNF-α recovered, to some extent, at ~30 d.p.i. A similar natural recovery of the damaged tissue has been noted in some animals with experimental allergic encephalomyelitis[31,32].

Gene delivery to the CNS is complicated by diverse cell types, some of which are postmitotic. The potency of many biological proteins that influence the CNS also necessitates development of a unique delivery vector that can target specific cells for transient gene expression. Our study establishes that replicons meet these criteria. Administration of replicons encoding mTNF-α to the spinal cord resulted in specific infection of motor neurons of the anterior horn. The high-level burst of mTNF-α expression in motor neurons influenced astrocytes (astrogliosis), microglia (microgliosis), and oligodendrocytes (MBP expression). These results clearly support the further evaluation of replicons for gene delivery to the CNS. There are many potential applications for replicon gene delivery to the CNS. For example, replicons encoding antiinflammatory cytokines such as IL-10 (ref. 33) could be evaluated to determine if administration for a short time following spinal cord trauma can reduce the severity of injury. Replicons encoding brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF) could be used to increase the concentration of these factors to facilitate neuronal recovery in the CNS (refs 34, 35). Combinations of replicons encoding cytokines or neurotrophic factors could also be formulated for specific applications in disease or trauma. The continued development of replicons for gene delivery holds promise for the generation of new therapeutics targeted to the CNS.

Experimental protocol

Tissue culture cells and viruses. HeLa HI cells were grown as described[7,8]. The recombinant vaccinia virus (VV-PI) was prepared as described[36].

Construction of replicons encoding mTNF-α. The complete cDNA for mTNF-α was purchased from R&D Systems (Minneapolis, MN). To subclone the gene into a replicon, mTNF-α was amplified by polymerase chain reaction (PCR) using the following primers: 5′-GTC GAC CTC AGA TCA TCT TCT CAA AAT TC-3′ and 5′-GTT AAC CAG AGC AAT GAC TCC AAA G-3′. The product was subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA), and the DNA sequence was determined. To generate a replicon containing the mTNF-α, a modified version of the poliovirus cDNA, pT7-lC was used that contains a unique *Xho*l restriction site between the VP2 and VP3 capsid genes and a unique *Sna*BI restriction site at nucleotide 3359, followed by the coding sequences for the 2A cleavage site[4]. The replicon was cloned downstream of a T7 promoter. The plasmid was transcribed in vitro, and the RNA transfected into HeLa H1 cells previously infected by VV-P1. The replicon was encapsidated by serial passage in HeLa H1 cells in the presence of VV-Pl (refs 3, 7). Cloning of the mTNF-α gene into the replicon cDNA was accomplished using standard methods[7].

Analysis of mTNF-α. The replicon encoding mTNF-α was propagated in the presence of VV-P1 and purified as described[3,7,8]. Similar procedures were used for the replicon encoding GFP (Jackson A. et al., in preparation). The replicons were tittered according to previous procedures[7]; the absence of poliovirus in the preparations was confirmed using a bioassay for infectious virus[8]. Replicons encoding either mTNF-α or GFP were used to infect HeLa H1 cells in 24-well plates for predetermined incubation times (4, 8, 12, and 24 h). The supernatants from the cells were removed at the designated times for the mTNF-α assay. The cells were lysed by three consecutive freeze/thaw cycles. Samples were microfuged for 20 min at maximum speed to pellet out cell debris. Supernatants or cell lysates were used in an ELISA assay (R&D Systems). Spinal cords from PVR mice inoculated with replicons were homogenized as reported[8], and assayed for mTNF-α expression by ELISA.

Biological assay for mTNF-α. HeLa HI cells were infected with replicons encoding either mTNF-α or as a control, GFP. After predetermined infection times (4, 8, 12, and 24 h), the supernatants were collected. Cell lysates were obtained by freeze/thaw cycles. The supernatants and cell lysates were then incubated in 96-well plates at 37°C overnight with WEHI cells treated with actinomycin D; MTT (3-[4,5-Dimethythiazol-2-yl]-2-5 diphenyltetrazolium bromide; Thiazolyl blue) was added after 24 h to each well (1.1 μg/μl). The cells were incubated for 7–8 h, and then lysed (50% dimethylformamide;

2.5% glacial acetic acid; 2.5% HCl (1 N); 10% (wt/vol) sodium dodecyl sulfate). Color change in the wells was measured at OD_{595} and values compared with those from a standard curve of known amounts of recombinant TNF-α.

Intraspinal administration of replicons. Mice were anesthetized by metofane inhalation (Pittman Moore, Mundelein, IL). Intraspinal inoculations were performed as previously described[8,37]. Briefly, the back of each mouse was disinfected with ethanol and a 2–3 cm incision was made in the skin in the curved thoracolumbar region. Replicons were loaded into 250 µl Hamilton syringes fitted with a 30-gauge needle attached to a repeating dispenser. The mouse was placed over a test tube and a 30-gauge needle was inserted between the spinous processes in the thoracolumbar region of the spine. Jerking of the hindlimbs was a sign of correct needle positioning. The skin was closed with sterile wound clips (Fisher Scientific, St. Louis, MO).

Tissue preparation and histochemical analysis. Mice were inoculated with the replicon encoding mTNF-α and killed 8 h later by CO_2 inhalation. The spines were removed and fixed in 4% paraformaldehyde at 4°C for at least 24 h. Spinal cord sections from mice inoculated with the replicon were extracted, paraffin embedded, and serially sectioned at 10 µm intervals, as described[8]. Sections were immunostained with antibodies to mTNF-α (R & D Systems, Inc.) and a rhodamine-conjugated secondary antibody. The neuronal marker NeuN and a fluorescein isothiocyanate isomer conjugated secondary antibody was used for identification of neurons (Chemicon International, Temecula, CA.). The antibodies were diluted 1:150 in PBS plus normal serum. Control experiments used the same protocol without primary antibody.

Acknowledgments

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