

*Journal of Neuroimmunology*, 88 (1-2), 1998, pp. 30-38.  
[http://dx.doi.org/10.1016/S0165-5728\(98\)00068-X](http://dx.doi.org/10.1016/S0165-5728(98)00068-X)

## Cytokine expression by inflammatory cells obtained from the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis induced by inoculation with myelin basic protein and adjuvants

P. A. McCombe, I. Nickson and M. P. Pender

Neuroimmunology Research Unit, Department of Medicine, The University of Queensland, Queensland, Australia

### Abstract

Inflammatory cells were obtained from the spinal cords of rats with acute experimental autoimmune encephalomyelitis EAE induced by inoculation with myelin basic protein MBP and adjuvants. Reverse transcriptase-polymerase chain reaction RT-PCR was used to investigate the expression of mRNA for interleukin-2 IL-2, IL-4, IL-10 and interferon- $\gamma$  (IFN- $\gamma$ ) by cells from groups of rats studied 10–21 days after inoculation. On all days of study, the inflammatory cells, which were predominantly lymphocytes, expressed mRNA for IL-2, IL-4, IL-10 and IFN- $\gamma$ . In the mRNA from normal rat spinal cord tissue, there was little expression of cytokine mRNA. Cells from a short-term MBP-reactive T cell line expressed all the cytokines. Densitometry was used to measure the products of PCR, to assess the expression of each cytokine relative to that of  $\beta$ -actin. IL-2 mRNA was expressed throughout the course of disease and reached a peak on day 18, during late clinical recovery. IFN- $\gamma$  was expressed throughout the course of the disease and was also high during late recovery. IL-4 mRNA was present in the spinal cord throughout the course of the disease, with a slight rise during late recovery. Relative expression of IL-10 rose to a peak on days 17–19, during late recovery from clinical disease. This study indicates that IL-2, IL-4, IL-10 and IFN- $\gamma$  are expressed by inflammatory cells in the spinal cord in EAE, with the relative expression of all cytokines being high during late clinical recovery.

**Keywords:** Cytokines; Encephalomyelitis; Immunoregulation; T lymphocyte

## 1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the nervous system. It can be induced by inoculation with myelin proteins, such as myelin basic protein (MBP), and adjuvants and can be passively transferred to naive animals by CD4<sup>+</sup> T cells (Pettinelli and McFarlin, 1981). EAE is characterized by infiltration of the nervous system with activated T lymphocytes and macrophages (Raine, 1984; McCombe et al., 1992). Cloned encephalitogenic T cells secrete interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) (Ando et al., 1989; Baron et al., 1993). Thus, encephalitogenic cells are Th1-like cells, as defined by the original concept of Mosmann and Coffman (1989) that CD4<sup>+</sup> T cells produce either IL-2 and IFN- $\gamma$  (Th1 cells) or IL-4, IL-5 and IL-10 (Th2 cells). Lewis rats spontaneously recover from acute EAE, with resolution of inflammation and remyelination of nerve fibres (Pender, 1989). During recovery from acute EAE, T cells and macrophages undergo apoptosis in the central nervous system (CNS) (Pender et al., 1992; Schmied et al., 1993; Nguyen et al., 1994; Tabi et al., 1994 and Tabi et al., 1995; McCombe et al., 1996a). While apoptosis of T cells in the CNS appears to play a major role in the resolution of inflammation in EAE, down-regulatory T cells and anti-inflammatory

cytokines may also be involved. Down-regulatory CD4<sup>+</sup> T suppressor cells have been described in rats that have recovered from EAE (Karpus et al., 1992; Varriale et al., 1994) and rats that have been tolerized with low doses of encephalitogenic antigen (Chen et al., 1994). Such regulatory T cells secrete anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Karpus et al., 1992; Chen et al., 1994). There is evidence that, during spontaneous recovery from EAE, anti-inflammatory cytokines such as IL-10 (Kennedy et al., 1992; Issazadeh et al., 1995a) are present in the CNS. IL-4 has also been found in the spinal cords of mice with EAE (Kennedy et al., 1992). The role of IFN- $\gamma$  in EAE requires clarification because, although IFN- $\gamma$  has been implicated in the pathogenesis of EAE, there is also evidence that it may play a protective role in EAE (Billiau et al., 1988; Voorthuis et al., 1990). We performed the present study to determine whether clinical recovery from EAE is associated with a selective increase in the expression of anti-inflammatory (Th2) cytokines, compared to that of pro-inflammatory (Th1) cytokines. We used semi-quantitative reverse transcriptase-PCR (RT-PCR) to assess the expression of IL-2, IL-4, IL-10 and IFN- $\gamma$  by inflammatory cells extracted from the spinal cord of rats with acute MBP-EAE, from days 10–21 after inoculation.

## 2. Materials and methods

### 2.1. Induction of EAE

EAE was induced in male Lewis rats (JC strain), aged 8–10 weeks, obtained from the Central Animal Breeding House of the University of Queensland. MBP was prepared from guinea pig brains by the method of Deibler et al. (1972). 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 1 hind footpad with 0.1 ml emulsion. The total dose of MBP was 50  $\mu$ g per rat. Rats were weighed on day 0 and daily from day 8. We assessed weakness of the tail, hindlimb and forelimb. The degree of weakness of each region was separately graded on a scale of 0 (no weakness) to 4 (total paralysis), as previously described (Pender, 1986). The scores from each region were added together to give a total clinical score (maximum total clinical score=12). For each experiment, we used the pooled spinal cords of groups of 4–6 rats that had been inoculated at the same time, and which were then sacrificed on the same day. Several groups of rats were used for each time-point, to provide replicates.

### 2.2. Extraction of cells from spinal cord

The cells were isolated from the spinal cords of anaesthetized rats perfused with ice-cold saline, as previously described (McCombe et al., 1996b). The spinal cord was removed by insufflation, weighed, and a single cell suspension in ice-cold RPMI 1640 containing 1% fetal 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 $\times$ g at 4°C. The myelin layer and supernatant were discarded, while the last 9 ml supernatant and the cell pellet were retained, resuspended, transferred to a conical 10 ml centrifuge tube, underlaid with 1 ml Ficoll and centrifuged for 20 min at 600 $\times$ g at 4°C. Cells were harvested from the interface above the Ficoll, resuspended in medium, washed once and counted. For flow cytometry and RNA extraction, the cells were then passed through a nylon wool column, using our previously described methods (Tabi et al., 1994). The number of cells remaining after passage through the nylon wool was counted, and the yield of cells after passage through the nylon wool was calculated.

### 2.3. Labelling of cells and flow cytometric analysis

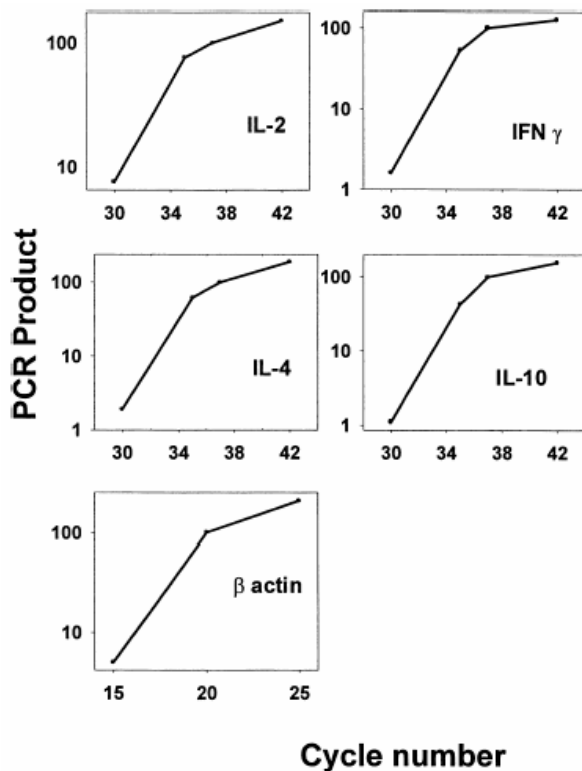
The cells were labelled for flow cytometric analysis using our previously described methods (McCombe et al., 1996b). Aliquots of  $10^5$ – $10^6$  cells were washed with a 1:1 solution of serum in phosphate-buffered saline (PBS) containing 1% fetal calf serum and 0.1% sodium azide, then incubated with primary antibodies in PBS/azide for 30 min at 4°C. Samples were washed and incubated with a secondary antibody for 30 min at 4°C in the dark. Cells were washed twice with PBS, then resuspended in 1 ml of ice-cold 50% ethanol and fixed overnight at 4°C. Samples were kept on ice, in the dark, and analyzed using a Becton Dickinson FACScan. Flow cytometric data were analyzed using Lysis II Software (Becton Dickinson). Background fluorescence, obtained by labelling the cells with isotype-control antibodies and FITC-conjugated secondary antibody, was subtracted from the test values. We used the following primary antibodies: OX19 (Dallman et al., 1984), which labels CD5; R73 (Hünig et al., 1989), which labels the  $\alpha\beta$  T cell receptor (TCR $\alpha\beta$ ); R78 (Torres Nagel et al., 1993), which labels V $\beta$ 8.2<sup>+</sup> T cells which are the predominant encephalitogenic cells in MBP-EAE (Imrich et al., 1995); OX1 (Sunderland et al., 1979), which labels the leukocyte common antigen (CD45) which is expressed in high levels on cells of haematogenous origin and in lower levels on microglia (Sedgwick et al., 1991). The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated F(ab')<sub>2</sub> rabbit anti-mouse IgG (STAR 41) (Serotec).

### 2.4. mRNA extraction and reverse transcriptase-PCR (RT-PCR)

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 yield of mRNA was 0.05–0.46  $\mu$ g mRNA/ $10^5$  cells. The yield was low when the number of cells was high, indicating that we had exceeded the capacity of the extraction kit. The mRNA was stored at –20°C before RT-PCR. For most samples, the mRNA was subdivided and the entire RT-PCR process was performed in duplicate or triplicate. Samples of mRNA (0.05  $\mu$ g) were reverse transcribed using random hexamers as primers and MuLV reverse transcriptase under the following conditions: 5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1 mM dNTPs, 5  $\mu$ M Random Hexamers (Gibco BRL), 1 U/ $\mu$ l RNase Inhibitor (Perkin Elmer), 2.5 U MuLV reverse transcriptase (Perkin Elmer) and 0.05  $\mu$ g poly(A<sup>+</sup>)mRNA, in a total volume of 20  $\mu$ l. Samples were incubated at room temperature for 10 min prior to reverse transcription. The reactions were mineral oil-overlaid and 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 MgCl<sub>2</sub>, 10 mM KCl, 62 mM Tris–HCl (pH 8.3), 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.008% (w/v) Tween, 0.2 mM dNTPs, 0.15  $\mu$ M of each primer and 0.02 U/ $\mu$ l *Red Hot Taq* DNA Polymerase (Integrated Sciences) in a total reaction volume of 100  $\mu$ l (that contained the entire 20  $\mu$ l RT reaction). The reactions were mineral oil-overlaid and 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  $\beta$ -actin]; then, 7 min at 72°C). **Fig. 1** shows the relationship of cytokine product to cycle number. Primers with the following sequences were synthesized by the Queensland Institute of Medical Research.

$\beta$ -actin sense	5' ATG CCA TCC TGC GTC TCG ACG TGG C 3'
$\beta$ -actin anti-sense	5' AGC ATT TGC GGT GCA CGA TGG AGG G 3'
IL-2 sense	5' GTC AAC AGC GCA CCC ACT TCA AGC 3'
IL-2 antisense * (2)	5' GCT TGT TGA GAT GAT GCT TTG ACA 3'
IL-4 sense	5' TGA TGG GTC TCA GCC CCC ACC TTG C 3'
IL-4 antisense	5' CTT TCA GTG TTFG TGA GCG TGG ACT C 3'
IL-10 sense	5' GAC TTT AAG GGT TAC TTG GGT TGC 3'
IL-10 antisense * (2)	5' CAC TGC GCT CTT ATT TTC ACA 3'
IFN- $\gamma$ sense * (1)	5' GAA AGC CTA GAA AGT CTG AAT AAC 3'
IFN- $\gamma$ antisense * (1)	5' GCA GCG ACT CCT TTT CCG CTT CCT 3'



**Fig. 1.** Relationship of cytokine product intensity (measured by densitometry) to number of cycles of PCR, for mRNA extracted from spinal cord inflammatory cells on day 14 after inoculation. This graph is a composite of 3 experiments which measured the increases in product intensity from 30–35, 35–37, and 37–42 cycles for IL-2, IL-4, IL-10, and IFN- $\gamma$  and from 15–20 and 20–25 for  $\beta$ -actin. We chose to use 37 cycles of amplification, which always gave readily visualized product from all cytokines studied. Because this degree of amplification is near the plateau phase of the reaction, we may have underestimated high levels of mRNA expression.

The primer sequences for IL-2, IL-10 and IFN- $\gamma$  were those used by Weinberg et al. (1993) in a study of MBP-EAE in the rat. The primers for IL-4 and  $\beta$ -actin were those used by McKnight et al. (1991). Except for those indicated with \*, the sequences are specific for the rat sequences as reported for  $\beta$ -actin (Nudel et al., 1983) (Genbank J00691), IL-2 (McKnight et al., 1989) (Genbank M22899), IL4 (McKnight et al., 1991) (Genbank X16058), IL-10 (Feng et al., 1993) (Genbank X60675) and IFN- $\gamma$  (Dijkema et al., 1985) (Genbank X0237, X0236, X0235). The sequences indicated by '\*' are specific for mouse sequences and differ from the rat sequences by the number of base pairs shown in parentheses. The predicted product sizes are:  $\beta$ -actin: 607 bp; IL-2: 449 bp; IL-4: 378 bp; IL-10: 201 bp; IFN- $\gamma$ : 383 bp. The sequences chosen all crossed exon boundaries. RT-PCR products (15  $\mu$ l of product loaded per lane) were visualized under ultraviolet light after electrophoresis on a 2% agarose gel in TBE buffer (10 mM Tris, 90 mM Boric acid, 1 mM EDTA) containing 0.5  $\mu$ g/ml ethidium bromide. Because RT-PCR was performed in duplicate or triplicates, 2–3 gels were obtained from each group of rats. To measure the intensity of product, gels were photographed using positive/negative Polaroid film, and negatives were analyzed to determine the intensities of cytokine bands and  $\beta$ -actin control bands, using a Molecular Dynamics densitometer and ImageQuant software.

## 2.5. Identification of products

RT-PCR products were dot-blotted onto positively-charged nylon membranes (Zetaprobe membrane, BioRad) using a Bio-Dot Microfiltration Apparatus (BioRad) according to the manufacturer's instructions. Twenty microliter aliquots of diluted cDNA, at dilutions ranging from 1 in 5 to 1 in 100, in 10 mM Tris-HCl, 1 mM EDTA (pH 8), were denatured by the addition of NaOH and EDTA to a final concentration of 0.4 M NaOH, 10 mM EDTA, in a final volume of 50  $\mu$ l. Samples were then heated at 100°C for 10 min, followed by rapid cooling on ice. After application of the samples to the membrane, 0.4 M NaOH was applied to promote binding of DNA to the membrane. Membranes were removed from the dot-blotting apparatus, rinsed in a solution of sodium chloride/sodium citrate (SSC) (0.03 M Na<sub>3</sub>Citrate, 0.3 M NaCl), and then air-dried. If membranes were not to be used the same day, they were baked at 80°C and then stored between 2 sheets of filter paper in a sealed plastic bag at room temperature. To identify the RT-PCR products bound to the membranes, we used the following probe sequences, which are specific for rat cytokines (Barton et al., 1995) and which were synthesized by Gibco BRL Life Technologies:

```

 $\beta$ -actin  5' CTT CAT GAG GTA GTC TGT CAG GT 3'
IL-2     5' GTT CAT CTT CTA GGC ACT G 3'
IL-4     5' GGG GTT CTC GGT GAA CTG AGG AAA
          CTC 3'
IL-10    5' GTT TTA CCT GGT AGA AG 3'
IFN- $\gamma$   5' CAG ATT ATC TCT TTC TAC CTC AGA
          C 3'

```

The probes were labelled, hybridized to the membranes and detected using a chemiluminescence system (ECL 3'-oligolabelling and detection system, Amersham). All reagents (except standard buffers) were supplied with the kit, and the suggested protocol was followed. Briefly, probes were labelled at the 3' hydroxyl terminal with fluorescein-11-dUTP, in a reaction catalyzed by terminal deoxynucleotidyl transferase. Probes were hybridized to the dot-blotted membranes during an overnight incubation at 42°C, at a concentration of 10 ng of probe per milliliter of

hybridization buffer. To allow only perfectly matched sequences to remain bound, we performed stringency washes at 5°C below the  $T_m$  of the probe, calculated according to the following formula:  $T_m = 81.5 + (16.6 \times [\text{moles Na}^+]) + (0.41 \times [\% \text{ GC bases}]) - (675 / \text{primer length}) - (0.61 \times [\% \text{ formamide}])$ . The following conditions were used:  $\beta$ -actin: 0.15×SSC, 0.1% sodium dodecyl sulphate (SDS), 42°C; IL-2: 0.4×SSC, 0.1% SDS, 42°C; IL-4: 0.1×SSC, 0.1% SDS, 47°C; IL-10: 1×SSC, 0.1% SDS, 42°C; IFN- $\gamma$ : 0.2×SSC, 0.1% SDS, 43°C. In a two-stage protocol, probe/DNA hybrids were detected. The membranes were incubated with horseradish peroxidase-conjugated antibody to fluorescein, followed by incubation with the supplied Detection Reagents. Light emission due to the enzymatic reduction of peroxide, coupled with luminol oxidation and subsequent breakdown was detected with blue light-sensitive film (Kodak Biomax XL).

## 2.6. Presentation of results

The results are mainly presented as the intensities of amplification product for each cytokine relative to that for  $\beta$ -actin. Such a comparison of results from different days and different samples assumes that  $\beta$ -actin expression is constant and that, as has recently been shown by Bishop et al. (1997), the RT-PCR reaction is reproducible. When possible, the RT-PCR reaction was performed in duplicate or triplicate for each sample of mRNA. The correlation co-efficient ( $r$ ) between paired samples was 0.70. Because the RT-PCR reaction may become nonlinear at the extremes of the range, the numerical values obtained for these ratios may not reflect the number of molecules of product. Because the number of cycles used (37) was near the plateau phase of the PCR reaction, we may have underestimated higher levels of mRNA expression. However, because the reactions were performed under the same conditions on all the days of study, a comparison of the relative expression of each cytokine can be made, with higher values indicating greater expression of cytokine. The results are also presented as cytokine mRNA expression per gram of spinal cord. This was calculated as cytokine expression relative to  $\beta$ -actin (an index of cytokine expression per cell, assuming that all cells have the same content of  $\beta$ -actin mRNA) multiplied by the yield of cells per gram of spinal cord. Data was analyzed with Kruskal–Wallis one-way analysis of variance (ANOVA) for ranks with Dunn's post-test comparing all other days with day 18, using Sigmastat Version 2 (Jandel Scientific software).

## 3. Results

### 3.1. Details of rats used for RT-PCR study

Table 1 shows the details of the rats used for the RT-PCR study. On each day of study, 3–5 groups of rats, each group comprising 4–6 rats, were used. For each group of rats, the spinal cords were combined before the extraction of cells. For controls, we studied total mRNA extracted from the spinal cord tissue of normal rats and mRNA obtained from a short-term MBP-reactive Lewis rat T cell line (kindly supplied by Ms. Jackie Harness).

Table 1  
Details of rats used in the study of cytokine RT-PCR

Day of study	Number of groups of rats	Total number of rats studied	Mean day of onset of disease	Mean total clinical score on day of study	Mean % yields of cells after nylon wool extraction	Mean of number cells per gram of spinal cord
10	3	15	9.2	1.9	40	$4.7 \times 10^5$
12	3	13	9.9	4.4	37	$9.2 \times 10^5$
13	3	12	10.4	5.1	34	$14.0 \times 10^5$
14	5	23	9.9	6.4	35	$13.0 \times 10^5$
15	4	18	9.8	4.9	21	$9.0 \times 10^5$
16	3	14	9.7	3.7	23	$7.4 \times 10^5$
17	4	16	10.2	2.6	17	$4.2 \times 10^5$
18	4	18	9.9	2.2	15	$4.0 \times 10^5$
19	3	15	10.0	2.0	17	$4.5 \times 10^5$
21	3	15	9.9	0.4	15	$9.9 \times 10^5$

### 3.2. Analysis of the cells obtained from the spinal cord

Table 1 shows the number of cells and the percentage of the original cells that remained after passage through nylon wool. Before nylon wool extraction, many macrophages could be seen by microscopy in the cell extract, whereas after nylon wool extraction, the number of macrophages visible by microscopy was reduced. The yield of cells after nylon wool extraction was lower on the later than on the earlier days of study, suggesting that the cells obtained in the later stages of the disease contained a higher proportion of macrophages. To compare the cell populations on different days after inoculation, we used flow cytometry analysis of another series of rats. Table 2 shows the percentage of cells (after passage through nylon wool) that were stained with antibodies to CD45, CD5, TCR $\alpha\beta$  and V $\beta$ 8.2. On days 12–21, the percentage of cells that were CD45<sup>+</sup> (cells of haematogenous origin) was around 90%. The CD45<sup>+</sup> cell population contained CD45<sup>high</sup> cells (including lymphocytes and macrophages) and CD45<sup>low</sup> cells which appear to be microglia (Sedgwick et al., 1991; McCombe et al., 1994). The percentages that were CD5<sup>+</sup> and TCR $\alpha\beta$ <sup>+</sup> remained constant from days 12–19, but declined on day 21, after recovery from the disease. The CD5<sup>-</sup> cells are, in part, accounted for by CD45<sup>low</sup> cells (microglia). The remaining CD5<sup>-</sup> cells are likely to be macrophages.

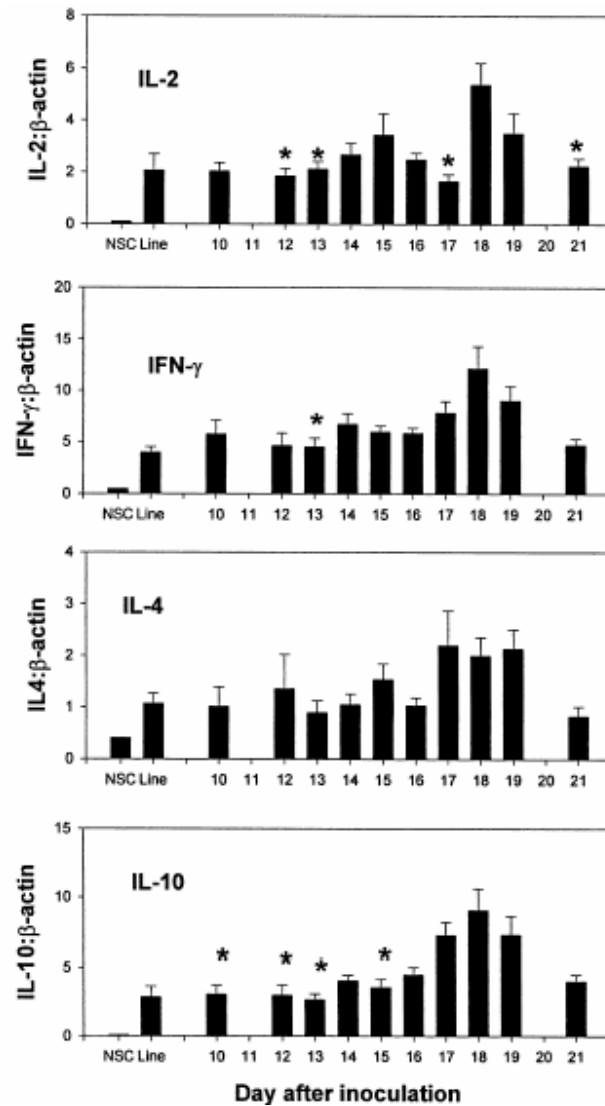
Table 2  
Characterization of cells extracted from the spinal cord of rats with EAE

Day of study	Mean % CD5 <sup>+</sup>	Mean % TCR $\alpha\beta$ <sup>+</sup>	Mean % CD45 <sup>+</sup>	Mean % V $\beta$ 8.2 <sup>+</sup>	Mean % CD45 <sup>low</sup>
12	67	54	97	9	4
13	65	44	91	8	3
14	75	51	91	12	8
15	74	37	86	8	4
16	70	37	87	5	10
17	61	37	84	5	11
18	66	43	88	6	10
19	54	45	91	5	16
21	10	9	88	1	60

### 3.3. Results of RT-PCR

In rats with EAE, we found intense product bands for IL-2, IL-10 and IFN- $\gamma$  on all days after inoculation. The band for IL-4 product was less intense. For a control, we studied mRNA extracted from spinal cord tissue of a normal rat. From this mRNA, we found only faint product bands for IL-2, IL-4 and IFN- $\gamma$  and there was no IL-10 product. Products for all cytokines were amplified from mRNA from the MBP-reactive T cell line. The cytokine products were of predicted size. Dot-blotting with labelled probes confirmed the identities of the cytokine products. In all cases, the PCR product gave a positive result with the specific probe and a negative result with the irrelevant probes. Fig. 2 shows the expression of IL-2, IL-10, IL-4 and IFN- $\gamma$ , measured by densitometry, and normalized against expression of  $\beta$ -actin. The mean density of  $\beta$ -actin product varied from 41–91 units. The level of expression of mRNA for IL-2, relative to that for  $\beta$ -actin, reached a peak on days 18–19 during late clinical recovery. IFN- $\gamma$  expression reached a peak on days 17–19. Relative expression of IL-4 did not vary greatly through the course of the disease but was highest on days 17–19. Relative expression of IL-10 rose from day 10 to reach a peak on days 17–19, during late clinical recovery. The observed increase in the relative expression of cytokines on days 17–19 could indicate either that individual cells from which the mRNA was extracted were producing relatively more cytokine mRNA, or that the proportion of cells producing cytokine mRNA was greater. Table 3 shows the expression of cytokine mRNA per gram of spinal cord. It can be seen that cytokine mRNA production per gram of spinal cord reached a peak on days 13–14 at the time of peak clinical disease.





**Fig. 2.** Mean ( $\pm$ SE) of the ratios of cytokine expression to  $\beta$ -actin expression from controls (NSC: normal spinal cord; Line: an MBP-reactive T cell line) and spinal cord inflammatory cells from rats with EAE on days 10–21 after inoculation. For cytokine mRNA, 37 PCR cycles were performed and for  $\beta$ -actin, 20 cycles were performed. The intensity of the product was measured by densitometry. With Kruskal–Wallis ANOVA for ranks,  $p < 0.05$  for each cytokine. The asterisks indicate values that were significantly different ( $p < 0.05$ ) from the value for day 18.

Table 3  
Cytokine expression per gram of spinal cord

Mean cytokine expression per gram of spinal cord ( $\pm$ SD)				
Day	IL-2	IFN- $\gamma$	IL-4	IL-10
10	1.1 (0.9)	2.9 (2.3)	0.6 (0.7)	1.9 (1.8)
12	1.4 (0.4)	3.2 (0.6)	0.8 (0.7)	2.2 (0.6)
13	3.3 (1.3)	7.0 (3.5)	1.5 (0.9)	4.1 (1.8)
14	3.6 (2.8)	7.8 (3.2)	1.1 (0.5)	4.7 (1.8)
15	3.0 (2.3)	5.3 (1.8)	1.2 (0.6)	3.1 (1.5)
16	1.7 (0.5)	4.0 (0.8)	0.7 (0.2)	3.1 (0.9)
17	0.8 (0.6)	4.1 (3.9)	0.7 (0.3)	3.7 (3.2)
18	2.1 (1.0)	4.7 (2.1)	0.8 (0.4)	3.5 (1.8)
19	1.6 (1.0)	4.1 (1.9)	0.9 (0.3)	3.4 (1.8)
21	2.6 (1.0)	4.6 (1.8)	0.9 (0.5)	4.0 (1.6)

#### 4. Discussion

We studied the expression of cytokine mRNA by inflammatory cells obtained from the spinal cords of Lewis rats with actively induced acute MBP-EAE. Cytokine mRNA expression does not necessarily reflect protein production by cells, but changes in mRNA expression through the course of the disease are likely to reflect modulation of the activity of cytokine-producing cells. Semi-quantitative RT-PCR was used to compare the results from rats studied on days 10–21 after inoculation. The technique we used may have underestimated higher levels of mRNA expression, because the number of cycles used was near the plateau phase. We have presented the results as cytokine mRNA expression relative to that of  $\beta$ -actin (Fig. 2). This gives an index of cytokine production per cell, and will increase if a higher proportion of the cells in the population express cytokine mRNA or if individual cells produce more cytokine mRNA. These results may be compared with those of Tanuma et al. (1997), who used competitive PCR in a study of cells extracted from the spinal cord of Lewis rats with EAE. We have also presented the results as cytokine mRNA expression per gram of spinal cord (Table 3), which can be compared with the results of Issazadeh et al. (1995a) and Issazadeh et al. (1995b), who used in situ hybridization to study cytokine mRNA expression per unit area of spinal cord section.

In our study, we used passage over nylon wool to enrich our population for lymphocytes. The study population was predominantly (70%) CD5<sup>+</sup>, indicating that the majority of the cells were T lymphocytes. Therefore, this study predominantly reflects cytokine mRNA expression by T lymphocytes. The CD5<sup>-</sup> cells were, in part, accounted for by CD45<sup>low</sup> cells that appear to be microglia (Sedgwick et al., 1991). Although we did not stain with a marker for macrophages, the remaining CD5<sup>-</sup> cells are likely to be macrophages. Microglia and macrophages may secrete IL-10, but do not secrete the other cytokines of interest in this study. The majority of T cells in the spinal cord of Lewis rats with acute MBP-EAE are CD45RC<sup>low</sup> (activated cells) (McCombe et al., 1992). The percentage of these cells that are V $\beta$ 8.2<sup>+</sup> (the predominant encephalitogenic cells) declines during clinical recovery from the disease (McCombe et al., 1996a). We found that IL-2, IL-4, IL-10 and IFN- $\gamma$  were expressed by inflammatory cells from the spinal cords of rats with MBP-EAE. In contrast, there was minimal expression of IL-2, IL-4 and IFN- $\gamma$  and no expression

of IL-10 in normal spinal cord. Messenger RNA from a short-term MBP-reactive T-cell line expressed all the cytokines studied. Thus, our study found that Th1 (IL-2 and IFN- $\gamma$ ) and Th2 (IL-4 and IL-10) cytokines are expressed by inflammatory cells in the CNS throughout the course of the disease.

Semi-quantitative studies were performed to examine the expression of cytokine mRNA from day 10 (early in the disease) until day 21 (when the majority of rats have completely recovered). Expression of IL-2 mRNA (relative to  $\beta$ -actin) reached a peak on day 18 during late clinical recovery. The peak of IL-2 production per gram of spinal cord was on day 14, at the peak of the clinical disease. Our findings of IL-2 mRNA expression throughout the clinical disease are consistent with previous studies indicating the importance of IL-2 in EAE. IL-2 is known to be present in the CNS in EAE (Baker et al., 1991; Merrill et al., 1992; Renno et al., 1994) and encephalitogenic T cell clones secrete IL-2 (Ando et al., 1989; Baron et al., 1993). Expression of IFN- $\gamma$  (relative to  $\beta$ -actin) was observed throughout the course of the disease, with a peak on days 17–19, during late clinical recovery. Our findings of IFN- $\gamma$  expression differ from those of Tanuma et al. (1997), who found peak expression on days 11 and 12, but did not study expression on days 17–19. We did not study day 11, and may have missed a peak at that time. Alternatively, our technique of semi-quantitative PCR may have underestimated such a peak. We found that the expression of IFN- $\gamma$  per gram of spinal cord reached a peak on day 14 and remained elevated until day 21. IFN- $\gamma$  has a wide range of activities and may contribute to the pathogenesis of EAE by the induction of major histocompatibility complex (MHC) antigen expression. IFN- $\gamma$  may also cause tissue damage by inducing NO synthase (Misko et al., 1995). The persistence of IFN- $\gamma$  expression during clinical recovery could be consistent with a down-regulatory role for this cytokine in EAE. Such a down-regulatory role is suggested by the observations that: (1) administration of antibody to IFN- $\gamma$  can enhance EAE (Billiau et al., 1988; Voorthuis et al., 1990), (2) intraventricular IFN- $\gamma$  can suppress EAE (Voorthuis et al., 1990), (3) abrogation of IFN- $\gamma$  expression makes BALB/c mice susceptible to EAE (Krakowski and Owens, 1996), and (4) EAE is more severe in IFN- $\gamma$  receptor deficient mice (Willenborg et al., 1996). IFN- $\gamma$  might down-regulate EAE through its ability to induce apoptosis of activated T cells (Liu and Janeway, 1990). During the later stages of the disease, the pro-inflammatory functions of IFN- $\gamma$  may be blocked by TGF- $\beta$  (Dore Duffy et al., 1996), thus allowing the protective role of IFN- $\gamma$  to predominate.

Expression of IL-4 relative to  $\beta$ -actin was observed on all days, with a rise in the levels on days 17–19. Issazadeh et al. (1995b) found only low numbers of IL-4 mRNA-expressing cells in the spinal cord of rats with EAE. However, in mice, Kennedy et al. (1992) found that IL-4 was present in the CNS and reached a peak before the peak of the clinical disease. Our study supports the view that IL-4-expressing cells are present in the CNS in EAE. IL-10 expression (relative to  $\beta$ -actin) increased throughout the course of the disease with maximum expression on day 18. IL-10 was first described as a product of Th2 cells that could inhibit cytokine synthesis by Th1 cells (Fiorentino et al., 1989). IL-10 is now known to have a wider biological role and is an anti-inflammatory cytokine produced by T cells, B cells and macrophages (Moore et al., 1993). These anti-inflammatory properties could contribute to the down-regulation of EAE. Kennedy et al. (1992), who studied mRNA from total spinal cord, showed that expression of IL-10 correlates with clinical recovery from EAE in mice. Issazadeh et al. (1995a) showed that IL-10 mRNA-producing cells are present in the CNS of rats during and after recovery from EAE. The techniques used by Issazadeh et al. (1995a) detect all cytokine-producing cells, and therefore would detect IL-10 mRNA expression by macrophages; however, we studied a predominantly lymphocyte population. It is also possible that astrocytes may produce IL-10 (Mizuno et al., 1994), but we did not find detectable IL-10 in mRNA extracted from normal spinal cord tissue.

Our findings of IL-10 expression differ from those of Tanuma et al. (1997), who found peak expression on days 11 and 12. We did not study day 11, and may have missed a peak at that time. We found a peak of IL-10 expression (relative to  $\beta$ -actin) on days 17–19, which were not studied by Tanuma et al. (1997). This peak of IL-10 expression occurred late in clinical recovery, and therefore is unlikely to play a primary role in the resolution of inflammation in EAE, although it may play a role in the subsequent regulation of inflammation. Previous studies are unclear about the role of IL-10 in recovery from EAE. One study in the rat (Rott et al., 1994) found that exogenous IL-10 reduced the severity of EAE, whereas another study, in mice, found the opposite (Cannella et al., 1996).

Thus, we found increased expression of all cytokines, relative to the expression of  $\beta$ -actin, during late clinical recovery, near the time that spontaneous relapses of EAE can occur. The alterations in cytokine expression may be due to changes in the population of cells in the spinal cord. We have previously shown that after the peak of clinical signs of acute MBP-EAE, there is a selective loss of V $\beta$ 8.2<sup>+</sup> cells (the predominant encephalitogenic cells) by apoptosis in the CNS (Tabi et al., 1994; McCombe et al., 1996a) and suggested that this is mediated through interactions between Fas and Fas ligand (White et al., 1998). Fas-mediated apoptosis occurs more rapidly in Th1 than Th2 cells (Zhang et al., 1997), so that selective apoptosis of Th1-like encephalitogenic cells, with sparing of Th2 cells, could explain the observed relative increase in expression of IL-10 in the later stages of the disease. However, this would not explain the increased expression of IL-2 and IFN- $\gamma$ . The increased relative expression of cytokines during late clinical recovery could also reflect an influx of activated T cells from the periphery. An influx of activated T cells could occur after a waning in the effects of endogenous corticosteroids which have a role in clinical recovery from EAE (MacPhee et al., 1989) and which may act by reducing influx of cells into the CNS.

In conclusion, we have found that the relative expression of the Th2 cytokine IL-10, and to a lesser extent the Th2 cytokine IL-4, is high in the CNS during late clinical recovery from EAE. However, the expression of the Th1 cytokines IFN- $\gamma$  and IL-2 is also high at this time. With further study, the original concept of clearly defined and highly polarized populations of Th1 and Th2 cells has been replaced by a more complex model of T cell cytokine production (Mosmann and Sad, 1996) in which the expression of different cytokines may be regulated independently. The alterations in cytokine expression in the spinal cord after the peak of the clinical disease may reflect the changes in the cell population in the spinal cord.

---

## Acknowledgements

We would like to thank Dr. T. Hunig and Dr. J. Sedgwick for kindly providing monoclonal antibodies. We are grateful to Mrs. J. Harness, who provided the MBP-reactive T cell line. The work was supported by the National Health and Medical Research Council of Australia NHMRC. Dr. P.A. McCombe is an NHMRC Senior Research fellow.

---

## References

- Ando, D.G., Clayton, J., Kono, D., Urban, J.L., Sercarz, E.E., 1989. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis EAE are of the Th1 lymphokine subtype. *Cell. Immunol.* 124, 132–143.
- Baker, D., O'Neill, J.K., Turk, J.L., 1991. Cytokines in the central nervous system of mice during chronic relapsing experimental allergic encephalomyelitis. *Cell. Immunol.* 134, 505–510.

- Baron, J.L., Madri, J.A., Ruddle, N.H., Hashim, G., Janeway, C.A.J., 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* 177, 57–68.
- Barton, K., McLauchlan, M.T., Calder, V.L., Lightman, S., 1995. The kinetics of cytokine mRNA expression in the retina during experimental autoimmune uveoretinitis. *Cell. Immunol.* 164, 133–140.
- Billiau, A., Heremans, H., Vandekerckhove, F., Dijkmans, R., Sobis, H., Meulepas, E., Carton, H., 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN- $\gamma$ . *J. Immunol.* 140, 1506–1510.
- Bishop, G.A., Rokahr, K.L., Lowes, M., McGuinness, P.H., Napoli, J., DeCruz, D.J., Wong, W.-Y., McCaughan, G.W., 1997. Quantitative reverse transcriptase-PCR amplification of cytokine mRNA in liver biopsy specimens using a noncompetitive method. *Immunol. Cell Biol.* 75, 142–147.
- Cannella, B., Gao, Y.L., Brosnan, C., Raine, C.S., 1996. IL-10 fails to abrogate experimental autoimmune encephalomyelitis. *J. Neurosci. Res.* 45, 735–746.
- Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A., Weiner, H.L., 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237–1240.
- Dallman, M.J., Thomas, M.L., Green, J.R., 1984. MRC OX-19: a monoclonal antibody that labels rat T lymphocytes and augments in vitro proliferative responses. *Eur. J. Immunol.* 14, 260–267.
- Deibler, G.E., Martenson, R.E., Kies, M.W., 1972. Large-scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep. Biochem.* 2, 139–165.
- Dijkema, R., van der Meide, P.H., Pouwels, P.H., Caspers, M., Dubbeld, M., Schellekens, H., 1985. Cloning and expression of the chromosomal immune interferon gene of the rat. *EMBO J.* 4, 761–767.
- Dore Duffy, P., Balabanov, R., Rafols, J., Swanborg, R.H., 1996. Recovery phase of acute experimental autoimmune encephalomyelitis in rats corresponds to development of endothelial cell unresponsiveness to interferon- $\gamma$  activation. *J. Neurosci. Res.* 44, 223–234.
- Feng, L., Tang, W.W., Chang, J.C., Wilson, C.B., 1993. Molecular cloning of rat cytokine synthesis inhibitory factor IL-10 cDNA and expression in spleen and macrophages. *Biochem. Biophys. Res. Commun.* 192, 452–458.
- F Fiorentino, D.F., Bond, M.W., Mosmann, T.R., 1989. Two types of mouse T helper cell IV Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170, 2081–2095.
- Hunig, T., Wallny, H.-J., Hartley, J.K., Lawetzky, A., Tiefenhaler, G., 1989. A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. *J. Exp. Med.* 169, 73–86.
- Imrich, H., Kugler, C., Torres-Nagel, N., Dorries, R., Hunig, T., 1995. Prevention and treatment of Lewis rat experimental allergic encephalomyelitis with a monoclonal antibody to the T cell receptor Vb8.2 segment. *Eur. J. Immunol.* 25, 1960–1964.
- Issazadeh, S., Ljungdahl, A., Hojeberg, B., Mustafa, M., Olsson, T., 1995a. Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA expression for interleukin-10, interleukin-12, cytolysin, tumor necrosis factor  $\alpha$  and tumor necrosis factor  $\beta$ . *J. Neuroimmunol.* 61, 205–212.
- Issazadeh, S., Mustafa, M., Ljungdahl, A., Hojeberg, B., Dagerlind, A., Elde, R., Olsson, T., 1995b. Interferon- $\gamma$ , interleukin-4 and transforming growth factor  $\beta$  in experimental autoimmune encephalomyelitis in Lewis rats: dynamics of cellular mRNA expression in the central nervous system and lymphoid cells. *J. Neurosci. Res.* 40, 579–590.
- Karpus, W.J., Gould, K.E., Swanborg, R.H., 1992. CD4<sup>+</sup> suppressor cells of autoimmune encephalomyelitis respond to T cell receptor-associated determinants on effector cells by interleukin-4 secretion. *Eur. J. Immunol.* 22, 1757–1763.
- Kennedy, M.K., Torrance, D.S., Picha, K.S., Mohler, K.M., 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol.* 149, 2496–2505.

- Krakowski, M., Owens, T., 1996. Interferon- $\gamma$  confers resistance to experimental allergic encephalomyelitis. *Eur. J. Immunol.* 26, 1641–1646.
- Liu, Y., Janeway, C.A. Jr., 1990. Interferon- $\gamma$  plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J. Exp. Med.* 172, 1735–1739.
- MacPhee, I.A., Antoni, F.A., Mason, D.W., 1989. Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on the regulation of the immune system by endogenous adrenal corticosteroids. *J. Exp. Med.* 169, 431–445.
- McCombe, P.A., Fordyce, B.W., de Jersey, J., Yoong, G., Pender, M.P., 1992. Expression of CD45RC and Ia antigen in the spinal cord in acute experimental allergic encephalomyelitis: an immunocytochemical and flow cytometric study. *J. Neurol. Sci.* 113, 177–186.
- McCombe, P.A., de Jersey, J., Pender, M.P., 1994. Inflammatory cells, microglia and MHC class II antigen-positive cells in the spinal cord of Lewis rats with acute and chronic relapsing experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 51, 153–167.
- McCombe, P.A., Nickson, I., Tabi, Z., Pender, M.P., 1996a. Apoptosis of V $\beta$ 8.2+ T lymphocytes in the spinal cord during recovery from experimental autoimmune encephalomyelitis induced in Lewis rats by inoculation with myelin basic protein. *J. Neurol. Sci.* 139, 1–6.
- McCombe, P.A., Nickson, I., Tabi, Z., Pender, M.P., 1996b. Corticosteroid treatment of experimental autoimmune encephalomyelitis in the Lewis rat results in loss of V $\beta$ 8.2+ and myelin basic protein-reactive cells from the spinal cord, with increased total T cell apoptosis but reduced apoptosis of V $\beta$ 8.2+ cells. *J. Neuroimmunol.* 70, 93–101.
- McKnight, A.J., Mason, D.W., Barclay, A.N., 1989. Sequence of rat interleukin-2 and anomalous binding of a mouse interleukin-2 cDNA probe to rat MHC class II-associated invariant chain mRNA. *Immunogenetics* 30, 145–147.
- McKnight, A.J., Barclay, A.N., Mason, D.W., 1991. Molecular cloning of rat interleukin-4 cDNA and analysis of the cytokine repertoire of subsets of CD4+ T cells. *Eur. J. Immunol.* 21, 1187–1194.
- Merrill, J.E., Kono, D.H., Clayton, J., Ando, D.G., Hinton, D.R., Hofman, F.M., 1992. Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice. *Proc. Natl. Acad. Sci. USA* 89, 574–578.
- Misko, T.P., Trotter, J.L., Cross, A.H., 1995. Mediation of inflammation by encephalitogenic cells: interferon- $\gamma$  induction of nitric oxide synthase and cyclooxygenase 2. *J. Neuroimmunol.* 61, 195–204.
- Mizuno, T., Sawada, M., Marunouchi, T., Suzumura, A., 1994. Production of interleukin-10 by mouse glial cells in culture. *Biochem. Biophys Res. Commun.* 205, 1907–1915.
- Moore, K.W., O'Garra, A., de Waal Malefyt, R., Vieira, P., Mosmann, T.R., 1993. Interleukin-10. *Annu. Rev. Immunol.* 11, 165–190.
- Mosmann, T.R., Coffman, R.L., 1989. Th1 and Th2 cells: different patterns of lymphokine secretion leads to different functional properties. *Annu. Rev. Immunol.* 7, 145–173.
- Mosmann, T.R., Sad, S., 1996. The expanding universe of T cell subsets: Th1, Th2 and more. *Immunol. Today* 17, 138–146.
- Nguyen, K.B., McCombe, P.A., Pender, M.P., 1994. Macrophage apoptosis in the central nervous system in experimental autoimmune encephalomyelitis. *J. Autoimmun.* 7, 145–152.
- Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z., Yaffe, D., 1983. The nucleotide sequence of the rat cytoplasmic b-actin gene. *Nucleic Acids Res.* 11, 1759–1771.
- Pender, M.P., 1986. Ascending impairment of nociception in rats with experimental allergic encephalomyelitis. *J. Neurol. Sci.* 75, 317–328.
- Pender, M.P., 1989. Recovery from acute experimental allergic encephalomyelitis in the Lewis rat. Early restoration of nerve conduction and repair by Schwann cells and oligodendrocytes. *Brain* 112, 393–416.

- Pender, M.P., McCombe, P.A., Yoong, G., Nguyen, K.B., 1992. Apoptosis of  $\alpha\beta$  T lymphocytes in the nervous system in experimental autoimmune encephalomyelitis: its possible implications for recovery and acquired tolerance. *J. Autoimmun.* 5, 401–410.
- Pettinelli, C.B., McFarlin, D.E., 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+2 - T lymphocytes. *J. Immunol.* 127, 1420–1423.
- Raine, C.S., 1984. Biology of disease. Analysis of autoimmune demyelination: its impact upon multiple sclerosis. *Lab. Invest.* 50, 608–635.
- Renno, T., Lin, J.Y., Piccirillo, C., Antel, J., Owens, T., 1994. Cytokine production by cells in cerebrospinal fluid during experimental allergic encephalomyelitis in SJL/J mice. *J. Neuroimmunol.* 49, 1–7.
- Rott, O., Fleischer, B., Cash, E., 1994. Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur. J. Immunol.* 24, 1434–1440.
- Schmied, M., Breitschopf, H., Gold, R., Zischler, H., Rothe, G., Wekerle, H., Lassmann, H., 1993. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am. J. Pathol.* 143, 446–452.
- Sedgwick, J.D., Schwender, S., Imrich, H., Dorries, R., Butcher, G.W., ter Meulen, V., 1991. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc. Natl. Acad. Sci. USA* 88, 7438–7442.
- Sunderland, C.A., McMaster, W.R., Williams, A.C., 1979. Purification with monoclonal antibody of a predominant leukocyte-common antigen and glycoprotein from rat thymocytes. *Eur. J. Immunol.* 9, 155–159.
- Tabi, Z., McCombe, P.A., Pender, M.P., 1994. Apoptotic elimination of V $\beta$ 8.2+ cells from the central nervous system during recovery from experimental autoimmune encephalomyelitis induced by the passive transfer of V $\beta$ 8.2+ encephalitogenic T cells. *Eur. J. Immunol.* 24, 2609–2617.
- Tabi, Z., McCombe, P.A., Pender, M.P., 1995. Antigen-specific downregulation of myelin basic protein-reactive T cells during spontaneous recovery from experimental autoimmune encephalomyelitis: further evidence of apoptotic deletion of autoreactive T cells in the central nervous system. *Int. Immunol.* 7, 967–973.
- Tanuma, N., Kojima, T., Shin, T., Aikawa, Y., Kohji, T., Ishihara, Y., Matsumoto, Y., 1997. Competitive PCR quantisation of pro- and anti-inflammatory cytokine mRNA in the central nervous system during autoimmune encephalomyelitis. *J. Neuroimmunol.* 73, 197–206.
- Torres Nagel, N.E., Gold, D.P., Hunig, T., 1993. Identification of rat Tcrb-V8.2, 8.5, and 10 gene products by monoclonal antibodies. *Immunogenetics* 37, 305–308.
- Varriale, S., Beraud, E., Barbaria, J., Galibert, R., Bernard, D., 1994. Regulation of experimental autoimmune encephalomyelitis: inhibition of adoptive experimental autoimmune encephalomyelitis by recovery-associated suppressor cells. *J. Neuroimmunol.* 53, 123–131.
- Voorthuis, J.A., Uitdehaag, B.M., De Groot, C.J., Goede, P.H., van der Meide, P.H., Dijkstra, C.D., 1990. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon- $\gamma$  in Lewis rats. *Clin. Exp. Immunol.* 81, 183–188.
- Weinberg, A.D., Wyrick, G., Celnik, B., Vainiene, M., Bakke, A., Offner, H., Vandenbark, A.A., 1993. Lymphokine mRNA expression in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis is associated with a host recruited CD45Rhi/CD4+ population during recovery. *J. Neuroimmunol.* 48, 105–118.
- White, C.A., McCombe, P.A., Pender, M.P., 1998. The roles of Fas, Fas ligand and Bcl-2 in T cell apoptosis in the central nervous system in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 82, 47–55.

Willenborg, D.O., Fordham, S., Bernard, C.C.A., Cowden, W.B., Ramshaw, I.A., 1996. IFN- $\gamma$  plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J. Immunol.* 157, 3223–3227.

Zhang, X., Brunner, T., Carter, L., Dutton, R.W., Rogers, P., Bradley, L., Sato, T., Reed, J.C., Green, D., Swain, S.L., 1997. Unequal death in T helper cell Th 1 and 2 effectors: Th1, but not Th2 effectors, undergo rapid Fas/FasL-mediated apoptosis. *J. Exp. Med.* 185, 1837–1849.