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Induction of Glial L-CCR mRNA Expression in Spinal Cord and Brain in Experimental Autoimmune Encephalomyelitis

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ABSTRACT Chemokines and chemokine receptors are important regulators of leukocyte trafficking and immune response. It is well established that chemokines and their receptors are also expressed in the central nervous system (CNS), where their expression has been associated with various neuroinflammatory diseases, such as multiple sclerosis (MS). One of the most important chemokines involved in MS pathology is CCL2 (previously known as MCP-1). CCL2, released by glial cells, activates the chemokine receptor CCR2, causing the infiltration of blood monocytes in tissues affected by MS. There is evidence, however, that CCL2 also has local effects on CNS cells, including induction or modulation of cytokine release and synthesis of matrix metalloproteinases, that might contribute to CNS pathology. These effects are most likely independent of CCR2, since CCR2 expression in glial cells is rarely observed. We have recently provided evidence for the presence of an alternative CCL2 receptor in glial cells called L-CCR and have investigated the expression of L-CCR mRNA in a murine EAE model. It is shown that L-CCR mRNA is expressed in infiltrating macrophages during EAE, but not in infiltrating T cells. Prominent expression of L-CCR mRNA was detected in astrocytes and microglia already at early time points throughout the brain and spinal cord supporting the hypothesis that L-CCR expression in glial cells is related to CNS inflammation.

INTRODUCTION

Chemokines and their receptors constitute an elaborate signaling system. Approximately 50 different human chemokines have been described. These chemokines interact with 18 different chemokine receptors (Murphy et al., 2000; Zlotnik and Yoshie, 2000).

Chemokines are classified by their structure based on the number and spacing of conserved cysteine motifs near the N-terminus. Thus, four groups, named the C, CC, CXC, and CX3C families, have been distinguished. The classification of chemokine receptors parallels the four subgroups designated for chemokines. These receptor subgroups have been designated XCR, CCR, CXCR, and CX3CR (Mackay, 2001). Most of these chemokine receptors recognize more than one chemokine. Since the different types of immune cells express multiple chemokine receptors that overlap in their ligand specificity, chemokine receptor pharmacology is complex. Several orphan receptors with chemokine-like structures can be found in databases (i.e., www.

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GPCR.org) suggesting that not all chemokine receptors have yet been described.

There is strong evidence that chemokines and their receptors are not only important in the peripheral immune system but also have numerous functions in the physiology and pathology of the CNS. A large number of detailed studies on CNS chemokines and chemokine receptors have been published, and it is now clear that most endogenous cell types within the CNS synthesize distinct chemokines and can also respond to chemokine stimulation (for review, see Ransohoff and Tani, 1998; Hesselgesser and Horuk, 1999; Asensio and Campbell, 1999; Mennicken et al., 1999; Bacon and Harrison, 2000; Bajetto et al., 2002; Biber et al., 2002).

The expression of chemokines in the CNS is generally induced by inflammatory and neurodegenerative events. Recent publications suggest, that chemokines are crucial mediators of leukocyte infiltration during CNS inflammation (Siebert et al., 2000; Izikson et al., 2000; Fife et al., 2000; Huang et al., 2001).

CCL2 is one of the most prominent chemokines associated with brain pathology and is expressed pronouncedly in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) (Sorensen et al., 1999, 2001). The importance of CCL2 for EAE was recently proved in mice deficient for CCL2 that developed a mild and slower form of EAE (Huang et al., 2001).

Infiltration of the brain by blood leukocytes is, however, only one component of the pathology of MS or EAE. Early activation of astrocytes and microglia (gliosis) plays an important role in the pathology of EAE and MS (for review, see Becher et al., 2000). It is known that glial cells respond to chemokines such as CCL2 with synthesis of matrix metalloproteinases and modulation of cytokine release, processes that are involved in EAE and MS (Cross and Woodroofe, 1999; Boddeke et al., 1999; Dorf et al., 2000). The expression of CCR2 in astrocytes or microglia is, however, questionable. This has led to the hypothesis that an additional chemokine receptor for CCL2 exists in glial cells (Heesen et al, 1996). We recently provided evidence for the expression of such a receptor in mouse glial cells, the orphan chemokine receptor L-CCR (Zuurman et al., 2003; Biber et al., 2003). Since expression of L-CCR is induced under pro-inflammatory conditions we have investigated the expression of L-CCR in spinal cord and brain from EAE animals.

MATERIALS AND METHODS

Animals and EAE Induction

All animal experiments have been approved by the animal experimental committee (DEC). Animals were handled and housed according to the guidelines of the central laboratory animal facility in Groningen, The Netherlands. Female C57BL/6 mice (8 weeks old) were immunized with an emulsion consisting of 200 μg myelin oligodendrocyte glycoprotein (MOG) peptide (35–55), emulsified in incomplete Freund's adjuvant (IFA; Difco) and supplemented with 60 μg killed mycobacteria (Difco) as described previously (Amor et al., 1996). On days 0 and 7 post-immunization, the animals were injected subcutaneously (s.c.) with 0.1 ml of this emulsion at two sites of the back (dorsal flank). Control mice received injections of the emulsion without the MOG peptide. Immediately and 24 h after immunization, all animals were injected (i.p.) with 200 ng pertussis toxin (Sigma) in phosphate-buffered saline (PBS).

Immunized animals were scored daily for clinical signs of EAE. The clinical stages were assessed as follows: 0 = normal; 1 = limp tail; 2 = impaired righting reflex; 3 = partial hindlimb paralysis; and 4 = total hindlimb paralysis. Animals exhibiting signs of a lesser severity than typically observed were scored as 0.5 less than the indicated grade. Animals reaching clinical scores of 3 and higher were terminated as described below, according to the rules of the local laboratory animal committee (DEC).

At different time points and various clinical scores, animals were decapitated under isoflurane anesthesia. Brains and spinal cords were removed and snap-frozen in liquid nitrogen; 20-μm-thick sections, cut using a cryostat, were lysed in guanidinium isothiocyanate/mercaptoethanol (GTC) buffer and used for RNA preparation. Sections of 10 μm were mounted on APES-coated slides and used for immunohistochemistry or in situ hybridization or both.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted with slight modifications according to Chomczynski and Sacchi (1987). For reverse transcription (RT), 1 μg of total RNA was transcribed into cDNA as described (Biber et al., 1997). Potential contamination by genomic DNA was checked by running the reactions without reverse transcriptase and using GAPDH primers in subsequent PCR amplification. Only RNA samples that showed no bands after that procedure were used for further investigations.

Polymerase Chain Reaction

In this study, 2 μl of the RT reaction was used in subsequent PCR amplification, as described (Biber et al., 1997). The following primers were used in PCR for L-CCR: 5′-CTGGCGGTGTTTATCTTGGT and 5′-AACCAGCAGAGGAAAAGCAA resulting in a 489bp PCR product. Primers for GAPDH were: 5′-CATCCTGCACCACCAACTGCTTAG and 5′-GCCTGCTTCACCACCTTCTTGATG, resulting in a 346-bp PCR product. Identity of all PCR products was checked by cloning into pCR2.1 (Invitrogen) and subsequent sequencing.

Quantitative Real-Time PCR (Q-PCR)

Generation of standard curves

The L-CCR plasmid DNA (L-CCR in pCRII, see next section) was quantified by spectrophotometry. Five serial 10-fold dilutions of plasmid DNA (from 2,000 fg to 0.2 fg/reaction) were prepared, amplified by PCR using L-CCR specific primers, and labeled with SYBR Green (Roche, Indianapolis, IN), which yields a bright fluorescence on binding of double-stranded nuclei acids; this fluorescence abruptly diminishes upon denaturation of DNA strands during the melting curve analysis. PCR reactions generating standard curves were performed on 20-μl reactions in glass capillaries, using a LightCycler (Roche) and subsequently analysed using LightCycler 3 software®. For each reaction, melting curve analysis was used to detect the synthesis of nonspecific products. Negative controls (omitting input cDNA) were also used in each PCR run, to confirm the specificity of PCR products. PCR standard curves were linear across serial 10-fold dilutions and the melting curve analysis indicated synthesis of a single homogeneous product of expected melting temperature.

PCR and real-time analysis

Standard curves were generated with each set of samples. The reactions were carried out in 20 μl containing 2.5 mM Mg^{2+}, 0.2 μM each forward and backward primer (identical to those used to generate the plasmid DNA template for standard curve), 1× DNA Master SYBR Green (LightCycler-DNA Master SYBR Green I kit, Roche) and 2 μl cDNA. Reaction conditions for PCR were: denature at 95°C for 1 min; 40 cycles of amplification by denaturing at 95°C for 15 s, annealing at 56°C for 5 s, and extending at 72°C for 20 s. The accumulation of products was detected as SYBR Green fluorescence at the completion of each cycle. Analysis was performed using LightCycler 3 software and results are expressed as the crossing point at which accumulation of PCR products became exponential. Using the standard curves, this value was converted to fg. The reaction conditions for melting curve analysis were: denaturation to 95°C at 20°C/s without plateau phase, annealing at 65°C for 15 s, denaturation to 95°C at 0.1°C/s, with continuous monitoring of SYBR Green fluorescence.

In Situ Hybridization

For in situ hybridization, the L-CCR PCR product was cloned into pCR II vector (Invitrogen) and linearized. L-CCR sense and antisense probes were synthesized by run-off transcription in the presence of the appropriate RNA polymerase and digoxigenin (DIG)-conjugated UTP according to the manufacturer's protocol (Roche).

Transverse sections (10 μm) of spinal cord and brain were collected on APES-coated slides and directly fixated in 4% paraformaldehyde (pH 9.5) for 30 min. After rinsing with KPBS (4 × 7 min), sections were permeabilized with 0.5% Triton X-100 in KPBS for 30 min, rinsed in 2× SSC for 5 min, dehydrated in an ethanol series, and air-dried.

Sections were hybridized overnight at 60°C in a freshly prepared hybridization mixture, containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 1× Denhardt's solution, and 10% dextran sulfate. Final probe concentration was 1 ng/μl hybridization mixture. Negative controls for in situ hybridization consisted of hybridization solution with DIG-labeled L-CCR sense probe. After hybridization, the sections were treated with RNase (10 μg/ml) for 30 min at 37°C and washed in 0.1× SSC at 65°C.

Immunodetection of the DIG-labeled RNA-RNA complex was preceded by preincubating the sections for 30 min in buffer 1 (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl), containing 5% blocking reagent (Roche). Subsequently, sections were incubated for 2 h at room temperature with anti-DIG-AP (Roche; diluted 1:500) in buffer 1, containing 1% blocking reagent. After thorough rinsing in buffer 1 and equilibration in an alkaline buffer solution (ABS: 0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2 \cdot 6H_2O$, pH 9.5), the alkaline phosphatase conjugate was revealed with a freshly prepared solution of 0.34 mg/ml nitroblue tetrazoleum (NBT; Roche) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphatase (Roche) in ABS. Endogenous nonintestinal phosphatase activity was inhibited by the addition of levamisole (Sigma) (0.24 mg/ml) to the staining solution. The color reaction was stopped by placing the slides in a buffer solution, consisting of 10 mM Tris-HCl, 1 mM EDTA, pH 8.5. Finally, sections were mounted using glycerol jelly or used for subsequent immunohistochemistry.

Immunohistochemistry

To identify the cells expressing L-CCR mRNA during EAE, the following antibodies were used: rat anti mouse-CD3 polyclonal antibody (Serotec) specific for T-cell identification, rat anti mouse F4/80 polyclonal antibody (Serotec) to identify monocytes/macrophages and activated microglia, rabbit anti-glial fibrillary acidic protein (GFAP), polyclonal antibody (DAKO) specific for astrocytes, or rabbit anti-MAP2 antibody (Chemicon) reactive with neurons.

Sections were incubated overnight with primary antibody at 4°C, and after several rinses, incubated with the appropriate biotinylated secondary antibodies (Vector; dilution 1:400). Sections were washed again, treated with an avidin-biotin-peroxidase complex (Vectastain ABC kit) according to the manufacturer's instructions, and followed by 0.04% DAB (Sigma) in 0.1 M Tris-HCl with 0.01% H_2O_2.

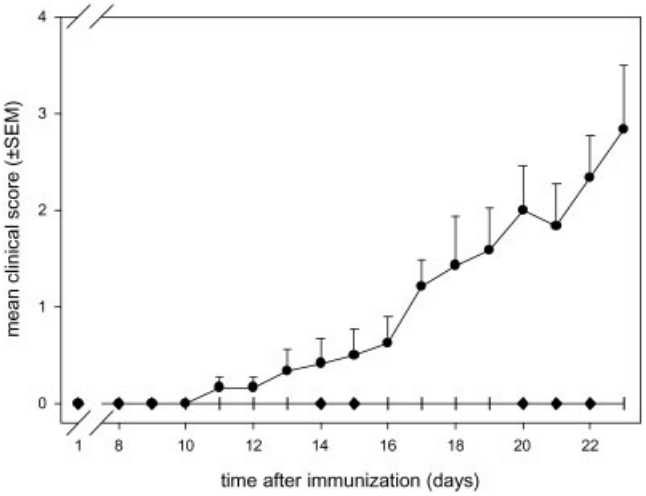

Fig. 1. Development of MOG-induced EAE in C57BL/6 mice. In MOG-injected mice (●, first signs were observed approximately 11 days after the first immunization with a progressive development reaching clinical score 2–3 after approx. 20 days. None of the control mice (◆) developed clinical signs of experimental autoimmune encephalomyelitis (EAE) (n = 6). Up to day 16 of the experiments, the number of MOG-injected animals was 14. Three animals that had developed mild EAE (score 1) in the previous night were terminated on day 16, and two more animals that developed clinical scores of 3 or higher according to the rules of the local laboratory animal committee (DEC) (see Materials and Methods). Of the nine animals left after day 16, those were terminated that developed clinical scores of 3 and higher; thus, five animals were left at the end of the experiment at day 23.

RESULTS

Development of Clinical Signs of EAE

Immunization of 8-week-old C57BL/6 mice with MOG peptide resulted in the development of clinical signs of EAE (Fig. 1). First signs were detectable around 11 days after the first immunization with a progressive development reaching clinical score 2–3 after approximately 20 days (Fig. 1); 12 of 14 mice (85%) displayed clinical signs during the observation period, whereas none of the 6 controls that received injections of the emulsion without MOG peptide developed clinical signs of EAE (Fig. 1).

Induction of L-CCR mRNA Expression

To investigate the expression of L-CCR mRNA, RT-PCR analysis was performed. No L-CCR mRNA was detected in the spinal cords of control animals (n = 3) or animals without clinical signs (n = 2) (Fig. 2A), but L-CCR mRNA expression was present in the spinal cords from animals that showed clinical signs of EAE (Fig. 2A). Interestingly, the mRNA expression level of L-CCR in spinal cord was already high at the onset of EAE (n = 3) (score1) and did not further increase with disease progression (n = 6) (Fig. 2A). Similar results were obtained in brain. L-CCR mRNA expression was barely detectable in control animals (n = 3) and in animals without clinical signs (n = 2) (Fig. 2A). Higher levels of L-CCR mRNA expression were observed after the onset of clinical signs of EAE (Fig. 2A). Additional Q-PCR experiments corroborated these data. Compared to control animals, approximately 13 and 15 times higher levels of L-CCR mRNA were detected in the spinal cord of animals showing clinical scores up to 1.5 or 3.5, respectively (Fig. 2B). The L-CCR mRNA expression in spinal cords from control animals (= 100%) did not differ from the L-CCR mRNA expression level in MOG injected animals without clinical scores (92%).

L-CCR mRNA Is Induced Throughout Spinal Cord and Brain

In situ hybridization studies also showed induction of L-CCR mRNA in spinal cord and brain of animals displaying clinical signs of EAE. Control hybridization in spinal cord tissue using the sense probe yielded unspecific staining (Fig. 3A) and was indistinguishable from the signal obtained with the anti-sense probe in control animals (n = 4) (Fig. 3B). Numerous L-CCR-positive cells were found in spinal cord of EAE animals at score 1 (Fig. 3C) (n = 3) or higher (n = 4) (data not shown). These positive cells were almost equally distributed throughout the spinal cord. Higher magnification showed typically cytoplasmatic localization of the L-CCR in situ signal (exemplified by arrowheads) (Fig. 3D).

Similar results were obtained in brain tissue. The signal obtained from control hybridization with the sense probe (data not shown) was indistinguishable from the in situ hybridization signal in control animals (n = 3) that only revealed unspecific staining (Fig. 3E). The development of clinical EAE signs was accompanied by the presence of numerous L-CCR mRNA-positive cells in most areas of the brain, exemplified for the cortex from an animal with clinical score 1 (n = 3) (Fig. 3F). Similar expression was also detected in animals with higher clinical scores (n = 4) and/or other areas of the forebrain like striatum or hippocampus (data not shown). Higher magnification showed the cytoplasmic expression of L-CCR mRNA (exemplified by arrowheads) (Fig. 3H) and corroborated the induction of L-CCR mRNA compared to control animals (Fig. 3G).

L-CCR mRNA Is Expressed Predominantly in Astrocytes and Microglia

To identify L-CCR-expressing cells in spinal cord and brain, in situ hybridization was combined with immunohistochemistry in score 1 (n = 2) and score 2–3 animals (n = 2). Combinations of in situ hybridization (purple reaction product) (Fig. 4) and immunohistochemistry (brown reaction product) (Fig. 4) showed that numerous GFAP-positive astrocytes express L-CCR mRNA in mouse cortex (see arrowheads in Fig. 4A). Combinations of immunohistochemistry and in

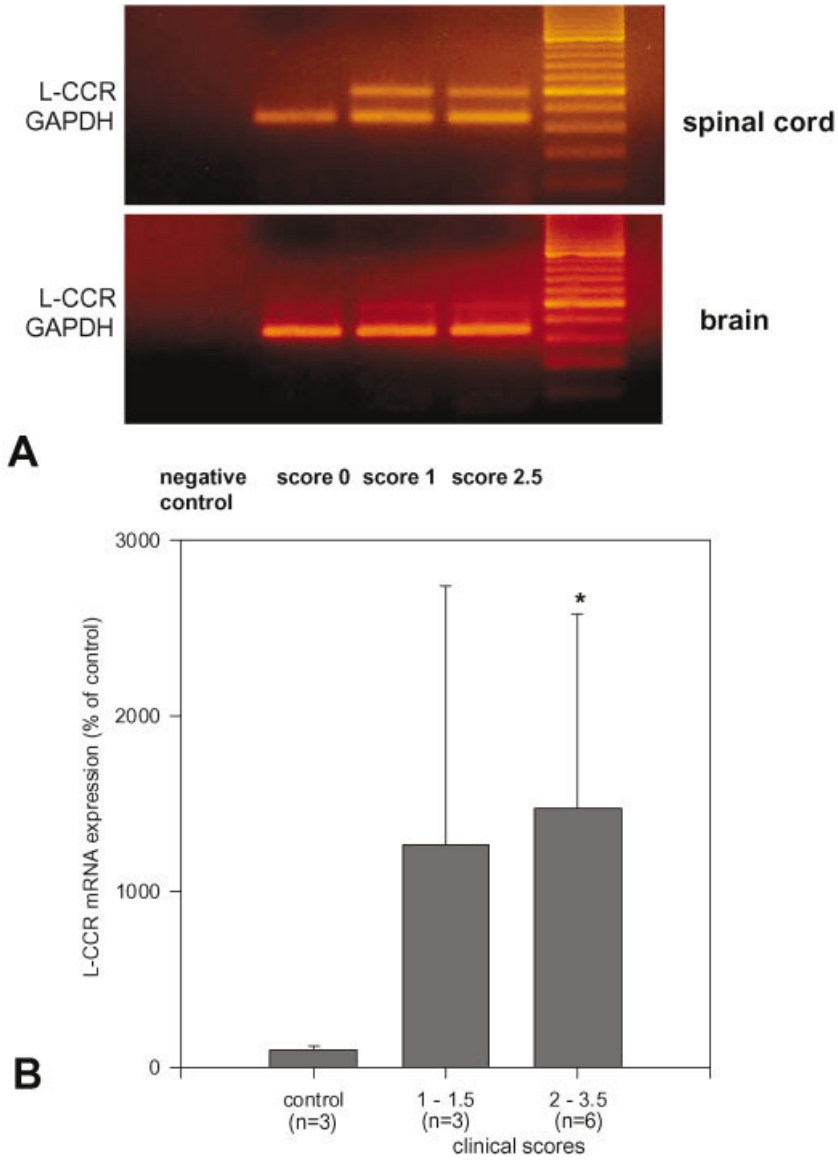

Fig. 2. Effect of experimental autoimmune encephalomyelitis (EAE) on L-CCR mRNA expression in mouse spinal cord and brain. **A:** Reverse transcription-polymerase chain reaction (RT-PCR) experiments revealed that L-CCR mRNA expression in mouse spinal cord (upper panel) and brain (lower panel) was accompanied by the development of clinical signs of EAE. L-CCR mRNA expression was not detected in tissue from control mice but was found in both spinal cord and brain of animals with signs of EAE. The number of cycles for GAPDH and L-CCR were 28 and 31 in spinal cord and 28 and 35 in brain tissue, respectively. MM, molecular weight marker, highlighted band is 500 bp. Both PCR products were run in the same gel. Negative control: PCR control without template. **B:** Q-PCR experiments showed 14–18 times more L-CCR mRNA in spinal cord samples from animals with clinical signs of EAE compared to control animals. *Significantly different from control value, $P \leq 0.05$, Student's t-test.

situ hybridization using the sense probe confirmed the specificity of the in situ signal in these double-labeling experiments. This has been depicted in Figure 4B, where only GFAP antibody staining is visible (brown reaction product). However, cells expressing L-CCR mRNA without GFAP staining were also detected (Fig. 4A, arrows). These cells might be astrocytes that express low amounts of GFAP or could be other cells that express L-CCR mRNA. Accordingly, more markers were used in double-labeling experiments. Using neuronal markers, it was found that the L-CCR in situ hybridization signal (purple reaction product) (see arrows) did not co-localize with MAP2-positive neurons (brown reaction product) (arrowheads) (Fig. 4C). When using the L-CCR sense probe only MAP2-positive cells were found, showing the specificity of the in situ hybridization signal (Fig. 4D). Thus, experiments with the neuronal marker MAP2 clearly showed that L-CCR mRNA is not neuronally expressed. Interestingly, L-CCR mRNA-positive cells were often found in intimate contact with MAP2-positive neurons (dashed arrows) (Fig. 4C). Similar results were obtained in animals with clinical scores 2–3 (data not shown).

For technical reasons, it was not possible to perform double-labeling experiments combining microglial markers like F4/80 with L-CCR in situ hybridization. We therefore performed L-CCR in situ hybridization analysis and F4/80 immunohistochemistry in serial sections. F4/80-staining was induced in animals showing clinical scores of EAE compared to control animals indicating the activation of microglia (data not shown). The comparison of serial sections indicated that various F4/80-positive cells in the spinal cord of score 3 animals (n = 3) (Fig. 5A) also showed L-CCR mRNA expression (Fig. 5B), which is depicted in the overlay (Fig. 5C) (compare the arrows for positive cells in Fig. 5A–C). Comparable results have been obtained

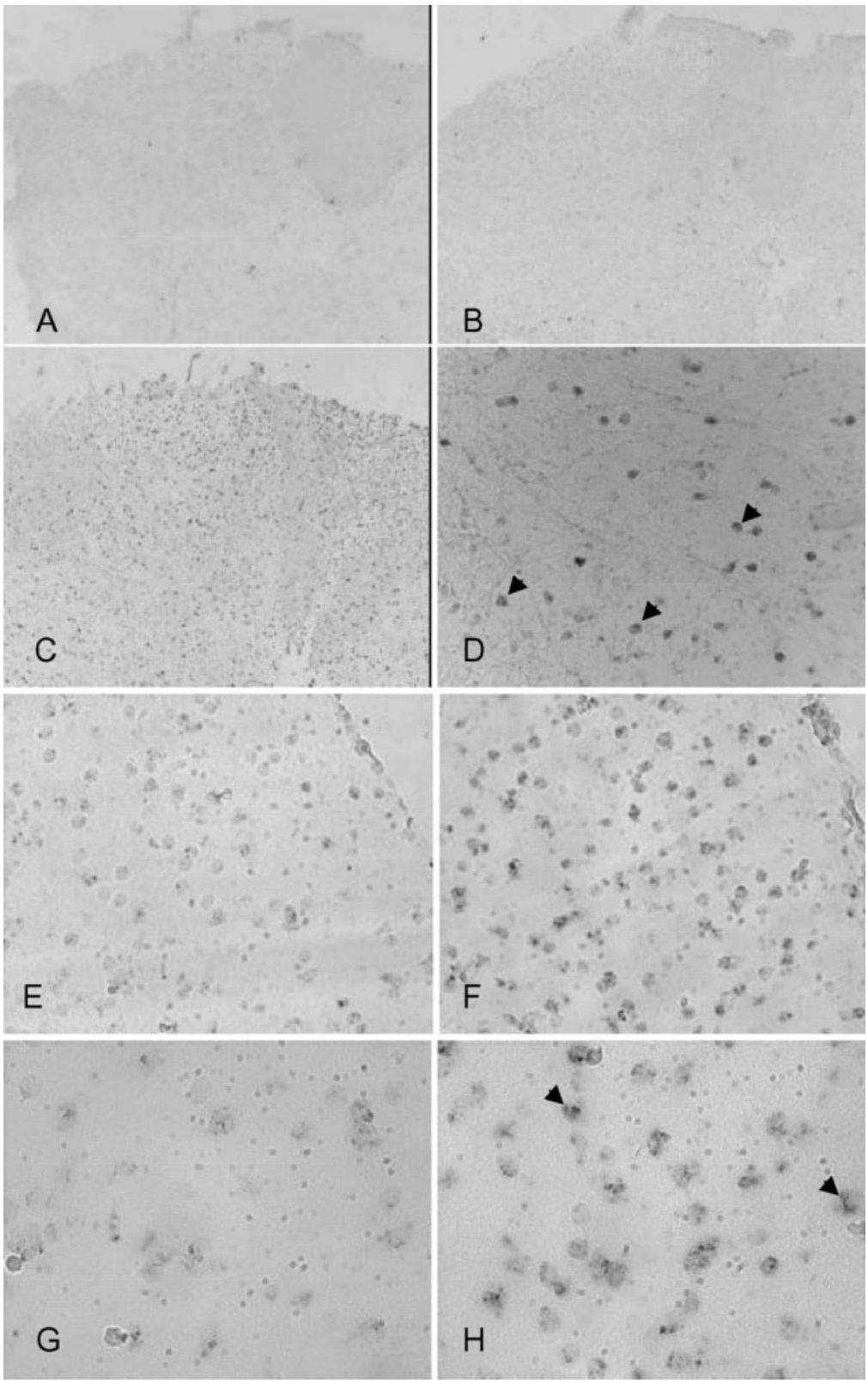

Fig. 3. L-CCR mRNA in situ hybridization in spinal cord and brain of mice with and without clinical signs of experimental autoimmune encephalomyelitis (EAE). **A:** In situ hybridization using L-CCR sense probe did not reveal any staining indicating the specificity of the observed signal. **B:** No in situ hybridization signal was observed in spinal cord sections of control mice. **C:** Numerous L-CCR-positive cells were found throughout the spinal cord of mice with clinical score 1. **D:** Higher magnification revealed the typically cytoplasmic pattern of the in situ hybridization signal (arrowheads). **E:** Lack of L-CCR mRNA expression in cortex of control mice. **F:** Numerous L-CCR mRNA-positive cells in the cortex of mice with clinical score 1. Higher magnification revealed the cytoplasmic expression of the L-CCR in situ hybridization signal in score1 animals (**H**) in clear contrast to the absence of any specific signal in control mice (**G**). ×25 in A–C; ×200 in D–F; ×400 in G,H.

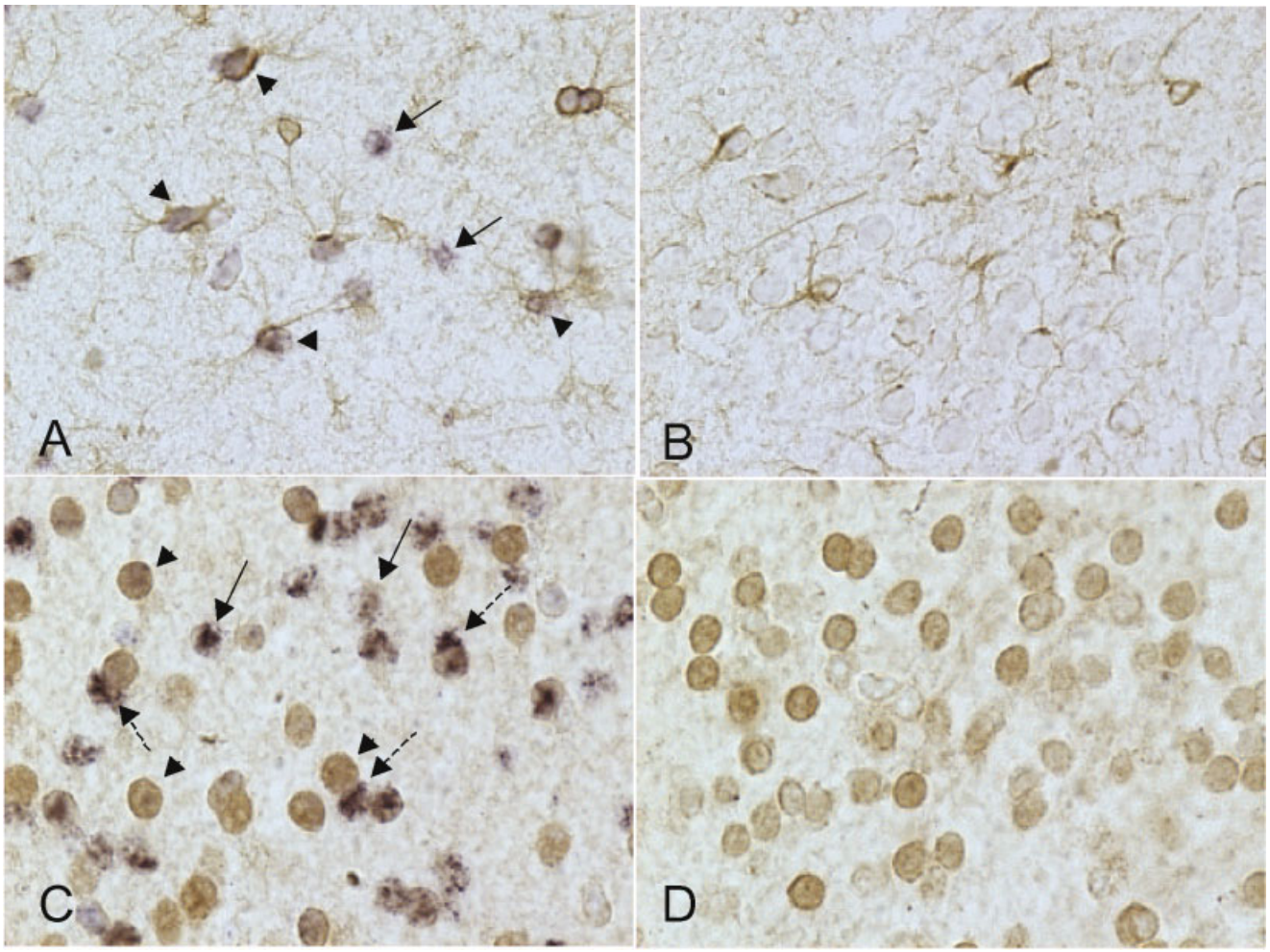

Fig. 4. L-CCR mRNA in situ hybridization in the cortex of score 1 animals and identification of astrocytes as L-CCR mRNA-expressing cells. **A:** L-CCR mRNA-positive cells (purple reaction product) were identified as astrocytes by glial fibrillary acidic protein (GFAP) staining (brown reaction product, exemplified by arrowhead). **B:** Control hybridization using the sense probe revealed the specificity of the in situ signal, here only the GFAP staining was found. **C:** MAP2-positive neurons (brown reaction product, exemplified by arrowheads) did not co-localize with staining for L-CCR mRNA (purple reaction product, exemplified by arrows). However, L-CCR-positive cells were often detected in intimate spatial relationship with MAP2-positive neurons (dashed arrows). **D:** Control hybridization using the sense probe revealed the specificity of the in situ signal, here only the MAP2 staining was found. Similar results were obtained in animals with clinical scores 2–3 (n = 2). ×400.

throughout the spinal cord and in the brain of animals showing clinical EAE scores (data not shown). Infiltrating F4/80-positive macrophages were found in particular in lumbar spinal cord of EAE mice (score 3, n = 3) (Fig. 5D). These cells were identified by their different morphology (compact round cells, exemplified by arrows) compared to F4/80-positive microglia showing a more ramified morphology (Fig. 5D). L-CCR in situ hybridization in serial sections (Fig. 5E) showed that several F4/80-positive macrophages also expressed L-CCR mRNA as depicted in the overlay (Fig. 5F) (compare the arrows in Fig. 5D–F).

Peripheral lymphocytes in the parenchyma of spinal cord were only detectable in animals with clinical score 3 (n = 3). Small infiltrates were observed after cresyl violet staining (arrows) (Fig. 6B). In animals with score 1 very few lymphocytes were detected in the meninges (n = 3) (arrow) (Fig. 6A) and were absent in spinal cord from control animals (n = 4) (data not shown). Antibody staining identified these cells in spinal cord as CD3-positive T cells. CD3-positive cells (arrows) were found in the meninges and parenchyma of score 1 animals (Fig. 6C) and score 3 animals (Fig. 6D), respectively. L-CCR in situ hybridization has been performed in serial sections (Fig. 6E). None of the numerous L-CCR mRNA-positive cells was found to co-localize with CD3-postive cells (Fig. 6D; overlay of Fig. 6D and E) as indicated by the arrows pointing to the place of CD3-positive cells.

DISCUSSION

The orchestration of leukocyte homing and the control of infiltration during inflammation is the major function of the chemokinergic system (Zlotnik and Yo-

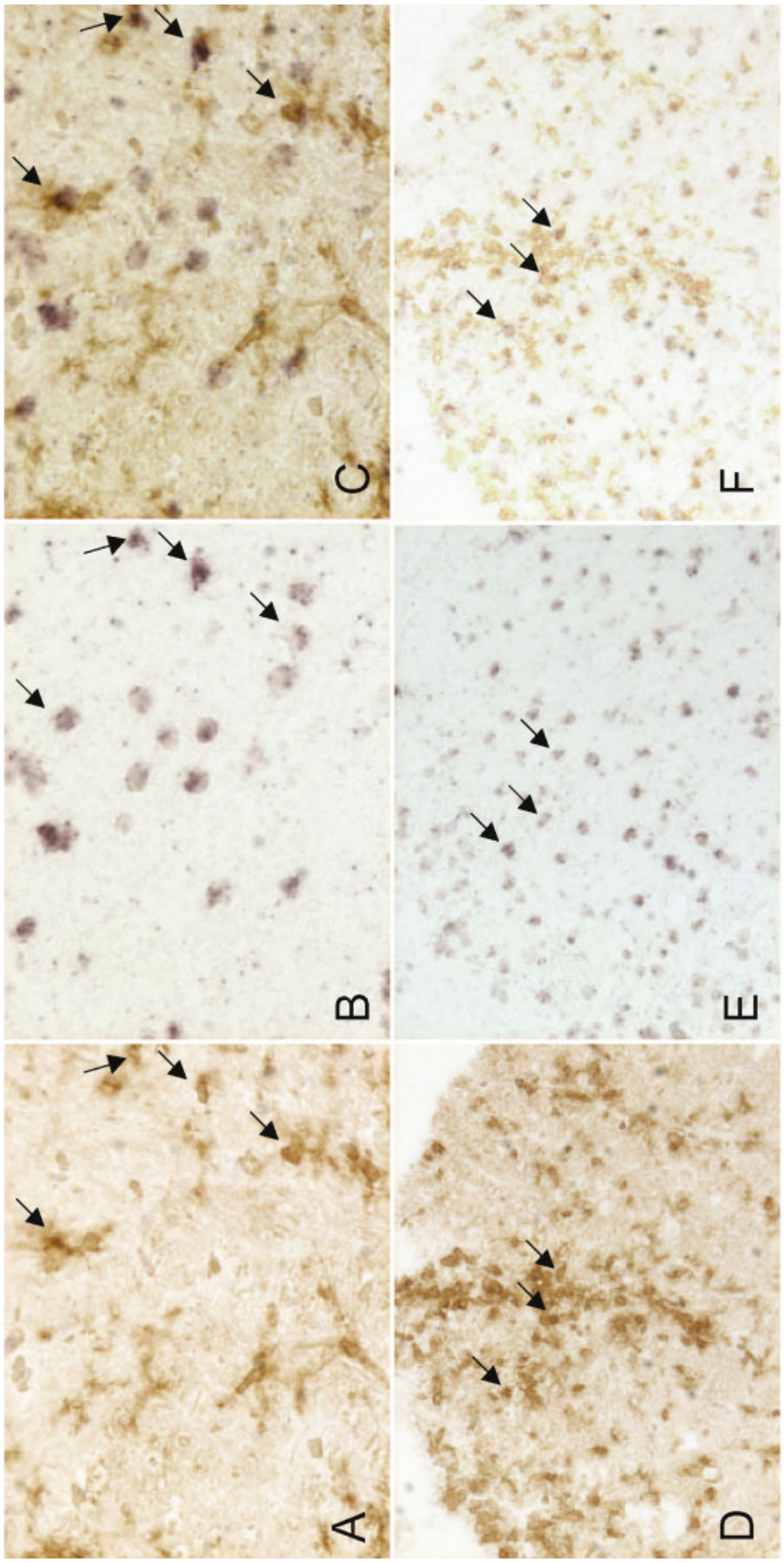

Fig. 5. Identification of L-CCR mRNA-expressing cells as microglia and macrophages in spinal cord of score 3 animals. **A:** F4/80-positive microglia were observed in spinal cord of score 3 animals (brown reaction product) exemplified by the arrows. **B:** L-CCR in situ hybridization (purple reaction product) in serial sections revealed numerous L-CCR mRNA-expressing cells some of which in similar places as the F4/80-positive microglia (exemplified by arrows). **C:** Electronic overlay of A and B showing that some F4/80-positive microglia express L-CCR mRNA (arrows). **D:** F4/80-positive macrophages (small round cells) in lumbar spinal cord of score 3 animals (brown reaction product) exemplified by the arrows. **E:** L-CCR in situ hybridization in serial sections revealed numerous L-CCR mRNA-expressing cells (purple reaction product) some of which in similar places as the F4/80-positive macrophages (exemplified by arrows). **F:** Electronic overlay of D and E showing that some F4/80-positive macrophages express L-CCR mRNA (arrows). ×400 in A–C; × 200 in D–F.

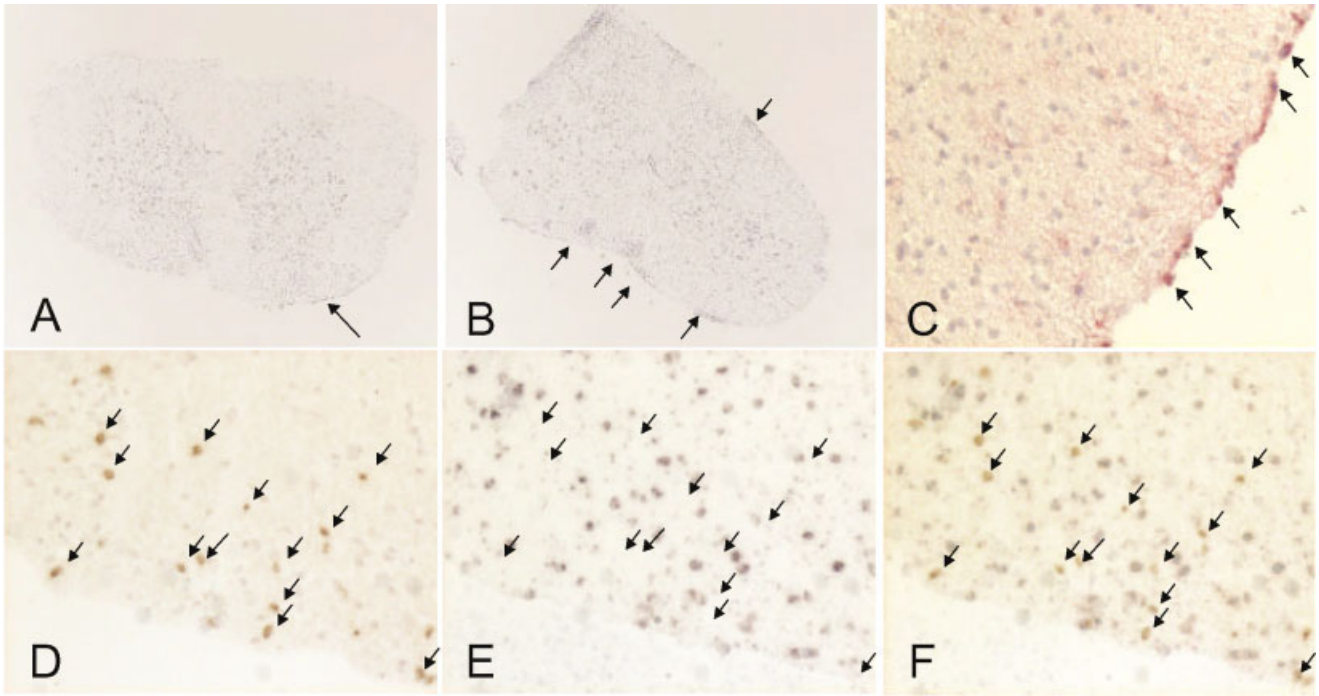

Fig. 6. Cresyl violet staining revealed subpial lymphocyte accumulation (**A**) and infiltration (**B**) in spinal cord of score 1 animals and score 3 animals, respectively. A population of these cells has been identified as T cells by CD3 staining (brown reaction product, arrows) in score 1 (**C**) and score 3 (**D**) animals. **E:** L-CCR in situ hybridization in serial sections revealed numerous L-CCR mRNA-positive cells. Comparing the positions of CD3-positive cells (indicated by the arrows) with L-CCR mRNA-positive cells revealed no overlap in the staining. **F:** Electronic overlay of D and E showed no co-localization of CD3 staining (brown reaction product) and L-CCR in situ hybridization signal (purple reaction product). ×12.5 in A,B; ×200 in C–F.

shie, 2000; Mackay, 2001; Gerard and Rollins, 2001). Since infiltrating leukocytes is of particular importance in the pathology of MS and EAE, the role of chemokines in these diseases has been investigated (Karpus and Ransohoff, 1998; Ransohoff, 1999; Sorensen et al., 1999, 2001, Boven et al., 2000; Trebst et al., 2001; de Groot and Woodroofe, 2001). Several attempts to inhibit leukocyte infiltration by blocking chemokine-chemokine receptor interactions have been made. Development of EAE is attenuated or blocked in chemokine-deficient mice and also after injection of chemokine blocking antibodies, suggesting that chemokines are crucial mediators of infiltration in CNS inflammation (Siebert et al., 2000; Izikson et al., 2000; Fife et al., 2000; Huang et al., 2001; Kivisakk et al., 2002). Accordingly, suppression of chemokine signaling in the CNS has been discussed recently as a therapy for MS (Ransohoff and Bacon, 2000; Kivisakk et al., 2001).

CCL2 is one of the most prominent chemokines in brain pathology and is strongly expressed in tissue affected by MS and EAE pathology. The importance of CCL2 for EAE has been demonstrated recently, thus mice deficient for CCL2 showed slower development of clinical signs of EAE, which were much milder than those in wild type controls (Huang et al., 2001). CCR2, the major chemokine receptor for CCL2, is prominently expressed in monocytes/macrophages. Macrophage infiltration is crucial for the onset of EAE and several reports indicate that mice deficient for CCR2 do not develop clinical signs of EAE, emphasizing the importance of CCL2-CCR2 signaling for the onset of EAE (Izikson et al., 2000; Fife et al., 2000). However, several lines of evidence suggest the existence of an alternative CCL2 receptor (Heesen et al., 1996; Rollins and Gerard, 2001; MacLean et al., 2000; Muessel et al., 2002). Furthermore, the importance of CCR2 in EAE has recently been challenged in a study showing that various CCR2-deficient mouse strains still develop EAE (Gaupp et al., 2003).

We have provided evidence for the expression of the orphan chemokine receptor L-CCR in glial cells and have shown that CCL2 is (among others) a potential chemokine ligand for this receptor (Zuurman et al., 2003; Biber et al., 2003). Since glial expression of L-CCR in vitro and in vivo is induced by pro-inflammatory conditions we have investigated its expression in EAE. Expression of L-CCR mRNA in unaffected spinal cord or brain is low. However, during the development of clinical symptoms of EAE expression of L-CCR is strongly induced. The results obtained with PCR techniques indicate that L-CCR mRNA is expressed more prominently in spinal cord. In contrast, in situ hybridization experiments showed robust L-CCR mRNA expression both in spinal cord and brain. The discrepancy between PCR and in situ hybridization in L-CCR mRNA expression levels observed in spinal cord and brain is most likely explained by a stronger dilution of glial RNA in the total RNA pool from brain material.

Interestingly, L-CCR mRNA expression was rapidly induced and accompanied early clinical signs of EAE. Thus, in mice with mild clinical scores (around 1) L-CCR mRNA expression levels were found at a degree similar to mice that had higher clinical scores. Thus, the onset of disease, but not the severity of clinical signs, correlated with L-CCR mRNA expression.

L-CCR mRNA expression was predominantly found in astrocytes throughout spinal cord and brain tissue and clearly no L-CCR mRNA expression was detected in neurons. Since it was not possible to combine in situ hybridization of L-CCR and immunohistochemistry with markers for microglia, macrophages, and T cells, immunohistochemical analysis in serial sections was performed. This more indirect technique revealed L-CCR mRNA expression in microglia, as well as in infiltrating macrophages. These results are corroborated by previous findings that L-CCR mRNA is expressed in cultured microglia (Zuurman et al., 2003) and in macrophages that infiltrate inflamed lung tissue (Oostendorp et al., in press). No co-localization between CD3-positive T cells and L-CCR mRNA-expressing cells was observable in serial sections, indicating that L-CCR mRNA is not expressed in T cells that infiltrate nervous tissue during EAE.

The rapid onset of L-CCR mRNA expression and its equal distribution throughout spinal cord and brain may suggest that an early and local neuroinflammatory signal triggers the expression of L-CCR in glial cells. Moreover, since EAE is a "white matter disease," it is remarkable that L-CCR mRNA expression was detected in gray matter structures like the cortex. Thus L-CCR mRNA expression was also induced in parts of the CNS that are not affected by EAE, indicating that L-CCR expression is plausible related to the overall reaction of the CNS to an inflammatory insult. The function of L-CCR expression in glial cells in currently unknown. It is known, however, that glial cells express various chemokine receptors and that glial cells significantly contribute to the pathology of neurodegenerative diseases (Dorf et al., 2000; Becher et al., 2000). Therefore, it has been proposed that glial chemokines and chemokine receptors are crucial elements for endogenous immune response in the CNS (Hesselgesser and Horuk, 1999; Biber et al., 2002; Bajetto et al., 2002). The early induction of L-CCR mRNA expression in EAE and our previous findings that L-CCR expression in brain is induced by lipopolysaccharide (LPS) (Zuurman et al., 2003) suggest that L-CCR is of pathophysiological relevance for CNS inflammation. This assumption, however, should be further investigated in L-CCR-deficient mice.

In summary, we have provided evidence for rapid and pronounced induction of L-CCR mRNA expression in CNS glial cells in EAE. The early overall expression of this chemokine receptor was not correlated to disease severity or places of leukocyte infiltration, indicating a local function of L-CCR in the endogenous immune response of the brain.

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