Cyclosporin A treatment modulates cytokine mRNA expression by inflammatory cells extracted from the spinal cord of rats with experimental autoimmune encephalomyelitis induced by inoculation with myelin basic protein

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Abstract

In Lewis rats, treatment with high doses of cyclosporin A (CsA) suppresses clinical signs of experimental autoimmune encephalomyelitis (EAE), although disease occurs when treatment is ceased. Treatment with low doses of CsA causes EAE to take a chronic relapsing course. We have previously shown that CsA treatment causes a decline in the number of T cells and increased inflammatory cell apoptosis in the spinal cord. The present study was undertaken to assess whether CsA therapy also modulates cytokine mRNA expression by inflammatory cells in the spinal cord of rats with EAE, looking for changes that might contribute to the observed effects of CsA on the course of EAE. EAE was induced in Lewis rats by inoculation with myelin basic protein and adjuvants. At the peak of neurological signs, on day 14 after inoculation, rats were given a single intraperitoneal injection of saline, or CsA at a dose of 8, 16, 32 or 64 mg/kg. The next day, rats were sacrificed, the spinal cords removed, inflammatory cells were extracted from the cords, and mRNA isolated from these cells. Expression of cytokine mRNA was assessed by semi-quantitative reverse transcription polymerase chain reaction (PCR) and by quantitative real-time PCR. With both techniques, we found that CsA suppressed the expression of interferon-γ mRNA and interleukin-2 (IL-2) mRNA. With real-time PCR, we found that CsA caused significantly increased expression of transforming growth factor-β mRNA. With the different techniques, we observed no consistent pattern of alteration of expression of interleukin-10 or interleukin-4 mRNA. It is possible that these changes in cytokine mRNA expression contribute to the modulation of the clinical course of EAE that is produced by CsA treatment.

Keywords: Cyclosporin A; Cytokines; Encephalomyelitis; Multiple sclerosis; Polymerase chain reaction
1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell mediated disease of the central nervous system (CNS) that can be induced by inoculation with CNS antigens, such as myelin basic protein (MBP), and adjuvants. It is widely studied as an animal model of multiple sclerosis. EAE is characterized by the infiltration of the nervous system with lymphocytes and macrophages, with subsequent demyelination of nerve fibres. In Lewis rats, EAE induced by inoculation with MBP (MBP-EAE) is an acute monophasic illness from which rats spontaneously recover. In various models of EAE, it has been shown that cytokines are involved in the pathogenesis and regulation of disease. Interleukin 2 (IL-2) is present in the CNS in EAE 1 2 3 4. Although interferon-γ (IFN-γ) is produced by encephalitogenic T cells, and thus may be involved in the pathogenesis of EAE, it may also play an immunoregulatory role in EAE 5 6 7 8. IL-4 has been found in the CNS in EAE in rats and mice 9 10. Expression of IL-10 mRNA in the CNS correlates with clinical recovery from EAE in mice 10 and cells producing IL-10 mRNA are present in the CNS of rats during recovery from EAE 11. Transforming growth factor (TGF)-β mRNA is expressed strongly in the CNS and may play a role in the recovery from MBP-EAE in Lewis rats 9 12. Using semi-quantitative reverse transcription PCR (RT-PCR), we have previously shown that mRNA of the pro-inflammatory cytokines (IL-2 and IFN-γ) and anti-inflammatory cytokines (IL-4 and IL-10) are expressed in the spinal cord during the course of acute MBP-EAE in the Lewis rat 13.

Cyclosporin A (CsA), described by Borel et al. 14 in 1976, is a cyclic peptide with immunomodulatory properties. CsA acts within the cell by binding to cyclophilin, which results in the inactivation of calcineurin, and subsequent suppression of transcription of genes, including those for cytokines and cytokine receptors 15 16. Treatment with CsA in high doses has been shown to suppress clinical signs of EAE in different species 17, including Lewis rats 18 19. Cyclosporin A also has a modest beneficial effect on the progression of disability in progressive multiple sclerosis 20. Treatment with low doses of CsA (4–8 mg/kg) converts acute EAE, induced in Lewis rats by inoculation with spinal cord or with myelin basic protein, into a chronic relapsing form of disease 18 19 21. There are other examples of the exacerbation of autoimmunity after CsA treatment—namely, the development of graft versus host disease after bone marrow transplantation 22 and development of autoimmunity after neonatal treatment with CsA 23.

The suppression of clinical signs of EAE by treatment with high doses of CsA is likely to be due to the immunosuppressive effects of CsA, which include suppression of early events in T cell activation 24 and suppression of antigen presentation by macrophages 25. These effects could occur in the CNS or in the periphery and lead to downregulation of disease in the CNS. We have shown that a single treatment with high dose CsA causes a significant reduction in the number of T cells in the spinal cord of rats with EAE, and an increase in the number of apoptotic lymphocytes in the spinal cord 19. CsA also inhibits the disruption of the blood–brain barrier that occurs in EAE 26. We have previously suggested that the production of relapsing disease after treatment with lower doses of CsA is likely to be due to the modulation of mechanisms that regulate the course of disease 19 27 28. Such mechanisms include apoptosis of T cells in the CNS 29 30 31 32 33 and production of regulatory cytokines in the CNS 13. We have studied the chronic relapsing forms of EAE induced by CsA therapy and shown that these are characterized by recurrent episodes of inflammation and demyelination of the nervous system 21 28.

Our previous studies of the effects of CsA on EAE have indicated that CsA therapy can modulate the numbers of cells in the inflammatory infiltrate. The present study was carried out to determine whether CsA treatment also alters cytokine mRNA expression by inflammatory cells in the CNS of rats with MBP-EAE. For simplicity, we chose to study the effects of a single dose of CsA. To assess the effects of CsA, we used semi-quantitative
reverse transcription (RT)-PCR, which we have previously used to study cytokine expression in this form of EAE. We also used quantitative real-time PCR, which is a new technique that has not previously been used to measure cytokine mRNA expression in EAE.

2. Materials and methods

2.1. Induction of EAE and CsA treatment

MBP was prepared from guinea pig brains by the method of Deibler et al. MBP in 0.9% saline was emulsified in an equal volume of incomplete Freund's adjuvant containing 4 mg/ml Mycobacterium butyricum. On day 0, anaesthetized rats were inoculated in one hind footpad with 0.1 ml emulsion containing 50 μg MBP per rat. Rats were weighed on day 0 and daily from day 8. We assessed the weakness of the tail, hindlimbs and forelimbs. The degree of weakness of each region was separately graded on a scale of 0 (no weakness) to 4 (total paralysis) as previously described. The scores from each region were added together to give a total clinical score (maximum total clinical SCORE=12). CsA or saline was administered by intraperitoneal injection on day 14, 16 h before study on day 15. Rats were treated with saline or CsA in groups of two or three. The spinal cords of all rats in the group were pooled for PCR studies.

2.2. Extraction of cells from the spinal cord and extraction of mRNA

As described previously, cells were isolated from the spinal cords of anaesthetized rats perfused with ice-cold saline. The entire spinal cords were removed by insufflation, weighed, and a single-cell suspension in ice-cold RPMI 1640 containing 1% foetal calf serum (FCS) was prepared by passage through a 200-size mesh stainless steel sieve. The cell suspension was mixed with isotonic Percoll (Percoll: Hank's balanced salt solution 9:1) in a 3:2 ratio (density 1.052) in 50 ml centrifuge tubes and centrifuged for 25 min at 640×g at 4°C. The myelin layer and supernatant were discarded, while the last 9 ml supernatant and the cell pellet were retained. The cell pellet and the last 9 ml supernatant were resuspended, transferred to a conical 10 ml centrifuge tube, underlaid with 1 ml Ficoll and centrifuged for 20 min at 600×g at 4°C. Cells were harvested from the interface above the Ficoll, resuspended in medium, washed once and counted. In the present study, cells were not passed over nylon wool. Messenger RNA was extracted from spinal cord inflammatory cells using Pharmacia QuickPrep Micro mRNA Purification Kit (a guanidinium thiocyanate-based extraction followed by oligo(dT) purification of mRNA). The mRNA was stored at −20°C before RT-PCR.

2.3. Semi-quantitative RT-PCR

Samples of mRNA (0.05 μg) were reverse transcribed using random hexamers as primers and MuLV reverse transcriptase under the following conditions: 5 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1 mM dNTPs, 5 μM Random Hexamers (GibCoBRL), 1 U/μl RNase Inhibitor (Perkin Elmer), 2.5 U MuLV reverse transcriptase (Perkin Elmer) and 0.05 μg poly(A⁺)mRNA in a total volume of 20 μl. Samples were incubated at room temperature for 10 min prior to reverse transcription. The reagents were overlaid with mineral oil and the reactions carried out in thin-walled tubes in a Perkin Elmer DNA Thermal Cycler (15 min at 42°C, 5 min at 99°C and 5 min at 5°C). PCR was carried out under the following conditions: 3.2 mM MgCl₂, 10 mM KCl, 62 mM Tris–HCl pH 8.3, 16 mM (NH₄)₂SO₄, 0.008% (w/v) Tween, 0.2 mM dNTPs, 0.15 μM of each primer and 0.02 U/μl Red Hot Taq DNA Polymerase (Integrated Sciences) in a total reaction volume of 100 μl (that contained the entire 20 μl RT products). The reagents were mineral oil-overlaid and the PCR reaction was carried out in thin-walled tubes in a Perkin Elmer DNA Thermal Cycler (2 min at 95°C; then
37 cycles of 1 min at 95°C and 1 min at 60°C 20 cycles for β-actin; then 7 min at 72°C). Primers with the following sequences were synthesized by the Queensland Institute of Medical Research:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin sense</td>
<td>5’ AIG CCA TCC TGC GIC TCG AGC TGG C 3’</td>
</tr>
<tr>
<td>β-actin antisense</td>
<td>5’ AGC ATT TGC GGT GCA CCG TGG AGG G 5’</td>
</tr>
<tr>
<td>IL-2 sense</td>
<td>5’ GTC AAC AGC GCA CCC ACT TCA ACC 3’</td>
</tr>
<tr>
<td>IL-2 antisense</td>
<td>5’ GCT TGT TGA GAT GAT GCT TTT ACA 3’</td>
</tr>
<tr>
<td>IL-4 sense</td>
<td>5’ TGA TGG GTC TCA GCC CCC ACC TGG C 3’</td>
</tr>
<tr>
<td>IL-4 antisense</td>
<td>5’ CTT TCA GTG TTP GAG GCC TGG ACT C 3’</td>
</tr>
<tr>
<td>IL-10 sense</td>
<td>5’ GAC TTT AAG GGT TAC TGG GGT TGC 3’</td>
</tr>
<tr>
<td>IL-10 antisense</td>
<td>5’ CAC TGC GCT CTT ATT TIC ACA 3’</td>
</tr>
<tr>
<td>IFN-γ sense</td>
<td>5’ GAA AGC CTA GAG AGT CTG AAT AAC 3’</td>
</tr>
<tr>
<td>IFN-γ antisense</td>
<td>5’ GCA GCG ACT CCT TTT CCG CTT C 3’</td>
</tr>
</tbody>
</table>

The primer sequences for IL-2, IL-10 and IFN-γ were those used by Weinberg et al. 36 in a study of MBP-EAE in the rat. The primers for IL-4 and β-actin were those used by McKnight et al. 37. The sequences of rat β-actin 38, IL-2 39 (Genbank M22899), IL-4 37 (Genbank X16058), IL-10 40 (Genbank X60675) and IFN-γ 41 (Genbank X0237, X0236, X0235) are known. The sequences used in the present study are specific for the known rat sequences, except for those indicated with asterisks, which are specific for mouse sequences and differ from the rat sequences by the number of base pairs shown in brackets. The predicted product sizes are: β-actin, 607 bp; IL-2, 449 bp; IL-4, 378 bp; IL-10, 201 bp; IFN-γ, 383 bp. The sequences chosen all crossed exon boundaries. We have previously shown that the products of these primers are labelled by probes specific for the cytokine sequences 15.

2.4. Real-time PCR

Real-time PCR using the ABI Prism 7700 Sequence Detection system (Perkin Elmer) was used to quantify cytokine expression. Primers and fluorogenic probes with the following sequences were custom designed using the ABI Prism Primer Express software (PE Biosystems), using the sequences of rat IL-2 39, rat IL-10 40, IFN-γ 41 42 and rat TGF-β 43.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 sense</td>
<td>CAGCATGCAAGCTGGAAC</td>
</tr>
<tr>
<td>IL-2 antisense</td>
<td>GTTGGGTGGCGGGAACA</td>
</tr>
<tr>
<td>IL-2 TM probe</td>
<td>TGGTGGCACTGACGCTTGTCCTC</td>
</tr>
<tr>
<td>IL-10 sense</td>
<td>TGGCAAAGCTGACGCA</td>
</tr>
<tr>
<td>IL-10 antisense</td>
<td>GTCAAGCTCGAGAGACTG</td>
</tr>
<tr>
<td>IL-10 TM probe</td>
<td>ATCCCTGCACTGACGTCA</td>
</tr>
<tr>
<td>IL-4 sense</td>
<td>AGTGGTTGACACAGCCTCTC</td>
</tr>
<tr>
<td>IL-4 antisense</td>
<td>TGCGAAAGCACTCTGGAA</td>
</tr>
<tr>
<td>IL-4 TM probe</td>
<td>CACGATGCAACAGCAGCC</td>
</tr>
<tr>
<td>IFN-γ sense</td>
<td>CTTGGAAGAGGAAAGAGCCG</td>
</tr>
<tr>
<td>IFN-γ antisense</td>
<td>TCCTGGCTCTAGGTGTTT</td>
</tr>
<tr>
<td>IFN-γ TM probe</td>
<td>TGGTTGGAAGAGGCAAACAG</td>
</tr>
<tr>
<td>TGF-β sense</td>
<td>AGAATACACCCCGGCTG</td>
</tr>
<tr>
<td>TGF-β antisense</td>
<td>TGGTTGATGAGTGTTTGGT</td>
</tr>
<tr>
<td>TGF-β TM probe</td>
<td>TGGTTGGAAGAGGCAAACAG</td>
</tr>
</tbody>
</table>

The primers and probes for GAPDH were purchased as predesigned endogenous control kit (PE Biosystems). The sequences are therefore not available.
Reverse transcription and PCR were performed in a single-step procedure using a Gold RT-PCR (one step) Kit (PE Biosystems). To allow fluorochrome detection, specifically designed optical tubes contained in a 96-well microtitre plate (PE Applied Biosystems) were used. Samples were tested in duplicate, with and without reverse transcriptase, and the results for the sample without reverse transcriptase were subtracted from the results of the sample with reverse transcriptase, to allow detection of DNA contamination. The method followed the manufacturer's instructions under the following conditions: 5.5 mM MgCl₂, 1×TaqMan Buffer, 300 μM dNTPs, 0.4 U/l Multiscribe Reverse Transcriptase, 0.25 U/l Amplitaq Gold DNA polymerase, 200 nM forward and reverse primers, 100 nM TaqMan probe and 0.05 μg of poly(A⁺)mRNA in a total volume of 50 μl. Tubes were sealed with optical caps, and a one-step RT-PCR was performed as follows: 30 min at 40°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 95°C.

Cytokine expression data obtained from the real-time PCR studies were analyzed quantitatively using a comparative CT method. For each cytokine, the threshold, or cycle number, at which product was detected, was compared to an endogenous (GAPDH) and exogenous control (saline-treated group). Prior to commencing studies, the comparative CT method was validated by ensuring that the efficiencies of both the target cytokine and GAPDH primers were approximately equal. Results in the first instance were calculated as ΔCT, i.e. the difference between the cytokine and GAPDH mean thresholds. A ΔΔCT was then determined by deducting the mean ΔCT saline result from the mean ΔCT result of each of the CsA treatment groups. Normalizing the thresholds of treatment groups to those of saline ensured that the standard deviations of the ΔCT and the ΔΔCT were the same. Results were finally expressed as 2−ΔΔCT which is an index of the amount of cytokine expressed in the spinal cord. These results were also expressed as cytokine expression per gram of spinal cord by multiplying the results by the number of cells/g of spinal cord.

2.5. Statistical methods

Results from different groups were compared using analysis of variance (ANOVA) with post-hoc comparison of means of single groups using Sigmastat (Jandel Scientific).

3. Results

3.1. Clinical details

Table 1 shows the clinical details of rats used in the study. Rats in the different groups did not differ significantly in the mean day of onset of disease or the clinical score on the day of injection (day 14). Table 1 also indicates that, although treatment with high dose CsA on alternate days can suppress clinical signs of MBP-EAE, treatment with a single injection of CsA did not alter the clinical course of disease.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of groups</th>
<th>Total no. of rats</th>
<th>Mean day of onset of disease</th>
<th>Mean (SD) clinical score on day of treatment (day 1)</th>
<th>Mean (SD) clinical score on day of study (day 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>9</td>
<td>26</td>
<td>9.6 (0.7)</td>
<td>3.7 (1.9)</td>
<td>3.2 (1.3)</td>
</tr>
<tr>
<td>CsA 8 mg/kg</td>
<td>3</td>
<td>18</td>
<td>9.2 (0.4)</td>
<td>7.0 (0.7)</td>
<td>6.7 (0.7)</td>
</tr>
<tr>
<td>CsA 16 mg/kg</td>
<td>5</td>
<td>24</td>
<td>9.8 (0.8)</td>
<td>6.7 (1.0)</td>
<td>6.8 (1.8)</td>
</tr>
<tr>
<td>CsA 32 mg/kg</td>
<td>5</td>
<td>18</td>
<td>9.6 (0.6)</td>
<td>6.2 (0.9)</td>
<td>6.0 (0.9)</td>
</tr>
<tr>
<td>CsA 64 mg/kg</td>
<td>4</td>
<td>13</td>
<td>9.6 (0.6)</td>
<td>6.4 (0.8)</td>
<td>6.4 (0.8)</td>
</tr>
</tbody>
</table>

Table 1. Clinical details of rats used in the study
3.2. Effect of CsA on number of cells per gram of spinal cord

The effect of CsA on the total number of cells obtained per gram of spinal cord, for all rats in the study, is shown in Fig. 1. It can be seen that a single dose of CsA caused a dose-dependent reduction in the number of inflammatory cells/g of spinal cord, with significant declines at doses of 16 and 64 mg/kg. Using this technique, we have previously shown that these cells extracted from the spinal cord of rats with MBP-EAE are predominantly T lymphocytes and macrophages/microglia and that CsA causes a decline in the number of T cells in the spinal cord.

Fig. 1. Mean (SD) number of inflammatory cells/g of spinal cord, for saline-treated rats and rats treated with CsA. The results from rats in the semi-quantitative RT-PCR study and in the real-time PCR study are combined. CsA caused a decline in the number of inflammatory cells/g of spinal cord. (Kruskal–Wallis ANOVA on ranks, \( p = 0.001 \)). Post-test calculations (Dunn's method) showed that the means represented by * differed significantly \( (p < 0.05) \) from the mean for saline-treated rats.

3.3. Semi-quantitative RT-PCR

The results of cytokine expression, normalized to \( \beta \)-actin, are shown in Fig. 2A. Because CsA therapy causes a reduction in the total number of cells in the spinal cord, the expression of cytokine per gram of spinal cord is shown in Fig. 2B. As we have previously shown with untreated rats with MBP-EAE, in the saline-treated rats there was an expression of all cytokines. Treatment with CsA significantly suppressed the expression of IL-2 and IFN-\( \gamma \) mRNA, both normalized to \( \beta \)-actin and when expressed as cytokine/g. There was no significant alteration of expression of IL-4 mRNA. With this technique, there was an apparent reduction in the expression of IL-10 mRNA, but this was significant only when expressed as cytokine/g.
Fig. 2. (A) Results of semi-quantitative RT-PCR showing mean (SEM) cytokine expression normalized to β-actin. CsA treatment was administered to rats with MBP-EAE as a single intraperitoneal injection on day 14, 16 h before sacrifice on day 15. Inflammatory cells were extracted from the spinal cord, and mRNA was extracted from the cells. Semi-quantitative RT-PCR was performed and the results are expressed as a ratio of cytokine expression to β-actin expression. Groups were compared using ANOVA. Post-test calculations (Dunn's method) showed that the means represented by * differed significantly (p<0.05) from the mean for saline-treated rats. CsA therapy caused a significant suppression of expression of IL-2 and IFN-γ. (B) Results of semi-quantitative RT-PCR showing mean (SEM) cytokine expression per gram of spinal cord. Groups were compared using ANOVA. Post-test calculations (Dunn's method) showed that the means represented by * differed significantly (p<0.05) from the mean for saline-treated rats. CsA therapy caused a significant suppression of expression of IL-2 and IFN-γ.

3.4. Quantitative real-time PCR

The results of real-time PCR are shown in Table 2. The threshold cycle (C_T) for GAPDH was constant in all groups and was not influenced by CsA therapy. The results of cytokine expression after CsA are given as mean ΔC_T. In the saline-treated rats, there was an expression of all cytokines. In the CsA-treated rats, there was a significant increase in the ΔC_T.
for IFN-\(\gamma\) and a significant decrease in the \(\Delta C_T\) for TGF-\(\beta\), compared to the saline-treated rats. CsA treatment caused an increase in the \(\Delta C_T\) for IL-2, in a dose-dependent manner, but this was not statistically significant. There was no significant change in the \(\Delta C_T\) for IL-10 or IL-4.

Table 2. Results of real-time PCR

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD) (\Delta C_T)</th>
<th>n</th>
<th>Mean (SD) (\Delta C_T)</th>
<th>n</th>
<th>Mean (SD) (\Delta C_T)</th>
<th>n</th>
<th>Mean (SD) (\Delta C_T)</th>
<th>n</th>
<th>ANOVA (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>3.8 (1.3)</td>
<td>14</td>
<td>4.3 (1.3)</td>
<td>14</td>
<td>5.4 (1.7)</td>
<td>9</td>
<td>4.8 (1.6)</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>-3.9 (2.1)</td>
<td>13</td>
<td>-1.7 (2.2)</td>
<td>13</td>
<td>-0.1 (3.1)</td>
<td>16</td>
<td>0.3 (4.3)</td>
<td>19</td>
<td>0.005</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.7 (1.0)</td>
<td>13</td>
<td>2.7 (1.2)</td>
<td>12</td>
<td>2.7 (1.9)</td>
<td>13</td>
<td>2.8 (3.9)</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>IL-10</td>
<td>-2.2 (1.8)</td>
<td>9</td>
<td>-2.2 (1.2)</td>
<td>9</td>
<td>-0.7 (2.9)</td>
<td>10</td>
<td>-1.0 (2.8)</td>
<td>18</td>
<td>NS</td>
</tr>
<tr>
<td>TGF-(\beta)</td>
<td>-1.2 (1.5)</td>
<td>15</td>
<td>-1.6 (1.9)</td>
<td>15</td>
<td>-0.4 (2.5)</td>
<td>15</td>
<td>-0.8 (2.6)</td>
<td>17</td>
<td>NS</td>
</tr>
</tbody>
</table>

The amount of mRNA in the starting sample can be calculated as \(2^{-\Delta\Delta C_T}\). Fig. 3 illustrates the results of these calculations showing the amount of each cytokine expressed as \(2^{-\Delta\Delta C_T}\). This reflects the expression of cytokine mRNA in equivalent amounts of total inflammatory cell mRNA, but is not adjusted for the numbers of cells present in the spinal cord. There was a decline in the amount of IL-2 and IFN-\(\gamma\) mRNA, but this was not statistically significant. There was a highly significant increase in the amount of TGF-\(\beta\) mRNA. There was a slight decline in the expression of IL-4 mRNA but this was not statistically significant.
Fig. 3. Results of real-time PCR showing mean cytokine mRNA expression, relative to saline. CsA treatment was administered to rats with MBP-EAE as a single intraperitoneal injection on day 14, 16 h before sacrifice on day 15. Inflammatory cells were extracted from the spinal cord, and mRNA was extracted from the cells. Real-time PCR was performed and the results are expressed as $2^{-\Delta\Delta C_{T}}$, which gives an indication of cytokine expression normalized to GAPDH and then normalized to the expression of cytokine in the saline-treated rats. Groups were compared using ANOVA. Post-test calculations (Dunn's method) showed that the means represented by * differed significantly ($p<0.05$) from the mean for saline-treated rats. CsA therapy caused dose-dependent suppression of expression of IL-2 and IFN-γ mRNA, and an increase in expression of TGF-β mRNA, but these results were statistically significant only for TGF-β.

Because CsA therapy causes a decline in the number of inflammatory cells obtained per gram of spinal cord, we also calculated the expression of cytokine mRNA per gram of spinal cord (Fig. 4). This result best expresses the amount of mRNA present in the spinal cord after treatment. There was a significant decline in the amount of IL-2 and IFN-γ mRNA per gram of spinal cord and a significant increase in the expression of TGF-β mRNA, although not at all doses. There was a decline in the expression of IL-4 mRNA, but this was not significant. There was no change in the IL-10 mRNA expression.

![Effect of CsA on cytokine mRNA expression per g of spinal cord (real-time PCR)](image)

Fig. 4. Results of real-time PCR showing mean cytokine mRNA expression per gram of spinal cord, relative to saline. Real-time PCR was performed as in Fig. 3 and the results have been adjusted for the number of cells/g of spinal cord. Groups were compared using ANOVA. Post-test calculations (Dunn's method) showed that the means represented by * differed significantly ($p<0.05$) from the mean for saline-treated rats. CsA therapy caused a significant dose-dependent suppression of expression of IL-2 and IFN-γ mRNA, and an increase in expression of TGF-β mRNA.
4. Discussion

In this study, we used PCR to study the effects of a single injection of CsA on cytokine mRNA expression by inflammatory cells from the CNS of rats with MBP-EAE. The cells extracted from the spinal cord comprise T lymphocytes and macrophages/microglia. We have previously shown that therapy with high dose CsA suppresses MBP-EAE, although rats have an episode of disease after therapy is ceased, and that low dose CsA causes chronic relapsing EAE. We have also demonstrated that a single dose of CsA leads to a reduction in the number and percentage of T cells, as well as increased T cell apoptosis, and an increase in the percentage of macrophages/microglia in the inflammatory infiltrate in the spinal cord in rats with EAE. The present study confirmed that a single dose of CsA causes a reduction in the number of inflammatory cells obtained from the spinal cord. Two PCR techniques were used, which gave similar results. Initially, a semi-quantitative RT-PCR was employed, extending previous studies by this laboratory which have demonstrated expression of IL-2, IL-4, IL-10 and IFN-γ mRNA in CNS inflammatory cells throughout the course of MBP-EAE in Lewis rats. We also used quantitative real-time PCR, a new technique that has not previously been used in studies of EAE. All cytokine mRNAs were detected with both methods, with the exception of TGF-β, which was not studied with the initial semi-quantitative experiments. Cytokine mRNA expression in the saline-treated control group was found to be similar to that previously found in untreated rats with MBP-EAE. Some variability was noted in the results obtained with real-time PCR, due in part to fluctuations in the individual C_T values for GAPDH. This was not attributed to an effect of CsA treatment as no dose-dependent changes in the mean C_T for GAPDH were observed. It has been suggested that GAPDH may vary in expression and ribosomal RNA may be a better internal standard than GAPDH for future studies.

With both techniques, CsA therapy suppressed expression of IFN-γ mRNA in inflammatory cells from the spinal cords of rats with MBP-EAE. There was also a dose-dependent reduction in expression of IL-2 mRNA. Real-time PCR showed a significant increase in the expression of TGF-β mRNA although this was not dose-dependent. There was some variability in the results of expression of IL-10 and IL-4 mRNA with the two techniques, and no consistent finding was demonstrated. As the experiments were performed on whole animals, these observations represent the final effect of CsA treatment on the expression of cytokine mRNA in the CNS in MBP-EAE. However, the results do not necessarily represent direct effects of CsA on cytokine expression in the CNS, as the alteration in the expression of cytokine mRNA may have occurred before the cells entered the CNS. We have previously shown that CsA treatment of MBP-EAE causes a decline in the proportion of T cells in the spinal cord inflammatory infiltrate. Such a decline could also contribute to a decrease in the expression of T cell cytokine mRNA in the inflammatory infiltrate. However, the present study was performed to measure the overall effect of CsA on total cytokine mRNA expression in the spinal cord rather than to measure the expression by specific cells.

Our observations are in keeping with the known effects of CsA. In vitro, CsA inhibits early events in T cell activation and suppresses IL-2 and IFN-γ expression in response to mitogen stimulation of T cells. TGF-β expression by epithelial cells in vitro is enhanced by CsA. In our study, with the different PCR techniques, we did not find consistent changes in IL-10 mRNA or IL-4 mRNA expression. It has been found that CsA suppresses IL-4 expression but has no effect on the IL-10 expression in mitogen-stimulated cells in vitro. However, in a study of cytokine expression in transplanted hearts in Wistar rats, CsA treatment for 7 days did not inhibit the expression of IL-4 but did inhibit IL-10 expression.

The modulation of cytokine expression that we observed in the present study may contribute to the ability of CsA therapy to modulate the course of EAE. The significant increase in the
expression of TGF-β mRNA by inflammatory cells in the CNS is likely to have a downregulatory role as numerous studies have revealed that TGF-β production correlates with the resolution of inflammatory responses, particularly in organ-specific autoimmune diseases. TGF-β has a wide range of immunosuppressive effects and is thought to downregulate EAE, so the increased expression of TGF-β would be expected to be associated with the suppression of disease. IL-2 and IFN-γ are secreted by encephalitogenic cells, and reduction in the expression of these cytokines would be expected to accompany a reduction in the inflammatory infiltrate. However, although IFN-γ involvement in the pathogenesis of EAE, it may also play a role in immunoregulation. Reduction in IFN-γ expression after treatment with CsA could therefore potentially enhance disease. Tanuma et al. have suggested that the suppression of IFN-γ by CsA may be involved in the induction of chronic relapsing EAE by CsA. They compared the first episode of CsA-induced chronic relapsing EAE with the second episode and found that IFN-γ expression by inflammatory cells in the CNS is impaired in the first compared to the second episode. In summary, we have shown that CsA therapy modulates the expression of cytokine mRNA in the CNS in MBP-EAE and suggest that these changes may contribute to the observed clinical effects of CsA on EAE.

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References


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