GENE-BASED INTRAMUSCULAR INTERFERON-BETA THERAPY FOR EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Ritika Jaini\textsuperscript{1}, Drew Hannaman\textsuperscript{2}, Justin M. Johnson\textsuperscript{1}, Robert M. Bernard\textsuperscript{2}, Cengiz Z. Altuntas\textsuperscript{1,3}, Maida M. de las Alas\textsuperscript{2}, Pavani Kesaraju\textsuperscript{1,3}, Alain Luxembourg\textsuperscript{2}, Claire F. Evans\textsuperscript{2,*,†}, and Vincent K. Tuohy\textsuperscript{1,3,*,*}\textsuperscript{,†}

\textsuperscript{1}Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio USA; \textsuperscript{2}Ichor Medical Systems, San Diego, California, USA; \textsuperscript{3}Department of Biology, Cleveland State University, Cleveland, Ohio USA

ADDRESS FOR CORRESPONDENCE: Dr. Vincent K. Tuohy, Cleveland Clinic, Lerner Research Institute, Department of Immunology, NB30, 9500 Euclid Avenue, Cleveland, Ohio, 44195 USA; Phone: 216-445-9684; Fax: 216-444-8372; E-mail: tuohyv@ccf.org; or Dr. Claire F. Evans, Ichor Medical Systems, 6310 Nancy Ridge Drive, Suite 107, San Diego, CA, 92121 USA; Phone: 858-550-2022; Fax: 858-550-2092; E-mail: cevans@ichorms.com

E-MAIL ADDRESSES FOR ALL AUTHORS: jainir@ccf.org (R.J.); dhannaman@ichorms.com (D.H.); johnsoj1@ccf.org (J.M.J.); rbernard@ichorms.com (R.M.B.); altuntc@ccf.org (C.Z.A.); mdelasalas@ichorms.com (M.M.D.); kesarap@ccf.org (P.K.); aluxembourg@ichorms.com (A.L.); cevans@ichorms.com (C.F.E.); tuohyv@ccf.org (V.K.T.)

RUNNING TITLE: Gene-Based IFNβ Therapy for EAE
FOOTNOTES

* Address correspondence to: Dr. Vincent K. Tuohy, Cleveland Clinic, Lerner Research Institute, Department of Immunology, NB30, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA; Phone: 216-445-9684; Fax: 216-444-8372; E-mail: tuohyv@ccf.org; or Dr. Claire F. Evans, Ichor Medical Systems, 6310 Nancy Ridge Drive, Suite 107, San Diego, CA, 92121, USA; Phone: 858-550-2022; Fax: 858-550-2092; E-mail: cevans@ichorms.com

† These authors contributed equally to this work.

‡ Nonstandard abbreviations used: 2′,5′-oligoadenylate synthetase (2′,5′-OAS); 9-fluorenylmethoxy-carbonyl (FMOC); base pairs (bp); central nervous system (CNS); electroporation (EP); enzyme-linked immunosorbant assay (ELISA); experimental autoimmune encephalomyelitis (EAE); interferon inducible gene 56 (IFI56); interferon regulatory factor-7 (IRF7); intramuscular (i.m.); magnetic resonance imaging (MRI); matrix metalloproteinases (MMP); million international units (MIU); multiple sclerosis (MS); recombinant interferon-beta (rIFNβ); reverse transcriptase polymerase chain reaction (RT-PCR); subcutaneous (s.c.); type 1 inflammatory helper T cell (Th1); type 2 regulatory helper T cell (Th2)
ABSTRACT

In contrast to serial injections of recombinant interferon-beta (IFNβ) for long-term therapy of multiple sclerosis (MS), prolonged systemic delivery of proteins derived through in vivo gene transfer may provide a more clinically relevant alternative. Here we compare the therapeutic efficacies of electroporation (EP) mediated intramuscular IFNβ gene transfer with repeated alternate day injections of recombinant IFNβ after onset of relapsing-remitting experimental autoimmune encephalomyelitis (EAE), an animal model widely used in MS research. We show for the first time that a single EP-mediated intramuscular administration of 20 μg of an IFNβ expressing plasmid provides long term expression of interferon inducible genes and is therapeutic in ongoing established EAE. The achieved therapeutic effects of IFNβ gene delivery are comparable to an eight week regimen of 10,000 IU rIFNβ injected every other day and involved a significant inhibition of disease progression and a significant reduction of EAE relapses compared to untreated or null vector treated mice. Our results indicate the viability of a convenient and effective gene-based alternative for long-term IFNβ protein therapy in MS.

KEYWORDS: EAE/MS, Gene Therapy, Autoimmunity, Cytokine, Interferon
INTRODUCTION

Recombinant interferon-beta (rIFN\(\beta\)) is currently the most common therapy for multiple sclerosis (MS). High dose, frequent administrations of rIFN\(\beta\) have been shown in MS to inhibit progression to disability, reduce exacerbation rates, and decrease occurrence of new enhancing central nervous system (CNS) lesions as determined by magnetic resonance imaging (MRI) [1-4].

Administration of a recombinant protein is typically associated with peak and trough kinetics governed by the biologic half-life of the protein. IFN\(\beta\) has a very short half life in serum [5] thus necessitating a regimen of frequent injections to compensate for rapid systemic inactivation and clearance. For instance, administration of Avonex\textsuperscript{\textregistered} (IFN\(\beta\)-1a) requires intramuscular (i.m.) injections of 6 million international units (MIU) of recombinant protein once weekly, while Rebif\textsuperscript{\textregistered} (IFN\(\beta\)-1a) requires subcutaneous (s.c.) injections of 6 or 12 MIU thrice weekly. On the other hand, Betaseron\textsuperscript{\textregistered} (IFN\(\beta\)-1b) requires a higher dosage of 8 MIU s.c. every other day which may account at least in part for inducing relatively high levels of neutralizing antibodies that predispose to decreased efficacy [6].

In addition to induction of neutralizing antibodies [6-11], long term repeated injections of rIFN\(\beta\) are associated with several clinically relevant side effects including depression, decreased production of red blood cells, leukocytes and platelets, as well as cellulitis and inflammation at sites of injection [12]. Moreover, liver toxicity possibly due to the sudden rise in serum levels of protein immediately after injection is also of great concern in patients undergoing long term rIFN\(\beta\) therapy [7,13]. As a result of the need for frequent dosing and the concurrent flu-like side effects that often occur following injection [14], non-compliance in patients is common particularly during periods of disease stabilization [15]. Unfortunately for the non-compliant patient, intermittent rIFN\(\beta\) dosage can
result in subsequent increased disease activity [16].

A single administration of the IFNβ gene offers the prospect of producing endogenous systemically distributed IFNβ protein over an extended period of time. The continuous protein synthesis and secretion characteristic of DNA-based delivery would eliminate the peak and trough kinetics characteristic of conventional rIFNβ injection regimens, thereby eliminating side effects and toxicity associated with bolus protein dosing. The dramatic reduction in dosing frequency possible with DNA-based delivery may also enhance patient acceptance and cost effectiveness of therapy. Recently, intracranial injection of plasmid DNA-liposome complexes [17,18] and intravenous injection of retrovirally transfected bone marrow cells [19] have been used to deliver the murine IFNβ gene in the CNS. Although these studies provide a proof of principle for IFNβ gene transfer for treating autoimmune demyelinating disease, approaches involving direct CNS delivery are unlikely to be practical in the clinical MS setting.

Given its relative abundance, accessibility, and mitotic stability, coupled with the ability to synthesize secreted proteins that are taken up into circulation, skeletal muscle provides the most suitable tissue target for DNA-based protein delivery. Several means for i.m. gene delivery exist, however, most methods have provided poor transfer efficiency and transient expression kinetics. One technique for DNA delivery, in vivo electroporation (EP), significantly enhances intracellular uptake of DNA and has been used to enhance the magnitude and duration of protein expression in muscle in a number of animal models [20-23]. In the current study we show for the first time that a single EP-mediated i.m. administration of a plasmid encoding IFNβ provides a therapeutic benefit in established EAE that is comparable to a regimen of s.c. rIFNβ at doses reflective of those currently used in the
treatment of MS patients. Compared to injection of null vector, the clinical treatment effect of gene-based IFNβ therapy involves a significant inhibition of disease progression and a significant reduction of clinical relapses. Our results indicate the efficacy and viability of a convenient gene-based alternative for long-term treatment of MS.
RESULTS

Intramuscular IFNβ gene transfer results in sustained upregulation of IFNβ inducible genes. Responses to IFNβ administration are often evaluated based on changes in the expression levels of IFNβ inducible genes, such as members of the 2',5'-OAS family [24]. To determine if a single administration of the murine IFNβ gene results in sustained systemic distribution of biologically active IFNβ protein, groups of six Swiss Webster mice received either 10 µg pORF-murine IFNβ plasmid administered i.m. into one tibialis anterior muscle with TriGrid™-mediated EP or 20 µg of pORF-murine IFNβ administered into both tibialis anterior muscles by conventional direct injection (40 µg total); six untreated mice served as naive controls. At various times after plasmid administration, splenic levels of 2',5'-OAS mRNA relative to naive controls were measured by real-time RT-PCR and normalized to GAPDH mRNA levels. We observed a sustained elevation in 2', 5'-OAS mRNA levels that persisted for more than 18 weeks after a single delivery of 10 µg of the IFNβ gene (Fig. 1A). Negligible induction of 2',5'-OAS mRNA was detected following administration of 40 µg of the pORF-murine IFNβ plasmid without EP (Fig. 1A). The 2',5'-OAS expression levels in mice treated with pORF-mIFNβ + EP were significantly higher (P<0.05) than levels measured in naive mice or in mice treated with pORF-mIFNβ without EP. Additionally, no induction of splenic 2',5'-OAS mRNA was observed following administration of 40 µg of noncoding plasmid (pORF-null) with EP (data not shown), indicating that the increased expression of 2',5'-OAS mRNA was due to EP mediated transfer of the IFNβ gene. Since expression of the interferon inducible genes IFI56 and IRF7 is induced in peripheral blood mononuclear cells of MS patients receiving rIFNβ therapy [25], we measured IFI56 and IRF7 mRNA levels in spleens at 12 weeks post-DNA transfer. Importantly, as shown in Fig. 1B, relative 2',5'-OAS, IFI56, and IRF7 expression levels were substantially higher at
12 weeks post-DNA transfer than those observed 6 hours after the fourth s.c. injection of normal mice with 10,000 IU rIFNβ, a regimen wherein upregulation of these genes had largely ceased by 24 hours following the fourth injection (Fig. 1B, right). Following IFNβ gene transfer, evaluation of IFNβ protein levels in serum by ELISA indicated that the levels of serum protein were below the level of detection of commercially available murine IFNβ ELISAs (data not shown). This finding is consistent with the short serum half-life of IFNβ protein that has made detection of recombinant IFNβ in serum very difficult [26,27].

**Inhibition of EAE progression in mice following IFNβ gene transfer.** Our prior studies have shown that treatment of SWXJ mice with s.c. injection of 10,000 IU/day rIFNβ over an eight week period starting on the day of EAE onset results in a significant inhibition of EAE progression [28,29]. To evaluate therapeutic effects of a gene-based therapy, we immunized eight week old female SWXJ with PLP 139-151 and randomized them on the day of EAE onset into four groups: 1) untreated mice; 2) mice treated in both anterior tibialis muscles with 10 μg pORF-null + EP; 3) mice treated in both anterior tibialis muscles with 10 μg pORF-murine IFNβ + EP; and 4) mice treated with 10,000 IU of rIFNβ injected s.c. every other day. Mice were weighed and evaluated daily for changes in neurologic signs over a period of 8 weeks after EAE onset and initiation of treatment. We found that compared to untreated mice, mice administered the pORF-murine IFNβ plasmid on the day of EAE onset and mice treated with rIFNβ every other day showed comparable clinical courses and highly significant inhibition of disease progression as determined by mean clinical scores over time ($P<0.0001$; Fig. 2). Mice treated with the pORF-null plasmid showed no significant therapeutic effect when compared to untreated EAE mice.
**Decreased exacerbation rates in mice following IFNβ gene transfer.** In addition to inhibition of EAE progression, treatment of EAE with rIFNβ causes a significant decrease in exacerbation frequencies [28,29]. To determine the relative impact on EAE exacerbation rates, the four groups of mice described above were evaluated for relapse frequencies over the last 6 weeks of the eight week observation period (i.e., following the initial acute attack). Exacerbations were assessed as an increase of at least one clinical score associated with at least a 7% decrease in body weight. Compared to untreated mice, the mean number of exacerbations per mouse (exacerbation rate) were significantly reduced in mice treated with pORF-murine IFNβ on the day of EAE onset and in mice treated with 10,000 IU of rIFNβ injected s.c. every other day ($P<0.01$; Fig. 3). Mice treated with the pORF-null plasmid showed no significant decrease in their exacerbation rate when compared to untreated EAE mice.

**Enhanced splenic expression of 2’,5’-OAS mRNA eight weeks after treatment of EAE with pORF-mIFNβ gene transfer.** To assess the induction of IFNβ responsive biomarker expression among the four mouse treatment groups, relative splenic mRNA levels of 2’,5’-OAS were measured by real-time RT-PCR eight weeks after EAE onset and initiation of treatment. 2’,5’-OAS mRNA levels were normalized to β-actin gene expression measured in parallel in each assay. We found that compared to untreated EAE mice, SWXJ mice treated at onset of EAE with pORF-murine IFNβ + EP showed highly significant enhanced splenic expression of 2’,5’-OAS eight weeks after onset of disease and initiation of treatment ($P<0.0001$; Fig. 4). Mice treated with pORF-null plasmid and mice sacrificed 48 hours after completion of a regimen of s.c. injection with 10,000 IU rIFNβ every other day for eight weeks did not show 2’,5’-OAS mRNA levels that were significantly different from those measured in spleens of untreated EAE mice. Thus, our data indicate that a single EP-mediated
administration of the murine IFN\(\beta\) gene leads to prolonged systemic availability of biologically active IFN\(\beta\), with corresponding enhanced expression of an IFN\(\beta\) responsive gene. In distinct contrast, periodic injection with rIFN\(\beta\) fails to maintain sustained levels of biologically active IFN\(\beta\) as evidenced by the lack of splenic 2’,5’-OAS mRNA induction in mice receiving rIFN\(\beta\).
DISCUSSION

Our study shows for the first time that gene-based i.m. IFNβ gene delivery for EAE is feasible and as effective as protocols currently used in the treatment of MS that require long term repeated injection with rIFNβ protein. Our data indicate that the treatment outcome following a single EP mediated i.m. administration of 20 μg of plasmid at the time of onset of clinical EAE is comparable to a regimen of s.c. IFNβ protein injection at 10,000 IU every other day for 8 weeks. Thus, our results provide a rational basis for the development of an effective, contemporary gene-based IFNβ delivery approach for the treatment of MS.

Much of the success of our study may be attributed to the TriGrid™ EP technology (Ichor) that provides an optimized electric field propagated at the site of DNA injection (Fig. 5A). An EP device suitable for gene delivery into large animals and humans has been developed that provides automated control of integrated delivery of the DNA agent and the EP procedure (Fig. 5B). This integrated injection/EP strategy provides spatial and temporal control of the injection process, thereby increasing the probability of high level vector uptake and gene expression and avoiding collateral damage to surrounding tissues. Coordinated injection and electric field propagation provide a convenient and effective way to achieve therapeutic levels of IFNβ using plasmid doses that compare favorably to those used in most other gene transfer protocols [22]. Thus, the efficiency of delivery and uptake may account for the observed long term elevation in splenic IFNβ biomarker activity consistent with prolonged expression and systemic circulation of IFNβ. This long term expression occurs even though the pORF-murine IFNβ contains CpG motifs often associated with early innate inflammatory responses at the sites of vector injection that can contribute to short term expression of
plasmid encoded genes [30].

In our study, the splenic expression levels of three IFNβ inducible genes (2′,5′-OAS, IFI56 and IRF7) 12 weeks after pORF-murine IFNβ delivery in healthy mice were significantly higher than those observed within the 24 hours following injection with rIFNβ protein (Fig. 1B). Extended observations of 2′,5′-OAS gene expression indicated that significant IFNβ bioactivity persisted for at least 18 weeks after a single EP mediated i.m. administration of the IFNβ gene (Fig. 1A). This prolonged elevation of IFNβ activity was also evident eight weeks after treatment of EAE mice with pORF-murine IFNβ EP, in contrast to mice receiving rIFNβ every other day for eight weeks (Fig. 4).

It should be noted that splenic levels of 2′,5′-OAS levels were measured in EAE mice that were euthanized almost 48 hours after their final injection with rIFNβ, and the lack of 2′,5′-OAS induction is consistent with the transient kinetics of injected protein. Nevertheless, the prolonged, high level of IFNβ activity achieved with EP mediated i.m. gene transfer suggests that regulated rather than constitutive production of the cytokine may be useful in future plasmid design. Such second generation constructs may incorporate several regulatory features including natural or synthetic polymers that control vector release and activity [31] or modified ‘latent’ proteins that may be activated at sites of inflammation [32]. In addition, regulation may be achieved using orally administered bioactive molecules that control inducible promoters linked to murine IFNβ [33-35] or that mediate disaggregation and secretion of aggregated IFNβ trapped in endoplasmic reticulum [36].

In addition, incorporation of "suicide genes" that produce prodrug mediated toxins or DNA crosslinking may provide a means to terminate plasmid gene expression even in non-dividing cells thereby offsetting unanticipated side effects that may emerge from long-term endogenous expression of IFNβ in muscle [37].
Several of the well documented side effects of current rIFNβ delivery regimens are likely to be avoided with a gene-based therapeutic approach. The ability to provide long-term production of protein from a single administration would avoid the development of cellulitis and inflammation often occurring at sites of repeated rIFNβ protein injection [12]. Steady state production of IFNβ is also likely to minimize flu-like side effects associated with transient spikes in IFNβ after protein administration, which should reduce patient non-compliance that often leads to tapered rIFNβ dosage and subsequent increased disease activity [15,16]. In addition, gene-based production of IFNβ also reduces the prospect of protein priming and subsequent development of neutralizing antibodies that predispose to decreasing biologic efficacy of rIFNβ injection therapy in MS [6-11]. Finally, long term, gene based production of IFNβ is likely to provide a significant improvement in the cost effectiveness of therapy, since the relatively expensive process of recombinant protein manufacture is replaced by endogenous production. Thus, IFNβ gene transfer offers several advantages over current rIFNβ therapy and with the anticipation of improvements in second generation vectors, will likely provide a viable, effective, and appealing treatment alternative for MS and possibly for other chronic inflammatory disorders.
MATERIALS AND METHODS

**Mice.** Female 8-10 week old ND4 Swiss Webster mice were obtained from Harlan (Indianapolis, IN). Female SWXJ (H-2q,s) mice were obtained from Jackson Laboratories (Bar Harbor, ME) by mating of SWR/J (H-2q) females with SJL/J (H-2s) males. All protocols for animal research met with the prior approval of the Institutional Animal Care and Use Committees (IACUC) of the Cleveland Clinic Foundation and/or Ichor Medical Systems, Inc. in compliance with the Public Health Service policy on humane care and use of laboratory animals.

**Peptide Synthesis.** PLP 139-151 HSLGKWLGHPDKF (substitution of serine for cysteine at residue 140) was synthesized at the Molecular Biotechnology Core Facility of the Lerner Research Institute using standard solid phase methodology and 9-fluorenylmethoxy-carbonyl (FMOC) side chain protected amino acids. The peptide was purified >97% by reverse phase HPLC, and amino acid composition was confirmed by mass spectrometry.

**Induction of EAE.** EAE was induced as previously described [29]. Briefly, 8 week old SWXJ mice were immunized s.c. in the abdominal flank on day 0 with 150 µg PLP 139-151 and 400 µg *Mycobacteria tuberculosis* H37RA (Difco, Detroit, MI) in 200 µl of an emulsion of equal volumes of water and Freund’s adjuvant (Difco). On days 0, 3, and 7 each mouse was injected i.v. with 0.2 µg of purified *Bordetella pertussis* toxin (List, Campbell, CA). Mice were weighed and scored daily for neurologic signs according to the following scale: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; 5, moribund state or death. Mice that became moribund were euthanized; all mice with clinical scores of 5 were included in daily group clinical evaluations as a clinical score of 5 for
the remainder of the study. All experiments were performed in a blinded manner. The blind was established and maintained throughout the course of experiments so that the investigator examining and clinically evaluating the mice was kept unaware of the treatment protocol used on each mouse throughout the experimental protocol. On the first day that clinical signs were detected, mice were incorporated randomly into different treatment protocol groups. As previously described [29], relapse was assessed when mice showed an increase in observed neurologic disability of at least one clinical score unit sustained for at least 2 days coupled with an abrupt substantial loss in weight (≥7% weight loss). Exacerbation rate or relapse frequency was assessed as the mean number of clinical exacerbations per mouse in each treatment group. Mortality within 14 days of EAE onset was considered the result of the primary attack. Thus, mice dying within 14 days of disease onset as well as all relapses occurring prior to day 14 were not included in the determination of exacerbation rate.

**Administration of Recombinant IFNβ.** Highly purified mouse IFNβ from *E. coli* expressing the murine IFNβ gene was purchased commercially (PBL Biomedical, Piscataway, NJ). The IFNβ was prepared fresh daily from frozen aliquots and was administered as previously described [29] under aseptic conditions on the day of EAE onset and every other day thereafter by s.c. injection of 10,000 IU (specific activity 3x10^7 IU/mg) in 0.1 ml of 0.01% mouse serum albumin in PBS. The dose of IFNβ selected represents approximately a 4-fold increase in effective dose/kg administered in human MS studies [1]. In a separate experiment, six Swiss Webster mice received four injections every other day of 10,000 IU IFNβ s.c. prior to euthanasia six hours (3 mice) and 24 hours (3 mice) after the last injection. Mice showed no apparent toxic side effects of any treatment protocols.
**IFNβ Expressing Plasmid.** The IFNβ gene expressing plasmid, pORF-murine IFNβ (pORF-mIFNβ) and its pORF-null version (pORF-mcs) were purchased commercially (InvivoGen, San Diego, CA). Each pORF is cloned in a mammalian expression cassette consisting of a composite promoter and a SV40 polyadenylation signal. The composite promoter consists of the human elongation factor-1 alpha core promoter coupled to the 5’ untranslated region of the human T cell leukemia virus type I long terminal repeat. The hEF-1α gene is abundantly expressed in a wide range of cells and its promoter facilitates persistent gene expression in vivo. The HTLV sequence enhances stability of generated mRNA. The plasmids were purified using the EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA).

**In Vivo Electroporation and Gene Delivery.** Plasmid vectors were delivered in vivo by EP-mediated i.m. injection. Hair around the anterior tibialis muscles of isoflurane anesthetized mice was clipped to expose the muscle and the overlying skin was disinfected using 70% alcohol swabs. Each target muscle was administered a single injection of 10-20 μg of plasmid DNA in an injection volume of 20 μl followed by the application of EP using the TriGrid™ Delivery System (Ichor Medical Systems, San Diego, CA). The EP protocol was completed within 0.5 seconds, comprising rectangular wave, direct current pulses applied at 220 volts/cm peak amplitude and 8% duty cycle. Control mice underwent the identical EP procedure for delivery of the pORF-null plasmid. The TriGrid™ EP system consists of an electric pulse generator and a TriGrid™ array comprising four electrodes 2 mm in length and 2.5 mm interelectrode spacing. A 0.3 ml syringe with a 0.5 inch 30 gauge needle (BD Ultra-Fine, 328431, Becton Dickinson, Franklin Lakes, NJ) is attached through the center of the electrode array (Fig. 5B). Integration of the means for DNA administration and electric field application into a single device provides spatial and temporal control over DNA delivery, ensuring that EP is applied at the site of DNA administration in a consistent fashion for all subjects.
Relative Quantitation of Gene Expression Using Real-Time RT-PCR. Systemic effects of plasmid IFNβ gene expression were determined by relative quantitation of splenic mRNA levels of interferon inducible genes including 2’,5’-oligoadenylate synthetase 2 (2’,5’-OAS), interferon inducible gene 56 (IFI56), and interferon regulatory factor-7 (IRF7). At various times post-DNA transfer, spleens were flash frozen and RNA was purified using the RNeasy Midi Kit (Qiagen, Valencia, CA). cDNA was prepared from 5 µg of RNA using the iScript Select cDNA Synthesis Kit (BioRad, Hercules, CA). Relative quantitation of gene expression was performed on the cDNA using reagents from Applied Biosystems (Foster City, CA) according to the manufacturer’s instructions. Predesigned and optimized TaqMan Gene Expression Assays for the detection of 2’,5’-OAS (Assay ID #Mm00460961_m1), IFI56 (Assay ID #Mm00515153_m1), IRF7 (Assay ID #Mm00516788_m1), GAPDH (catalog #4308313), and β-actin (catalog #4352933E) were purchased from Applied Biosystems. qPCR was carried out in a final volume of 25 µl in 1X TaqMan Universal Master Mix using Applied Biosystems Universal Cycling Conditions (50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation and 60°C anneal/extension). The PCR was performed using the Applied Biosystems 7900 Real-Time Fast PCR System at the University of California, San Diego Cancer Center Molecular Pathology Shared Resources Lab (Swiss Webster mice experiments) or 7300 Real-Time PCR System (EAE experiments) at Ichor. Levels of 2’,5’-OAS, IFI-56, and IRF-7 mRNA were normalized to mRNA levels of the endogenous control gene, GAPDH (Swiss Webster mouse experiments) or β-actin (EAE experiments), measured in parallel in each assay. Relative quantitation of expression of mRNA was calculated as the fold increase in expression in experimental versus naive control or untreated EAE mice.
**Statistical Analysis.** Time course differences in 2’5’-OAS mRNA expression levels were compared using Wilcoxon rank sum statistic. Disease progression curves were compared using one way analysis of variance (ANOVA). Differences in exacerbation frequencies and in static expression levels of IFNβ inducible genes were compared using the unpaired Student’s t-test.
ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants NS-37476, NS-36054, AI-51837, DC-006422 (V.K.T.), R44-NS-043829 (C.F.E.) and R43-NS-043829 (M.M.D.), and by National Multiple Sclerosis Society fellowship FG-1606 (R.J.). The authors wish to acknowledge Barry Ellefsen, Gerald Nakamura, and Lillian Chau for technical assistance; Iveta Kalcheva for assistance with the real-time PCR studies, and Karen Dolter for critical review of the manuscript.
REFERENCES


20. Mir, L. M., Bureau, M. F., Gehl, J., Rangara, R., Rouy, D., Caillaud, J. M., Delaere, P.,

    efficiency growth hormone-releasing hormone plasmid vector administration into skeletal


23. Rizzuto, G., Cappelletti, M., Maione, D., Savino, R., Lazzaro, D., Costa, P., Mathiesen, I.,

    pharmacokinetics and pharmacodynamics of recombinant human interferon beta-1a after


    in the absence of measurable serum concentrations: a comparative trial of subcutaneous and


FIGURE LEGENDS

FIG 1. **IFNβ gene transfer results in sustained induction of IFNβ inducible genes.** (A) Spleens of Swiss Webster mice show sustained elevated expression of mRNA for the interferon inducible gene, 2′,5′-OAS, for more than 18 weeks after a single *i.m.* administration of 10 μg of pORF-mIFNβ + EP. Negligible induction of 2′,5′-OAS mRNA occurs when 40 μg of the pORF-mIFNβ plasmid is injected without EP. Fold induction of 2′,5′-OAS mRNA in spleens was calculated relative to naive mice. The 2′,5′-OAS expression levels in mice treated with pORF-mIFNβ + EP were significantly higher (*P*<0.05) than levels measured in naive mice or in mice treated with pORF-mIFNβ without EP. (B) In addition to 2′,5′-OAS, enhanced splenic expression of mRNA for the interferon inducible genes, IFI56 and IRF7, is evident 12 weeks after a single *i.m.* transfer of 10 μg of pORF-mIFNβ + EP in normal Swiss Webster mice (n=6). 2′,5′-OAS, IFI56 and IRF7 mRNA levels are substantially higher than those observed 6 (n=3) and 24 (n=3) hours after the fourth *s.c.* injection of Swiss Webster mice with 10,000 IU rIFNβ (see methods). Error bars show ±SE.

FIG 2. **Inhibition of EAE progression in mice treated with IFNβ gene therapy.** Eight week old female SWXJ mice were immunized with PLP 139-151 and randomized at EAE onset into four groups: 1) untreated; 2) treated with pORF-null + EP; 3) treated with pORF-mIFNβ + EP; or 4) treated with 10,000 IU of rIFNβ injected *s.c.* every other day. Mice were weighed and evaluated daily for neurologic signs over a period of 8 weeks after EAE onset and initiation of treatment. Compared to untreated mice, mice inoculated with the pORF-mIFNβ plasmid on the day of EAE onset and mice treated with rIFNβ every other day showed highly significant inhibition of disease progression (*P*<0.0001) as determined by decreased mean clinical scores over time. Treatment with the pORF-
null plasmid showed no significant therapeutic effect. Error bars show ±SE.

**FIG 3. Decreased exacerbation rates in mice treated with IFNβ gene therapy.** SWXJ mice typically undergo cycles of EAE exacerbations and remissions following recovery from the initial EAE onset. The four treatment groups described in Fig. 2 were assessed for exacerbation frequencies over the eight week period of clinical evaluation following onset of disease. Exacerbations were assessed as an increase of at least one clinical score associated with at least 7% decrease in body weight. Compared to untreated or pORF-null + EP treated mice, the mean number of exacerbations per mouse (exacerbation rate) were significantly reduced in mice treated with pORF-mIFNβ + EP on day of EAE onset and in mice treated with 10,000 IU of rIFNβ injected s.c. every other day (P<0.01). Treatment with the pORF-null plasmid showed no significant therapeutic effect. Error bars show ±SE.

**FIG 4. Enhanced splenic expression of 2′,5′-OAS eight weeks after treatment of EAE with pORF-mIFNβ gene therapy.** When compared to untreated EAE mice, female SWXJ mice treated at onset of EAE with pORF-mIFNβ + EP (n=10) show highly significant enhanced splenic expression of 2′,5′-OAS eight weeks after onset of disease and initiation of treatment (P<0.0001). Mice treated with pORF-null plasmid (n=8) and mice injected s.c. with 10,000 IU rIFNβ (n=6) every other day for eight weeks did not show 2′,5′-OAS mRNA levels that were significantly different from those measured in spleens of untreated EAE mice (n=11). Splenic levels of 2′,5′-OAS mRNA were analyzed by real-time RT-PCR and data presented as fold increased mRNA levels compared to untreated EAE mice. Error bars show ±SE.
FIG 5. TriGrid™ intramuscular electroporation delivery device. (A) Ichor’s proprietary arrangement of electrodes in the form of trigrids, with electrodes separated by a predefined distance (L) and with the z dimension defined by electrode length allows generation of an optimum electric field for maximum efficiency of gene uptake on electroporation. (B) The integrated rodent applicator comprises an array of four electrodes interfaced with a 0.3 ml injection syringe. The electrodes surround a recessed central injection needle (inset), ensuring that the electroporation effect is achieved at the site of DNA administration.
FIGURE 2

Mean Clinical Score vs. Day After EAE Onset

- Untreated (n=14)
- pORF-null (n=12)
- pORF-mIFNβ (n=13)
- rIFNβ (n=13)
FIGURE 4

Fold Increased 2',5'-OAS Gene Expression Relative to Untreated EAE Mice

- Untreated
- pORF-null
- pORF-mIFNβ
- rIFNβ

*p < 0.05