Cytokine Gene Therapy in Experimental Allergic Encephalomyelitis by Injection of Plasmid DNA-Cationic Liposome Complex into the Central Nervous System

J. Ludovic Croxford, Kostas Triantaphyllopoulos, Osvaldo L. Podhajcer, Marc Feldmann, David Baker and Yuti Chernajovsky

*J Immunol* 1998; 160:5181-5187; ;
http://www.jimmunol.org/content/160/10/5181

References
This article cites 48 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/160/10/5181.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cytokine Gene Therapy in Experimental Allergic Encephalomyelitis by Injection of Plasmid DNA-Cationic Liposome Complex into the Central Nervous System\textsuperscript{1}

J. Ludovic Croxford,\textsuperscript{2*} Kostas Triantaphyllopoulos,\textsuperscript{3} Osvaldo L. Podhajcer,\textsuperscript{3} Marc Feldmann,\textsuperscript{3} David Baker,\textsuperscript{*} and Yuti Chernajovsky\textsuperscript{†}

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the central nervous system with many similarities to multiple sclerosis. The main effector cells involved are CD4\textsuperscript{+} T cells, recognizing encephalitogenic epitopes within the central nervous system, and macrophages, both of which secrete proinflammatory cytokines, such as IFN-\(\gamma\) and TNF. Studies have shown that immunomodulation of this inflammatory response by anti-inflammatory cytokines (IL-4, IL-10, IFN-\(\beta\), and TGF-\(\beta\)) can reduce clinical severity in EAE. The importance of TNF in EAE has been demonstrated by using soluble TNF-receptor molecules to inhibit EAE. However, the limitation of this type of therapy is the necessity for frequent administration of cytokine proteins due to their short biologic half-life. This study demonstrates that EAE can be inhibited by a single injection of therapeutic cytokine (IL-4, IFN-\(\beta\), and TGF-\(\beta\)) DNA-cationic liposome complex directly into the central nervous system. DNA coding for a novel, dimeric form of human p75 TNF receptor also ameliorated clinical EAE. Local administration of DNA-cationic liposome complex has identified gene targets that may be more efficiently exploited using vectors producing more stable expression for effective treatment of neuroimmunologic disease. The Journal of Immunology, 1998, 160: 5181–5187.

\textsuperscript{1}Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; CNS, central nervous system; TNFR, tumor necrosis factor receptor; dTNFR, dimeric TNFR; SCH, spinal cord homogenate; p.i., postinoculation; MMelLV, LTR, Moloney murine leukemia virus long terminal repeat; NSE, neuron-specific enolase; i.e., intracerebral; DNA-CLC, deoxycytosinucleic acid cationic liposome complex; m, murine.

In an attempt to convert the pathogenic Th1 response occurring in lymphoid tissue to an inhibitory Th2 response, studies have shown that EAE can be suppressed by systemic administration of inhibitory cytokines, such as IL-4, TGF-\(\beta\), and IL-10. IFN-\(\beta\) has been shown to be an effective therapy for MS and is thus another candidate for immunomodulation of EAE (5). Soluble cytokine receptors for TNF (p55 and p75 TNFR) and anti-TNF Abs have also been shown to inhibit EAE by antagonizing pathogenic cytokine activity (6). There is evidence to suggest that both TNF-\(\alpha\) and TNF-\(\beta\) are involved in disease processes such as EAE, in which encephalitogenic T cells secrete TNF, which may cause cell infiltration and demyelination by attacking oligodendrocytes and the myelin sheath surrounding neuronal axons (7, 8). Such immunotherapy given systemically, however, is limited by the short biologic half-life of cytokines, and disease returns following cessation of treatment. Therefore, gene therapy of EAE by inhibitory cytokines (IL-4, TGF-\(\beta\), IFN-\(\beta\), and IL-10) or cytokine inhibitors (p75 dimeric TNFR (dTNFR) (9) and p55 TNFR-Ig (10)), given locally as plasmid DNA, could provide an extended expression of cytokine at physiologic concentrations from a single injection.

Currently, viral vectors available for gene therapy are replication-deficient retrovirus and adenovirus, both of which have limitations for use in the treatment of inflammatory CNS disorders. Cells within the CNS are in a postmitotic state and therefore are refractory to infection by retrovirus, which integrates into the host genome of dividing cells. Adenoviral vectors have the capacity to infect a broad range of cell type, both dividing and nondividing, with high efficiency. However, these virus particles have been shown to be immunogenic, thus limiting longevity of expression and repeat administrations (11). Plasmid DNA exists epichromosomally and therefore can be used to transfect most cell types. It has also been reported to be nonimmunogenic when delivered in vivo to nonhuman primates (12). Although not as efficient in transfection as adenoviral vectors, studies have shown expression for up to 19 mo in skeletal muscle (13). Therefore, gene therapy by the
Inhibition of EAE by Local Cytokine Gene Therapy

Materials and Methods

**Animals and disease induction**

Biozzi ABH mice were bred at the Institute of Ophthalmology (London, U.K.). Mice (6–8 wk old, typically male) were injected in the flank with 1 mg of ABH mouse spinal cord homogenate (SCH) in IFA supplemented with 60 µg of mycobacteria (days 0 and 7 as described previously (14)). Animals were monitored up to day 24 postinoculation (p.i.), and clinical signs were assessed: 0 = normal, 1 = flaccid tail, 2 = impaired righting reflex, 3 = partial paralysis, and 4 = complete paralysis. Clinical signs of a lower severity than typically observed were scored 0.5 lower than the grade indicated as described previously (15).

**Cytokine plasmids**

Plasmids and retroviral vectors expressing cytokines and cytokine inhibitors were as follows:

- Human p55 TNFR (extracellular domain) fused to mouse IgG1 backbone was provided by Prof. B. Beutler (Southwestern University, Dallas, TX) and was driven by the CMV promoter (10).
- The human p55 TNFR fusion protein was then cloned into pBabe-blo, driven by the MuLV LTR promoter as previously described (16).
- The cDNAs for mouse mIL-4 and human TGF-β were obtained from the American Type Culture Collection (Rockville, MD) and cloned into the pBabe-neo retrovector vector (17), mIL-4 (0.59 kb BamHI fragment) was cloned into the BamHI site of pBabe-neo; the cloning of TGF-β has been previously described (18).
- Dimeric human p75 dTNFR was cloned from the Trip-4 plasmid (9) into the retrovector vector MFG (19), provided by Dr. P. D. Robbins (University of Pittsburgh), as described previously (20).
- A genomic clone of mouse TNF (provided by Dr. N. Sarvetnick, Scripps Institute, La Jolla, CA) was cloned into pBabe-blo, mIL-10 cDNA was provided by Dr. K. Moore (DNAX, Palo Alto, CA) and was cloned (as a 1.2-kb XhoI fragment) into the Sall site of pBabe-puro (17).
- Mouse IFN-β, driven by the MuLV LTR in pBabe-blo or the neuron-specific enolase (NSE) promoter were previously described (16).
- The plasmid pCH110 expressing the bacterial β-galactosidase gene under the early SV-40 promoter was purchased from Pharmacia Biotech (Uppsala, Sweden).

**Expression of cytokine plasmids**

Plasmid DNA was prepared as described previously (9). Briefly, *Escherichia coli* cultures containing the plasmids of interest were grown at 37°C. Following lysis of bacteria and addition of RNase A (10 mg/ml), samples were centrifuged at 25,000 × g at 4°C for 40 min and filtered through sterile gauze. DNA was precipitated with 60% propan-2-ol at −20°C for 30 min. Samples were centrifuged for 20 min at 1,500 × g, and pellets were washed with ethanol and resuspended for 5 min at 1,500 × g before being applied to Qiagen columns (Qiagen, Chatsworth, CA) and eluted with wash buffer. After precipitation, plasmids were resuspended in normal saline before injection of 100 µg of the naked DNA. Plasmids were transfected into COS-7 cells for transient expression assays or were permanently transfected using the GPeN-AM12 packaging cell lines (21) and were used to confirm the functional activity of the plasmid constructs using cytokine ELISA or biologic assays and Western blots. The IFN-β-NSE plasmid was assessed following transfection into neuroblastoma cells (16).

**Intramuscular injection of cytokine plasmid DNA**

Mice were anesthetized by i.p. injection of 0.3 ml/kg of a mixture (1:1) of Hypnorm (Janssen, Wantage, U.K.) and Hypnovel (Roche, Welwyn Garden City, U.K.) diluted in H2O. These received a single dose of DNA (50 µl), injected i.m. in the right tibialis anterior muscle. In some instances 50 µl of 1.2% BaCl2 (gift from Dominic Wells, Charing Cross and Westminster Medical School, London, U.K.) in PBS was injected into the same muscle 5 days before plasmid injection to induce myoproliferation, thus facilitating the uptake of plasmid into the regenerating muscle tissue (22). Cytokine plasmid DNA (100 µg in saline) was injected i.m. on day 0 of EAE induction.

**Intracerebral (i.c.) injection of cytokine protein**

Murine cytokine proteins IL-4 (5 µg) and IL-10 (20 µg) in 30 µl of PBS were injected i.c. into the right frontal cortex of mice anesthetized with halothane (May and Baker, Dagenham, U.K.) at the onset of disease (clinical grade 1) using 27-gauge insulin syringes (Surgecon, Leeds, U.K.) as described previously (15). Generally, i.c. injections were well tolerated by the mice. Any mice showing evidence of brain damage as a result of the i.c. injection were excluded from the study.

**Intracerebral DNA injection**

Animals were injected i.c. with 100 µg of plasmid DNA dissolved in 30 µl of saline as described above. In some instances this was mixed with 10% lipofectin reagent (Life Technologies, Grand Island, NY; 1 mg/ml) according to the manufacturer’s instructions. The plasmid-cationic liposome complex was allowed to stand at room temperature for 15 min before i.c. injection. These were administered, shortly before the anticipated onset of disease, on day 12 p.i. Controls were either untreated or injected with lipofectin in saline.

**Immunohistochemistry**

Muscle and brain tissue from treated and control mice were removed at various time points up to 18 days postinjection of plasmid DNA complex and embedded in OCT compound (Raymond A. Lamb, London, U.K.). These were snap-frozen in liquid nitrogen-cooled isopentane (BDH, Poole, U.K.). Cryostat sections were cut at a thickness of 8 µm, air-dried, fixed in acetone for 10 min at room temperature, and stored at −20°C. Endogenous peroxidase activity was blocked by incubating the sections with 0.03% hydrogen peroxide (Sigma, Poole, U.K.) in PBS for 10 min (14). The sections were washed in PBS, then incubated with 5% normal mouse serum in PBS for 30 min. Tissue culture supernatant of rat mAb specific for mouse IL-4 (BVD4−1D11, Pharmingen, San Diego, CA) and mouse IL-10 (J555-2A5, Pharmingen) (23), rabbit polyclonal antibodies specific for mouse IFN-β (Lee Biomolecular, San Diego, CA) and TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated mouse mAb reactive with human p55 (CD120a) TNFR (Htr-9, Hoffmann-La Roche, Basel, Switzerland), and biotinylated mouse anti-CD120b p75 (4D1B10 MR2-1). Caltag, San Francisco, CA) were incubated at room temperature for 1 h and then washed in PBS. Rat mAb and rabbit Ab were detected with peroxidase-conjugated polyclonal antisera as described previously (14). Peroxidase activity was developed using diaminobenzidine substrate (0.06% 3,3-diaminobenzidine and 0.018% H2O2 in PBS for 1 min. Sections were counterstained with hematoxylin and mounted in DPX (BDH, Poole, U.K.). Positive controls for the Ab staining were cytokine retrovirus-transfected Biozzi ABH mouse fibroblast cytokines and human PBL cytokins. Cross-reactivity of the human-specific mAb was checked using mouse lymph node cell cytokins. Negative controls consisted of omission of primary Ab or irrelevant isotype-matched control as described previously (14). Controls also consisted of staining with mAb of CNS and muscle tissue injected with plasmid DNA of a different specificity to the primary Ab.

**β-Galactosidase analysis**

Muscle and brain tissue injected with the lacZ construct (pCH110) were removed between 7 and 10 days p.i. and fixed in a 0.005% glutaraldehyde/2% paraformaldehyde solution (Agar Scientific, Cambridge, U.K.) in sodium phosphate buffer (pH 7.3) for 30 min. Tissue was then washed in PBS and placed into a staining solution containing 50 mM Tris-HCl (pH 7.5), 2.5 mM ferro-ferricyanide, 15 mM NaCl, 1 mM MgCl2 (Sigma), and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Life Technologies) in X,N-dimethylformamide (Sigma) in PBS. The tissue block was incubated at 37°C for 1 to 2 h. Tissue was then washed in PBS and placed into formalin (Agar Scientific). Expression of LacZ construct was seen by blue staining in the tissue block by light microscopy. Negative controls were either untreated tissue or tissue injected with cytokine plasmid DNA.

**Statistical analysis**

Results were presented as the mean clinical score or onset ± SEM, and statistical difference was determined using the Mann-Whitney U nonparametric ranking test.

**Results**

**Systemic cytokine gene therapy**

A single i.m. injection of 100 µg of cytokine DNA in saline administered on the day of immunization to target the induction phase of EAE failed to ameliorate disease severity or the onset of disease (Table I). Immunohistochemical staining of the injected muscle failed to show any increase in cytokine protein production beyond endogenous levels observed within the tissue (data not shown). Injection of 1.2% BaCl2 solution into the mouse tibia...
anterior muscle 5 days before DNA injection induces myoproliferation, to increase plasmid uptake (22), at the time of DNA injection. Again, the majority of injected cytokine constructs failed to make an impact on disease compared with that in untreated or BaCl2-pretreated animals, except for the p55 TNFR-Ig fusion protein DNA, which significantly reduced clinical disease ($p < 0.05$) and appeared to delay, although nonsignificantly, the onset of disease (Table II). Although using a reporter gene LacZ construct (pCH110), expression of $\beta$-galactosidase could be seen in some muscle fibers following the route of injection, again immunohistochemistry could not demonstrate significant cytokine production above that seen endogenously or within invading inflammatory cell components (data not shown).

### CNS-directed cytokine DNA gene therapy

Having established that systemic delivery of plasmid cytokine DNA during sensitization did not significantly alter EAE disease course, but that local delivery of the cytokine protein could have a transient effect (6), the potential of local gene delivery to the CNS was studied. One hundred micrograms of cytokine DNA was injected i.c. on day 12 p.i. shortly before the onset of disease (Table III) in an attempt to modulate the effector phases of the disease course. The majority of these failed to make any significant impact on the disease course (Table III). Following i.c. injection of the IL-4 plasmid DNA construct, there was a reduction in disease incidence, by 20%, compared with that in SCH control animals, which all developed disease. However, the effect of the IL-4 construct on disease severity within the whole group (Table III) was just beyond statistical significance ($p = 0.055$), as the animals that developed disease exhibited severity comparable to that in control animals.

### Immunostaining of CNS-directed gene expression from cytokine DNA-cationic liposome complexes

Cationic liposomes facilitate the uptake of DNA into cells (24), and injection of DNA-CLC increases the CNS expression of reporter gene constructs (25). Using immunocytochemistry, it was difficult to definitively detect gene-delivered protein expression following injection of the DNA construct. Mouse IFN-$\beta$ appeared to be present around the myelin sheath of axons in the CNS of untreated mice, although at a low levels. Following i.c. injection of the IFN-$\beta$ DNA-CLC, the intensity of this staining appeared higher than that in controls (data not shown), although it is not clear whether this was biologically significant. The Ab reactive with human TGF-$\beta$ also detected mouse TGF-$\beta$ within the extracellular matrix, and thus it was not possible to distinguish any expression above that normally expressed endogenously. Likewise, IL-10 protein could be readily detected in normal CNS. This was present in astrocytes, axons within the white matter, and neural cell bodies within the gray matter (Fig. 1). This staining was inhibited by coinubcation of the mAb with rIL-10 protein. Interestingly, injection of two different batches of 100 to 500 $\mu$g of IL-10 mAb directly into the CNS of mice either at the onset of EAE or in normal animals proved fatal. IL-4 protein expression was below the level of detection. The biotinylated mouse mAb (Hr-9) specific for human p55 TNFR1 (CD120a) cross-reacted with mouse CD120a at all dilutions (1/50 to 1/16,000 of 1 mg/ml) tested when used to stain mouse lymph node cell cytopsins and was therefore not used for CNS staining. Mouse Ig is readily detected within normal mouse CNS tissue (14) and consequently could not be used to detect the Ig portion of the p55 fusion protein.

### Table I. Failure of systemic injection of cytokine DNA construct to inhibit EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score (±SEM)</th>
<th>Mean Day of Onset (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH</td>
<td>21/21</td>
<td>3.5 ± 0.2</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>TGF-$\beta$</td>
<td>7/7</td>
<td>2.9 ± 0.4</td>
<td>15.6 ± 0.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>8/10</td>
<td>2.5 ± 0.5</td>
<td>16.1 ± 0.4</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>7/7</td>
<td>3.6 ± 0.2</td>
<td>16.4 ± 0.6</td>
</tr>
<tr>
<td>IFN-$\beta$</td>
<td>6/6</td>
<td>3.4 ± 0.3</td>
<td>15.9 ± 0.4</td>
</tr>
<tr>
<td>IFN-$\beta$ [NS]</td>
<td>5/5</td>
<td>3.1 ± 0.2</td>
<td>15.6 ± 0.5</td>
</tr>
</tbody>
</table>

*Animals were injected with SCH in Freund's adjuvant on days 0 and 7. A total of 100 $\mu$g of naked DNA coding for TGF-$\beta$, IL-4, IFN-$\beta$, IFN-$\beta$ [NS], and TNFR-Ig p55 were injected i.c. on day 12. The results represent the mean ± SEM maximum clinical score of all animals within the group and the mean day of onset of the animals that developed EAE.

### Table II. Partial inhibition of EAE by systemic administration of cytokine DNA constructs into myoproliferating muscle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score (±SEM)</th>
<th>Mean Day of Onset (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH</td>
<td>22/22</td>
<td>3.4 ± 0.2</td>
<td>16.4 ± 0.3</td>
</tr>
<tr>
<td>BaCl$_2$</td>
<td>10/10</td>
<td>3.5 ± 0.2</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>p75 $\Delta$TNFR</td>
<td>5/5</td>
<td>3.6 ± 0.3</td>
<td>17.6 ± 1.0</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>13/14</td>
<td>2.6 ± 0.3*</td>
<td>16.0 ± 0.4</td>
</tr>
<tr>
<td>IL-10</td>
<td>9/9</td>
<td>3.1 ± 0.3</td>
<td>18.3 ± 0.8</td>
</tr>
<tr>
<td>TNF</td>
<td>9/9</td>
<td>3.6 ± 0.3</td>
<td>17.0 ± 0.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>6/6</td>
<td>2.8 ± 0.5</td>
<td>18.3 ± 1.0</td>
</tr>
<tr>
<td>TGF-$\beta$</td>
<td>15/15</td>
<td>3.0 ± 0.2</td>
<td>16.2 ± 0.4</td>
</tr>
</tbody>
</table>

*Animals were injected with SCH in Freund’s adjuvant on days 0 and 7. BaCl$_2$ solution (1.2%) was injected i.m. into the anterior tibialis 5 days prior to 100 $\mu$g naked DNA coding for p55TNFR-Ig, p75 $\Delta$TNFR, IL-10, IL-4, TNF, and TGF-$\beta$ injected i.m. on day 0. The results represent the mean ± SEM maximum clinical score of all animals within the group and the mean day of onset of the animals that developed EAE.

$p < 0.05$ compared to animals injected with SCH only.
In contrast, it was possible to distinguish the expression of the human p75 TNFR from the endogenously expressed mouse p75 TNFR using biotinylated anti-human CD120b. Human p75-positive resident cells were detected around the injection site within 3 days p.i. (Fig. 2). Sparse expression could be detected by 6 days p.i., but by day 18 p.i., no positive staining was detected (n = 5; data not shown). Sections of normal brain taken between 24 h and 21 days after i.c. DNA-CLC injection failed to show any abnormality associated with CNS cytokine production or any evidence of leukocyte infiltration (data not shown).

**Therapeutic effect of CNS-directed gene transfer using cytokine DNA-CLC**

Although protein expression following plasmid DNA-CLC was clearly at low levels, these were examined as a means of controlling EAE. Again, mice received a single i.c. injection of 100 μg of cytokine, but with 3 μg of lipofectin (total volume, 30 μl), on day 12 p.i. Mice injected i.c. with CLC alone showed no significant difference from untreated controls (p > 0.05) in disease incidence, clinical severity, or disease onset (Table IV). In contrast to that observed following injection of naked DNA alone (Table III), cytokine DNA-CLC coding IFN-β, IL-4, TGF-β, p55 TNFR-Ig, and p75 dTNFR all significantly (p < 0.001) reduced the severity of disease compared with that in either untreated or CLC-treated controls (Table IV). Injection i.c. with IFN-β-NSE DNA-CLC also significantly (p < 0.02) inhibited the severity of EAE compared with that in the CLC control group, although this was to a lesser degree than that observed following injection of IFN-β DNA-CLC, which was driven by the MMuLV LTR (Table IV). Mice injected with p75 dTNFR DNA-CLC showed the greatest inhibition of disease course compared with untreated (p < 0.001) and CLC-treated (p < 0.005) animals (Fig. 3) and reduced the disease incidence by 56% (Table IV). The disease frequency following injection of IL-4 TGF-β and IFN-β was similarly reduced by 30 to 40%. In contrast, injection of LacZ or IL-10 DNA-CLC failed to affect the disease incidence or severity (Table IV). This study was designed to examine the clinical course of nondemyelinating acute EAE (14), and animals were allowed to remit. The lesion load in

**FIGURE 1.** Immunohistochemical staining for endogenous IL-10 in the normal mouse spinal cord. Endogenous IL-10-positive astrocytes (a; →) and IL-10-positive axons (b) within the white matter.

**FIGURE 2.** Mouse brain cells positive for human p75 dTNFR 3 days after DNA-CLC injection into right frontal lobe. The arrow shows human p75 dTNFR-positive cells around the site of injection 3 days postinjection of 100 μg of human p75 dTNFR DNA-CLC (30 μl) into the right cortex.

Table IV. Inhibition of EAE by local administration of cytokine DNA-CLC to the CNS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score (±SEM)</th>
<th>Mean Day of Onset (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLC</td>
<td>8/8</td>
<td>3.8 ± 0.1</td>
<td>15.0 ± 0.3</td>
</tr>
<tr>
<td>SCH</td>
<td>23/23</td>
<td>3.7 ± 0.1</td>
<td>15.1 ± 0.3</td>
</tr>
<tr>
<td>LacZ</td>
<td>7/7</td>
<td>3.2 ± 0.2</td>
<td>14.7 ± 0.3</td>
</tr>
<tr>
<td>TGF-β</td>
<td>10/15</td>
<td>2.0 ± 0.4***</td>
<td>16.4 ± 0.5</td>
</tr>
<tr>
<td>IL-4</td>
<td>9/13</td>
<td>2.3 ± 0.4***</td>
<td>16.2 ± 0.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>11/11</td>
<td>3.6 ± 0.2</td>
<td>16.2 ± 0.3*</td>
</tr>
<tr>
<td>p75 dTNFR</td>
<td>5/9</td>
<td>1.0 ± 0.3***</td>
<td>17.2 ± 0.6**</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>11/11</td>
<td>3.0 ± 0.3*</td>
<td>16.4 ± 0.2***</td>
</tr>
<tr>
<td>IFN-β</td>
<td>4/7</td>
<td>1.6 ± 0.6**</td>
<td>14.8 ± 0.3</td>
</tr>
<tr>
<td>IFN-β [NSE]</td>
<td>16/18</td>
<td>2.7 ± 0.3*</td>
<td>15.4 ± 0.3</td>
</tr>
</tbody>
</table>

*Animals were injected with SCH in Freund’s adjuvant on days 0 and 7. A total of 100 μg of naked DNA coding for LacZ, TGF-β, IL-4, IFN-β, IFN-β [NSE], TNFR-Ig p55, and d-TNFR p75 were mixed with lipofectin and injected i.c. on day 12. The results represent the mean ± SEM maximum clinical score of all animals within the group and the mean day of onset of the animals that developed EAE.

*p < 0.05; **p < 0.01; ***p < 0.005 compared to animals injected with CLC only.
ABH mice correlates with the progression of clinical disease, and the number of infiltrating cells is rapidly diminished as animals begin to remit (14, 26). Therefore, once control animals begin to remit, lesions are lost, and thus histologic analysis of such animals is uninformative.

Discussion

This study demonstrates that local gene delivery of therapeutic cytokine DNA-CLC to mice with established EAE can significantly reduce the severity and onset of clinical disease.

Systemic cytokine gene therapy targeting the induction phase of EAE in lymphoid tissue by a single i.m. administration of cytokine plasmid DNA to the anterior tibialis muscle failed to influence the clinical progression of EAE, even following administration of a myotoxic agent that has been shown to produce a 5- to 40-fold increase in efficiency of gene transfer in the regenerating anterior tibialis and quadricep muscle fibers (22, 27, 28). This ineffectiveness in EAE treatment is probably due to inefficient gene transfer of the cytokine DNA, as immunohistochemical analysis of injected tissue did not show levels of cytokine above endogenous levels. However, when DNA coding for p55 TNFR-Ig was delivered into myoproliferating tissue on day 0, there was a slight reduction in clinical severity of EAE and may relate to a prolonged systemic half-life mediated by the Ig domains of the immunoadhesin. DNA plasmid DNA has shown success in the lupus-prone mouse (MRL/lpr/lpr) model in which RSV-driven TGF-β cytokine genes can be expressed for prolonged periods and mimic the beneficial characteristics of the cytokine itself injected in vivo (29). However, DNA was administered in five separate injections to the quadriceps muscle of BALB/c mice (28) compared with a single injection in the anterior tibialis muscle in this ABH mouse EAE model, thus targeting a larger area of muscle fibers. Although the efficiency of uptake may be related to the mouse strain used (28, 30), efficacy at inhibition of EAE compared with that following similar i.c. injection of naked DNA alone, although it was still difficult to detect protein expression, again pointing toward a low transfection efficiency or low levels of expression. Similar i.c. injection of a fluorescent dye showed expression throughout the frontal lobe, around the meninges, and pial surfaces, and this can reach the spinal cord as shown previously (33). It is likely that the DNA-CLC also disperses in this manner, and this coupled with the relatively low transfection efficiency would mean a few positive cells, possibly at some distance from the site of injection, making immunohistochemical analysis of construct expression difficult to quantify. This contrasts with that observed following similar i.c. injection of adenovirus.

Previous studies have demonstrated the increased efficacy of therapeutic agents when delivered locally to the site of inflammation (6). Therefore, cytokine DNA was delivered into the CNS during the effector phase of EAE. Such gross tissue remodeling as those occurring after the i.m. injections (induced by the myotoxic agent) and after repeated injection is not feasible in the CNS. As with systemic administration, cytokine plasmid DNA injected into the CNS has no mechanism by which to target the nucleus of resident CNS cells, unlike virus, and results in poor gene transfer, which may account for the relative lack of effect. However, studies have shown that an increase in uptake efficiency of reporter gene constructs can be achieved by forming a DNA-CLC, producing a lipid complex that fuses with the plasma membrane of target cells and facilitates the uptake of DNA (25, 32). Such reporter gene constructs have been shown to transfect neuronal cells, astrocytes, the myelin-forming oligodendrocytes, and ependymal cells (25, 32) and was detected for at least 9 days following reporter gene DNA-CLC injection (25). However, DNA-CLC can produce biologically significant expression, as shown by the ability of tyrosine hydroxylase gene therapy to control a rat model of Parkinson’s disease (32). In this study cytokine DNA-CLC had efficacy at inhibiting EAE compared with that following similar injection of naked DNA alone, although it was still difficult to detect protein expression, again pointing toward a low transfection efficiency or low levels of expression. Similar i.c. injection of a fluorescent dye showed expression throughout the frontal lobe, around the meninges, and pial surfaces, and this can reach the spinal cord as shown previously (33). It is likely that the DNA-CLC also disperses in this manner, and this coupled with the relatively low transfection efficiency would mean a few positive cells, possibly at some distance from the site of injection, making immunohistochemical analysis of construct expression difficult to quantify. This contrasts with that observed following similar i.c. injection of adenovirus.

vectors, when widespread expression of protein throughout the CNS can readily be detected (see Footnote 4).

Injection of plasmid IL-4, IFN-β, TNFR, and TGF-β DNA-CLC into the CNS inhibited the development of clinical EAE, which correlates with lesion load (26). This supports the inhibitory action seen in EAE when these proteins are administered systemically (14, 34–38). However, a therapeutic effect may be observed with much lower doses when administered locally within the CNS (6). Although the efficacy of particular cytokines in different EAE models may be controversial, TGF-β and TNF inhibitory molecules have most consistently shown inhibitory activity (36, 38). Although both p55-TNFR-Ig and p75 dTNFR gene therapy exhibited a therapeutic effect, this was particularly evident with the p75 dTNFR. The p75 dTNFR dimer has a 100-fold greater affinity for TNF than the monomeric TNFR, which is effective in collagen-induced arthritis (9, 39), and this may also have increased tissue penetration compared with that of the p55 TNFR-Ig fusion protein due to its smaller size. In vivo immunogenicity of the construct protein will also be decreased, as the human Ig tail, which is highly immunogenic in mice, is not present (40) (see Footnote 4). Although the mechanism of action has yet to be elucidated, it has been shown using inhibitory proteins that TNF neutralization during effector phase EAE may inhibit leukocyte accumulation along the neuroaxis during mouse EAE (8, 41) and may also inhibit damage to myelin (8). Studies in TNF gene-targeted mice suggest that TNF-induced chemotactic factors however may be a more important target for TNF inhibition than vascular extravasation in EAE (42). Gene therapy with TNFR may also indicate additional activities of TNF in leukocyte activation (39).

TGF-β is involved in the natural resolution of clinical EAE in mice (36) and is thought to be a major mediator of oral tolerance, which can effectively inhibit clinical EAE (43). Although it has recently been reported that constitutive expression of active TGF-β by astrocytes in the CNS of a TGF-β transgenic mouse under control of the glial fibrillary acidic protein promoter can enhance the clinical expression of EAE (44), this was not noted in this study following TGF-β DNA-CLC injection. While this may relate to different levels of production of the cytokine, importantly the TGF-β construct used here would be secreted in the inactive procytokine form. This would be cleaved at the site of inflammation to form an active compound. Local delivery of this construct using lymphocyte cell vectors has been shown to be effective at inhibiting collagen-induced arthritis (18) and indicates that local TGF-β immunotherapy may be effective in the control of organ-specific autoimmune disease.

The first cytokine to show significant positive benefit in the progression control of MS is IFN-β. Although s.c. administration of IFN-β protein has shown a significant reduction in the frequency of relapse in some patients (5), initial studies also suggested possible efficacy following intrathecal IFN-β administration (45). This study indicates that mouse IFN-β gene vector delivered locally to the CNS also exhibits immunoinhibitory effects. While both treatment with IFN-β and IFN-β driven by the neuron-specific NSE promoter reduced clinical severity, MMuLV LTR-driven IFN-β was more effective. This difference is probably due to the more selective expression of the NSE promoter vector to CNS neurons, whereas the MMuLV LTR-driven IFN-β could transfect other resident CNS cells, such as glia. While the mechanism of action requires elucidation, the modest efficacy of gene therapy shown here and that observed in Lewis rat and murine EAE following prolonged IFN-β protein administration (35, 46) indicate that this mechanism is in part mediated by immunomodulatory effects that may be independent of any potential antiviral effects. Although efficacy in rat EAE was noted following continued s.c. treatment with rat IFN-β, short term s.c. administration exacerbated disease (35), which was not observed with a single i.c. administration of IFN-β gene vectors.

EAE is transferable with neuroantigen-specific Th1 T cells, and MS is exacerbated by treatment with IFN-γ (47). In mice at least, Th1 responses may be suppressed by immune deviation by Th2 cytokines such as IL-4 and IL-10. At present there are conflicting data using IL-10 to treat EAE, and it has been reported that IL-10 mRNA is up-regulated during the recovery phase of EAE in mice (3). Although IL-10 protein has been shown to suppress EAE in Lewis rats (48), and in mice a genetically modified T cell clone for IL-10 has ameliorated disease (49), other studies in mice using IL-10 protein and IL-10 transduced T cell hybridomas suggest either no effect or disease exacerbation (50, 51).

Surprisingly, this study does not support a major role for CNS-directed IL-10 therapy in the control of murine EAE, using either rIL-10 or IL-10 DNA-CLC and supports the observed lack of efficacy following IL-10 adenovirus administration (see Footnote 4). However, it was readily possible to detect high levels of IL-10 protein in the CNS of ABH mice. It is therefore possible that the exogenously delivered IL-10 failed to exert any influence above that already present in the CNS, which was insufficient to prevent EAE from developing. The significance of such local IL-10 requires further elucidation. The rapid death of animals following local anti-IL-10 Ab treatment suggests that this may be physiologically important. That IL-10 protein given locally into the CNS also has no inhibitory effect suggests that IL-10 may act differently depending on the dose and route administered. This study suggests, however, that IL-4 is the major Th2 cytokine that can inhibit the expression of EAE and is supported by another recent study that demonstrated that local gene delivery into the CNS of IL-4, but not IL-10, infiltrating T cell hybridomas can inhibit the development of clinical EAE (51).

Direct injection of genetic material has some advantages over other methods of gene therapy, in that it avoids the use of infectious virus, such as adenovirus vectors, that have been proved to be immunogenic in mice, thus preventing repeat administration and reduced expression (11). Plasmid DNA exists epichromosomally and, compared with other methods of delivery uptake, is less efficient, and expression of DNA is transient, although delivery of naked DNA has been shown to persist for up to 19 mo in muscle cells (13, 52–54). With the current understanding of the cytokine network, however, the appropriate levels of expression for optimal efficacy are currently unknown and will require controllable expression to identify. The limited control of transfection efficiency by the plasmid DNA constructs seen here suggests that other gene-targeting methods may be more suitable to resolve this issue.

While this study indicates that plasmid DNA injections in this current protocol may be too inefficient for further clinical development, this study highlights some potential gene targets for therapy that probably influence different pathways within the progression of disease. It has also been revealed that cytokine DNA can produce therapeutic cytokines in vivo in the CNS, which can influence the clinical severity and course of EAE and which may be developed further with more suitable long term cellular gene vectors.

Acknowledgments
We thank the various cited people for providing access to and donation of reagents.

References