Enhanced functional recovery from spinal cord injury following intrathecal or intramuscular administration of poliovirus replicons encoding IL-10

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Abstract

Poliovirus-based vectors (replicons) have been shown to maintain the in vitro tropism of poliovirus for motor neurons of the CNS. To determine if replicons could be effective for delivery of potentially beneficial proteins to the CNS, we have constructed and characterized a replicon encoding IL-10. IL-10 was rapidly produced in tissue culture cells following in vitro infection with replicons encoding IL-10. Intrathecal inoculation of replicons encoding IL-10 into the non-injured CNS of mice transgenic for the poliovirus receptor resulted in expression of IL-10 within motor neurons at 24–48 h post-inoculation, which subsided by 72–96 h post-inoculation. Single intrathecal or intramuscular injections of replicons were given following spinal cord trauma. Animals receiving replicons encoding IL-10 demonstrated a greater functional recovery in the first 24 h after injury that was maintained throughout the testing period. Compared to animals given replicons encoding gfp, CNS tissue from animals given replicons encoding IL-10 revealed extensive expression of IL-10 from astrocytes around the CNS lesion during the first week following injury. The expression of IL-10 from astrocytes also correlated with more resting microglia as opposed to the rounded activated microglia seen in animals given replicons encoding gfp. Results of these studies establish that replicons can be used to express biologically active molecules in motor neurons of the CNS and these biologically active molecules can have a direct effect on the CNS or induce a cascade of molecules that can influence the cellular composition and activation state of cells within the CNS.

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Introduction

Poliovirus belongs to the family Picornaviridae, a family of small RNA viruses, and is the causative agent of poliomyelitis. Poliovirus infection in primates and humans results in destruction of motor neurons at all levels of the spinal cord, brain stem, and motor cortex (Bodian, 1949; Bodian and Howe, 1955). As a consequence of this destruction, an acute paralytic disease of the central nervous system occurs which is referred to as poliomyelitis (Bodian, 1949; Bodian and Howe, 1955). Studies on the pathogenesis of poliovirus have been facilitated by the development of a small animal model (transgenic mice) that expresses the poliovirus receptor (PVR). Infection of these animals by a variety of routes leads to the generation of symptoms including paralysis, respiratory difficulties, and death (Deatly et al., 1998, 1999; Ren and Racaniello, 1992a, 1992b).

The in vivo tropism of poliovirus for motor neurons has been exploited by our laboratory in the development of recombinant poliovirus genomes to deliver and express foreign proteins in motor neurons (Bledsoe et al., 2000a,
Previous studies from this laboratory have described the construction and characterization of poliovirus genomes in which the capsid genes in poliovirus have been substituted with a variety of foreign genes (Ansardi et al., 2001; Novak et al., 2003; Porter et al., 1995; Porter et al., 1996, 1998). These RNA genomes, referred to as replicons, still retain the capacity to undergo self-amplification and expression of foreign genes in the cytoplasm of cells. Because the replicons are generated by providing the capsid proteins in trans, they can undergo only a single round of replication in the infected cell and do not spread to infect other cells. Consequently, replicons encoding reporter genes such as luciferase or green fluorescent protein (gfp) are safe when given directly into the CNS of transgenic mice or through peripheral routes (Bledsoe et al., 2000a, 2000b; Jackson et al., 2001, 2003).

In previous studies, we have characterized the in vivo expression of foreign proteins from poliovirus replicons following infection of motor neurons in PVR transgenic mice (Bledsoe et al., 2000a, 2000b; Jackson et al., 2001, 2003). Our results demonstrated that following infection, motor neurons expressed the foreign proteins for up to 72 h post-inoculation; after that time, protein expression within motor neurons dramatically decreased. Poliovirus replicons maintained the tropism for motor neurons within the CNS, and infection of other resident cell types was not seen. The transient, high-level expression of foreign proteins in the CNS using poliovirus replicon vectors prompted us to assess the effects of replicons encoding biologically active proteins within the CNS. Towards this goal, in a previous study, we reported the effects of administration of replicons encoding TNF-α into the CNS (Bledsoe et al., 2000a, 2000b). The expression of TNF-α within the CNS of these mice resulted in pleotropic effects, including behavioral abnormalities and CNS tissue damage. The results of this study established that replicons could be used to express biologically active molecules within the CNS.

In the current study, we have further exploited the capacity of replicons to deliver biologically active molecules to the CNS. For these studies, we have constructed replicons which encode the gene for IL-10, since IL-10 is a potent anti-inflammatory cytokine that reduces inflammation and improves functional outcome in human and animal models of inflammatory disease (Abraham et al., 2004; Bethea et al., 1999; Pearse et al., 2004; Plunkett et al., 2001; Takami et al., 2002). Within the CNS, IL-10 reduces TNF-α production by astrocytes and antigen presentation by both astrocytes and microglial cells (Balasingam and Yong, 1996). In vivo studies have demonstrated that a single, systemic dose of IL-10 blocks cytokine production and infiltration of inflammatory cells in an experimental model of CNS inflammation (Crisi et al., 1995; Fiorentino et al., 1991). Systemic administration of IL-10 following spinal cord injury (SCI) has been shown to result in enhanced sensorimotor recovery and a decrease in pain responses (Abraham et al., 2004; Bethea et al., 1999; Pearse et al., 2004; Plunkett et al., 2001; Takami et al., 2002). We have utilized a spinal cord injury model in the PVR transgenic mice to determine whether administration of replicons encoding IL-10 would provide a similar therapeutic benefit following injury. We found that animals given replicons encoding IL-10 following injury exhibited a greater functional recovery than those given control gfp replicons. The recovery correlated with an increased expression of IL-10 and reduction in the inflammatory response near the site of the CNS damage. The results of our study demonstrate the capacity of replicons to deliver biologically active molecules to the CNS and support the concept that early intervention to prevent inflammation can exert long-term benefits in functional recovery from spinal cord injury.

Results

Construction and characterization of a replicon encoding IL-10

In previous studies, we have described the methods for construction and characterization of replicons encoding foreign genes (Jackson et al., 2001; Novak et al., 2003). The gene encoding murine IL-10 was inserted into the poliovirus genome between the VP2 and 2A genes (Fig. 1A). The IL-10 gene was inserted downstream from the coding sequences for the FMDV self-cleaving peptide and upstream of a series of amino acids encoding the 2A protease cleavage site (Fig. 1B). The construct was designed to maintain a translational reading frame between the VP4/VP2 genes and the remaining non-structural poliovirus genes. Replicon RNA genomes encoding the IL-10 gene were generated using an in vitro transcription system. In preliminary studies, we found that transfection of this RNA into cells resulted in the expression of the appropriate virus proteins (i.e., 3Dpol) as determined by immunoprecipitation assays; IL-10 expression was confirmed using an antibody specific for IL-10 (data not shown).

The replicon was encapsidated into poliovirions using a complementation system, and encapsidated replicons were purified by ultra-centrifugation followed by titer determination as previously described (Novak et al., 2003). Expression of IL-10 was demonstrated following infection of HeLa cells for 6 h with encapsidated replicons encoding IL-10; encapsidated replicons encoding gfp served as a control for these experiments. The IL-10 expression within the cells was analyzed using an ELISA, which detects biologically active IL-10 (Fig. 1C). Cells infected with the replicon encoding IL-10 yielded an average of 200 pg/mL of IL-10 over background activity (three independent experiments).

To further analyze the expression of IL-10 in replicon-infected HeLa cells, we analyzed expression using immuno-
fluorescence with antibodies specific for IL-10. At 24 h post-infection, HeLa cells infected with the replicon encoding IL-10 exhibited a strong immunofluorescence using antibodies specific for IL-10 (Fig. 1D). At longer times of infection (72–96 h), we observed extensive cell death (Ansardi et al., 2001). As expected, the expression was contained mainly within the cytoplasm of the replicon-infected cells, consistent with the exclusive cytoplasmic location of poliovirus replication. Thus, the IL-10 expressed from the replicons maintains the capacity to be recognized by both an ELISA and by immunohistochemistry performed on replicon-infected cells.
Expression of IL-10 within the spinal cord following administration of replicons

The distribution of IL-10 expression following replicon infection was analyzed in mice transgenic for the poliovirus receptor (PVR mice) that received a single intrathecal injection of replicons encoding IL-10 or gfp. The replicons were injected into the cerebrospinal fluid (csf) to access the subarachnoid space at the level of the cauda equina. This technique causes minimal damage to the spinal cord as assessed by behavioral and histological analysis (Jackson et al., 2001). A second series of PVR mice received intramuscular injections of replicons encoding IL-10 or gfp into the semitendinosus muscle of the right hindlimb, which is innervated by the sciatic nerve. Transport of replicons via the sciatic nerve results in infection of motorneurons within the spinal cord (Jackson et al., 2003). The spinal cords of two mice were analyzed at each survival time for both replicons. Consistent with previous studies, histological processing and routine hematoxylin and eosin stains did not reveal any influx of inflammatory cells due to the injection procedures or the replicon infection (data not shown).

Previous studies utilizing a replicon encoding gfp have shown that intrathecal administration results in foreign gene expression localized to neurons throughout all levels of the spinal cord and brainstem (Jackson et al., 2001). Intramuscular injection of replicons encoding gfp into the hindlimb muscles resulted in expression within motor neurons primarily in the lumbar cord (Jackson et al., 2003). In the normal CNS, however, IL-10 and IL-10 receptor expression has been found mainly in astrocytes and microglia and rarely in neurons (Hulfshof et al., 2002). To determine whether IL-10 expressed by replicons was localized in neurons, we analyzed IL-10 expression following intrathecal or intramuscular inoculation of replicons (Fig. 2). A low power view of a frozen section through the lumbar enlargement of the spinal cord in a PVR transgenic mouse that had received a single intrathecal injection of replicons encoding IL-10 24 h earlier revealed abundant IL-10 expression in both the white and grey matter (Fig. 2a); an adjacent tissue section processed without primary antibody revealed only diffuse autofluorescence (Fig. 2b). A higher power view of sections from the same animal revealed extensive IL-10 expression in the white matter in PVR mice.
receiving intrathecal injections of replicons encoding IL-10 (Fig. 2c); intramuscular injections produced a similar, but less, pronounced result (Fig. 2d). Limited expression of IL-10 in subpial astrocytes is seen in the spinal cords of PVR mice that received injections of gfp replicons (Fig. 2e) or artificial cerebrospinal fluid (csf) (Fig. 2f).

Cellular localization of IL-10 expression within the grey matter revealed IL-10 expression within the cytoplasm of large triangularly shaped cells characteristic of motorneurons (Fig. 3a). The same cells were labeled using an antibody specific for neurons (NeuN; Fig. 3b). IL-10 expression in neurons from replicons was only found 24–48 h post-infection, was entirely cytoplasmic, and does not co-localize with the Hoechst nuclear counter stain (Figs. 3c and 3d). No IL-10 expression was seen at this time in microglia or oligodendrocytes (data not shown).

Previous studies have shown that IL-10 can induce expression of IL-10 within the CNS due to the autocrine expression of IL-10 in astrocytes (Balasingam and Yong, 1996). A longitudinal frozen section through the lumbar enlargement of the spinal cord in a PVR transgenic mouse that had received a single intrathecal injection of replicons encoding IL-10 24 h earlier revealed extensive IL-10 expression in the subpial and fibrous astrocytes of the white matter and occasionally in the protoplasmic astrocytes of the grey matter (Fig. 3e). Co-localization of the IL-10 expression with staining for GFAP, a marker for astrocytes (Fig. 3f and nuclear counterstain, Fig. 3g), revealed IL-10 expression was evident throughout the entire process of each astrocytic cell type, and not just the cell body (Fig. 3h).

Although the astrocytes of the spinal cord were not infected by replicons, they demonstrated marked IL-10 expression throughout the first week after injection of replicons encoding IL-10; this was presumably due to an autocrine effect following exposure to the IL-10 produced initially by replicon-infected motorneurons. This conclusion is further supported by analysis of the extent and distribution of the patterns of IL-10 activity seen following injections of the control gfp replicon or artificial csf, where little IL-10 expression was induced in astrocytes.
Effects of IL-10 replicons following spinal cord injury

Recombinant IL-10 protein has been analyzed previously for its therapeutic effects following spinal cord injury and has been shown to improve functional outcome (Abraham et al., 2004; Bethea et al., 1999; Pearse et al., 2004; Plunkett et al., 2001; Takami et al., 2002). To ascertain whether the IL-10 produced by the replicons would exhibit a similar or more marked effect on functional outcome after injury, severe contusive spinal cord injuries were performed on PVR transgenic mice using a weight drop method (Kuhn and Wrathall, 1998). Immediately following injury, intrathecal injections of replicons encoding gfp ($N = 10$) or IL-10 ($N = 10$) were made using a 30-gauge needle inserted into the csf in the subarachnoid space (Jackson et al., 2001). Additional groups of PVR mice received replicons encoding IL-10 ($N = 10$) or gfp ($N = 10$) via intramuscular injection.

All animals were tested before and after surgery with a series of tests designed to assess a range of sensory and locomotor skills. Testing was performed prior to surgery (Day 0), the day following surgery (Day 1), and at weekly intervals for 8 weeks. The tests included a free field observation scale modified from the Basso, Beattie, and Bresnahan Score (BBB), a reflex test for toe spread, an inclined plane test (Rivlin and Tator, 1977), a rope hang, platform hang, and wire mesh test, a reversal test, a timed distance test, a free field swimming test, and an evaluation of overall health. The twelve tests are weighted to compose a 100-point scale, the Combined Mouse Behavioral Score (CMBS) (Fig. 4). The differences in behavior in mice receiving IL-10 replicons were evident as early as 24 h after injury in the first testing session and were maintained throughout the 8 weeks of testing. Statistical analyses utilizing the Kruskal–Wallis nonparametric analysis of variance test and an ANOVA with the Bonferroni post hoc correction revealed that the difference between the IL-10 group and the gfp group was statistically significant at the $P < 0.05$ level at all time points tested. The increase in functional recovery with the IL-10 replicons was evident when the replicons were given by either intrathecal or intramuscular routes and was significant using BBB subscore or CMBS.

![Fig. 4](image-url)
Immunofluorescent analysis of spinal cord tissue sections taken from injured mice treated with IL-10 or gfp replicons was performed to determine the cell types involved in mediating the functional improvement seen with the IL-10 replicons in the chronic injury groups described above. Two groups of PVR mice were given spinal cord contusions and intrathecal injection of replicons and were allowed to survive for 24, 72, and 96 h. Two animals were examined at each time interval for each group. All animals were tested the day after surgery to confirm the severity of the injury, and scores ranged from 29 to 35 on the CMBS scale. Within the primary site of injury (i.e., injury center), IL-10 expression was found within astrocytic processes extending throughout the entire extent of the white matter. Co-localization of IL-10 expression and markers for microglia/macrophages revealed CD11b expression in small rounded cells, whose size and shape were characteristic of activated microglia or infiltrating blood-borne macrophages; additional experiments will be needed to ascertain the origin of these cells. Differences in the pattern of IL-10 expression and the morphology of cell types within the spinal cord were more remarkable in tissue located approximately 1 mm or more away from the injury center. This tissue is less affected by the necrotic changes, but is susceptible to damage by secondary processes such as inflammation, reactive oxygen species, and nitric oxide production. IL-10 expression in this area of the injured spinal cord was more pronounced in PVR mice that received replicons encoding IL-10. Visualization of CD11b from animals given IL-10 replicons revealed highly ramified (resting) microglial profiles (Fig. 5a). In contrast, tissue taken from a similar location in the spinal cord of a PVR mouse that had received an injection of gfp replicons 72 h earlier contained rounded cell profiles characteristic of activated microglia which are associated with deleterious inflammatory responses (Fig. 5b). The CD11b-positive cells were distributed throughout the white matter and the grey matter and were particularly numerous surrounding blood vessels. Thus, the primary morphological difference between the injured spinal cords of animals receiving IL-10 or gfp replicons was the appearance of ramified (normal, resting) microglia surrounding the injury site for a period of 1–3 days after injury in those animals receiving IL-10 replicons.

**Discussion**

In this study, we have further investigated the potential of poliovirus replicons encoding foreign proteins for gene delivery to the CNS. Poliovirus replicons encoding IL-10 were constructed and shown to express this protein following injection of tissue culture cells in vitro. The cellular specificity of the replicons within the spinal cord was analyzed following single intrathecal or intramuscular injections of poliovirus transgenic mice. Within 24 h after inoculation, the IL-10 co-localized with motor neurons of the spinal cord. At later intervals, enhanced IL-10 expression was evident in astrocytes, presumably due to an autocrine effect of neuronally produced IL-10 on surrounding astrocytes. The biological function of IL-10 expressed within the CNS from poliovirus replicons was evaluated using a spinal cord injury model. In this case, expression of IL-10 in the CNS from replicons inoculated via the intrathecal or intramuscular routes resulted in enhancement of functional recovery from spinal cord injury. The recovery correlated with a reduction in the inflammatory response in the spinal cord surrounding the injury site that occurs in the first week following injury.

The results of our former and current studies support the concept that poliovirus replicons have the capacity to safely deliver biologically active molecules to the CNS. Previously, we have analyzed poliovirus replicons encoding gfp or luciferase for the capacity to express these proteins within the CNS (Bledsoe et al., 2000a, 2000b; Jackson et al., 2001). The results of those studies, as well as our current study, establish that poliovirus replicons maintain their tropism for motor neurons of the CNS. Expression of IL-10 within motor neurons of the CNS following inoculation with replicons did not appear to have any deleterious effect as measured from behavioral testing or histological analysis.

Our study is the first demonstration of the use of poliovirus replicons to express a biologically active molecule that exerts a therapeutic effect. We chose IL-10 for expression within the CNS because previous studies have demonstrated that exogenous IL-10 has therapeutic potential to improve functional recovery following spinal cord injury (Abraham et al., 2004; Bethea et al., 1999; Pearse et al., 2004; Plunkett et al., 2001; Takami et al., 2002). IL-10 suppresses the production of inflammatory cytokines such as TNF-α and mediates the functional improvement seen with the IL-10 replicons in the chronic injury groups described above.
as TNF-α, matrix metalloproteinases, and other chemokines (Balasingam and Yong, 1996; Fiorentino et al., 1991). IL-10 also prevents glutamate-induced neurotoxicity (Bachis et al., 2001; Brodie, 1996; Grilli et al., 2000) and promotes NGF production by astrocytes (Brodie, 1996). Reduction in pain behavior was noted in rats following spinal cord injury and a single systemic application of IL-10 (Plunkett et al., 2001). Other studies, though, have shown that IL-10 treatments following spinal cord injury decreased the amount of damaged tissue and reduced macrophage infiltration but did not result in functional recovery. These discrepancies regarding the effectiveness of IL-10 are not only due to the timing of the treatments during the disease injury process but also to the route of administration of this potent cytokine. Since IL-10 does not normally cross the blood–brain barrier, it can only reach the CNS once the blood–brain barrier has been disrupted (Kastin et al., 2003). Due to the short half-life of cytokines in the body, supraphysiologic amounts of IL-10 must be given to achieve a therapeutic effect in the CNS. In addition, the pleotropic nature of IL-10 may cause additional effects on other organs and the immune system. The use of replicons for the delivery of IL-10 to the CNS could circumvent the problems associated with the systemic administration of IL-10. Indeed, our studies provide two routes in which IL-10 could potentially be safely administered to the CNS (Jackson et al., 2003). Direct intrathecal inoculation following damage would ensure the access of IL-10 to the CNS. It is also possible to deliver IL-10 via intramuscular inoculation following CNS damage. With respect to this issue, it is important to note that while most of the human population has been immunized against poliovirus to protect against poliomyelitis, a previous study from this laboratory has shown that animals previously immunized to poliovirus are susceptible to infection with replicons (Porter et al., 1997). Thus, intramuscular inoculation of replicons even into poliovirus-immune individuals should be feasible.

An additional, important point of using replicons for delivery of biologically active molecules is the rapid and short time frame for foreign gene expression within neurons following inoculation. The kinetics of IL-10 expression within the CNS from the IL-10 replicon was also similar to that seen for other proteins expressed from replicons (Bledsoe et al., 2000a, 2000b; Jackson et al., 2001). Peak expression occurred at 24 h post-inoculation of the replicons and was confined exclusively to motor neurons; expression of IL-10 within motorneurons was absent at approximately 96 h post-inoculation. Indeed, previous studies have shown that the effects of IL-10 are manifested only when given early following CNS injury (Bethea et al., 1999). At later times, IL-10 may have no effect, or possibly be detrimental (Bethea et al., 1999; Pearse et al., 2004). In contrast to expression of IL-10 from DNA-mediated gene delivery vectors, the rapid and short-term expression of IL-10 from poliovirus replicons probably facilitates the beneficial outcome of this treatment. In support of this conclusion, we have found that additional administration of replicons encoding IL-10 to injured animals during the first week after injury did not significantly improve the functional recovery of these animals. Clearly, additional experiments will be required to establish the conditions to further improve the functional recovery of animals following spinal cord injury. The transient expression from replicons could permit the sequential use of replicons encoding different molecules, resulting in a cascade of potentially beneficial cytokines/biologically active molecules in the CNS. One possibility would be to give replicons encoding IL-10 early after injury to prevent inflammation, followed later by replicons encoding growth factors such as NT3 or NGF to encourage axonal outgrowth and neuronal recovery. Since replicons do have the potential to affect non-neuronal cells within the CNS, as shown in this study where a cytokine cascade occurred in astrocytes as a result of IL-10 expression in motor neurons, a therapeutic strategy could be formulated to affect different CNS cell populations at critical points in the recovery process to further enhance functional recovery from spinal cord injury.

Materials and methods

Replicon encoding IL-10

The complete cDNA of poliovirus in the plasmid designated pT7-IC is positioned downstream from a promoter for the bacteriophage T7 RNA polymerase, which allows for in vitro transcription of full-length RNA when templates are linearized at the Sal restriction site (Fig. 1A). Taq polymerase was used to amplify the mouse IL-10 cDNA from PcD (SR alpha)-F115 (ATCC, #68027) using primers: 5′-CCCTCAGCGGTCGCTGATGCCTAGTGC-3′ and 5′-ACGTAAGTTTCTATTTTATGATCATGATG-3′. The PCR product was subcloned into the vector pGEM-T Easy (Promega) and sequenced. The oligonucleotides were chosen so that unique XhoI and SnaBI restriction endonuclease sites were added to the 5′ and 3′ end of the gene, respectively. The IL-10 gene was isolated by restriction digestion with XhoI and SnaBI and subcloned into the poliovirus cDNA, resulting in the deletion of the coding region for VP3 and VP1 in the poliovirus genome. In addition, a sequence encoding the 20 amino acid self-cleavage peptide of FMDV was cloned between the VP2 and IL-10 genes. The resulting plasmid contained the IL-10 gene positioned between the genes encoding VP2 self-cleavage and 2A, thereby conserving the translational reading frame (Fig. 1B). A replicon encoding green fluorescent protein (gfp) was constructed to serve as control (Novak et al., 2003). The replicons were serially passaged to high titer and used for transfection in HeLa cells, were negative.
**In vitro assay for IL-10**

Replicons encoding IL-10 and gfp were used at a similar multiplicity of infection to infect HeLa cells in 6-well plates. After 6 h, supernatants and cells were collected; cells were lysed by three consecutive freeze–thaw cycles. The IL-10 expression was analyzed using an ELISA assay (RD Systems). The results shown are representative of the cell values for three independent experiments.

**Immunofluorescent analyses of replicon-infected HeLa cells**

HeLa cells were infected with the replicon encoding IL-10 and incubated overnight at 37 °C. For immunofluorescent analysis, cells were fixed with 2% paraformaldehyde for 2 h at 4 °C and rinsed with phosphate-buffered saline (PBS). IL-10 expression in cells was examined using a monoclonal antibody against murine IL-10 (Chemicon, Inc) at a concentration of 1:500 at 4 °C overnight followed by a secondary antibody conjugated to Alexa 594 (Molecular Probes, Inc) at a concentration of 1:400 for 1 h at room temperature. After rinsing with PBS, the cells were examined using a DMIRBE Leica confocal microscope and a Krypton laser. Control slides were imaged at the same laser intensity levels to assess the level of background fluorescence due to fixation and tissue processing.

**Surgical procedures**

PVR transgenic mice were anaesthetized with a mixture of 3% Halothane with oxygen at a rate of 1 L/min, followed by a maintenance dose of 1.5% Halothane. Corneal reflex and breathing rates were continuously monitored throughout the surgery. Following exposure of the spinal column, a laminectomy was performed at the level of the dural sac near the cauda equina. In this region, the spinal cord has narrowed and the cerebrospinal fluid bathing the cord is readily accessible. A 30-gauge needle attached to a micropipette was used for intrathecal injections of 10 μl of 10^8 replicons encoding IL-10 (N = 8), gfp (N = 8), or artificial csf (N = 4), and the animals were sacrificed at 24, 48, 72, or 96 h after injection. Two animals were analyzed at each survival time. Replicons have also been shown to infect motor neurons via the intramuscular route (Jackson et al., 2003). A second group of PVR mice received single intrathecal injections of 20 μl of 10^8 replicons encoding IL-10 (N = 10) or IL-10 (N = 10) were made into the right semitendinosus muscle using a micropipette attached to a 30-gauge needle (Jackson et al., 2003).

**Behavioral assessment**

Sensorimotor function of the spinal cord in all animals was assessed using a series of behavioral tests that evaluate a range of functional activities from reflex behavior to coordinated locomotion. The 12 tests included a free field observation scale modified from the Basso, Beattie, and Bresnahan (BBB) scale used in rats, wire mesh, rope hang, and platform hang (Kuhn and Wrathall, 1998), a reflex test for toe spread, a timed distance walk, a reversal test, a free-field swimming test, and an evaluation of overall health. The tests are weighted on a 100-point scale, the Combined Mouse Behavioral Score (CMBS). The tests were performed the day after surgery in all animals and for the following 8 weeks in the long-term survival groups. In animals receiving intrathecal injections only, the tests served to ensure that the injection was performed in a non-invasive manner, while in the injured mice, the tests served to chart the time course of recovery and the differences between treatment groups. Comparisons between weekly scores for animals receiving IL-10 replicons and those with control gfp replicons were analyzed using the Kruskal–Wallis nonparametric analysis of variance. ANOVA tests followed by a Bonferroni post hoc test were also made at each interval after injury. Differences were significant at the P < 0.05 level for both CMBS and the BBB subscore.

**Histological and immunofluorescent analyses**

The mice were sacrificed using an overdose of ketamine and xylazine followed by cardiac perfusion with saline and 4% paraformaldehyde. After 1–4 h of post-fixation at 4 °C, the spinal cords were removed from the spinal column and transferred to PBS with 0.1% paraformaldehyde or to 30% sucrose for cryoprotection. The tissue was embedded in OCT compound and sectioned at 16 μm on a cryostat in the longitudinal plane. All sections were mounted on gelatin-coated slides, air dried, and stored at 20 °C. Representative sections were OCT compound and sectioned at 16 μm on a cryostat in the longitudinal plane. All sections were mounted on gelatin-coated slides, air dried, and stored at 20 °C. Representative
sections throughout the injury site were stained with hematoxylin and eosin to compare the inflammatory cell response and the extent of damaged tissue following replicon injection and/or spinal cord injury.

Sections throughout the spinal cord were rinsed three times in 0.1 M phosphate-buffered saline (PBS) and placed in 10% normal donkey serum (NDS) diluted in a 0.3% Triton X-100/PBS mixture for 1 h at room temperature to prevent non-specific binding. Experimental slides were incubated at 4 °C overnight in a 1:500 concentration of a polyclonal murine IL-10 antibody (RD) in 0.3% Triton X-100/PBS. Control slides were processed without the addition of the primary antibody. Sections were rinsed three times in PBS followed by a 1-h incubation at room temperature with a biotinylated donkey anti-rabbit secondary (Jackson Immunoresearch) diluted 1:400 with 0.3% Triton-X100/PBS. After rinsing with PBS three times, sections were incubated for 1 h at room temperature in Alexa 488 streptavidin (Molecular Probes, Inc.) diluted 1:200 in 0.1 M PBS. Following two rinses in PBS, the slides were placed in distilled water and coverslipped in Perma-fluor or processed for co-localization.

Co-localization of neuronal and glial antigens was performed using monoclonal antibodies and a 1:400 concentration of donkey anti-mouse or donkey anti-rat secondaries conjugated with Alexa 594 (Molecular Probes, Inc.). Neurons were identified using a 1:500 concentration of NeuN (Chemicon, Inc.), a primary antibody directed against neuronal nuclei. A 1:500 concentration of an antibody against glial fibrillary acidic protein (GFAP) (Sigma) was used to localize astrocytes: while anti-MAB328 (Chemicon, Inc.) and anti-CNPase (Sigma) were used to identify oligodendrocytes (1:500). Microglia were characterized using an antibody against CD11b at 1:300 dilution (Serotec) or anti-F4/80 at a 1:50 dilution (RD). Similar patterns of co-localization were obtained with both microglial antibodies. Slides were counterstained for visualization of the nucleus with a 1:2000 dilution of Hoechst 33258 (Sigma).

All slides were examined for IL-10 expression using a Leitz Aristoplan light microscope. Fluorescent images were recorded using a Leica DMRBE confocal microscope equipped with an ultraviolet laser for short wavelength images, an argon laser for intermediate wavelengths, and a krypton laser for long wavelength fluorochromes. Control slides for each animal were shot at the same laser settings to determine the level of background fluorescence resulting from fixation and tissue processing. The images were processed using Adobe Photoshop 6.0.

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References


