GLIA 41:327–336 (2003)

LPS-Induced Expression of a Novel Chemokine Receptor (L-CCR) in Mouse Glial Cells In Vitro and In Vivo

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KEY WORDS neuroimmunology; astrocytes; microglia; chemotaxis; chemokines

ABSTRACT There is increasing evidence that chemokines, specialized regulators of the peripheral immune system, are also involved in the physiology and pathology of the CNS. It is known that glial cells (astrocytes and microglia) express various chemokine receptors like CCR1, -3, -5, and CXCR4. We have investigated the possible expression of the known CC chemokine receptors (CCR1–8 and D6) in murine glial cells. In addition, we examined possible glial expression of the orphan CC chemokine receptor L-CCR that has been identified previously in murine macrophages. We report here expression of L-CCR mRNA in murine astrocytes and microglia. Furthermore, L-CCR mRNA expression was strongly induced after application of bacterial lipopolysaccharide (LPS), both in vitro and in vivo. Functional studies and binding experiments using biotinylated monocyte chemoattractant protein (MCP)-1 (CCL2) indicate that CCL2 could be a candidate chemokine ligand for glial L-CCR. Based on the data presented, it is suggested that L-CCR is a functional glial chemokine receptor that is important in neuroimmunology. *GLIA 41:327–336, 2003.*

INTRODUCTION

Chemokines are small chemotactic cytokines of approximately 10 kDa, which orchestrate the inflammatory response by attracting leukocytes to sites of inflammation and by controlling the homing of lymphocytes (Rollins, 1997; Baggiolini, 1998; Murphy et al., 2000). Chemokines and their receptors, all of which are G-protein–coupled, are subdivided into four families: CXC, CC, C, and CX3C chemokines (Murphy et al., 2000). Chemokine signaling is often nonspecific; most chemokines activate more than one chemokine receptor and vice versa (Devalaraja and Richmond, 1999; Mantovani, 1999). In humans, more than 25 CC chemokines and 10 CC chemokine receptors (CCR) have been cloned (Murphy et al., 2000).

Chemokines and their receptors are not only present in the peripheral immune system. It has recently become clear that chemokines are also expressed in brain during development and pathology (Asensio and Campbell, 1999; Asensio et al., 1999; Glabinski and Ransohoff, 1999; Oh et al., 1999; Biber et al., 2002). The cellular location of chemokine receptors in the central nervous system is still a subject of investigation, but it is clear that all types of endogenous brain cells express chemokine receptors. Concerning the CC chemokine receptor family in glial cells, specific expression of the CC chemokine receptors CCR1, CCR3, and CCR5 has been reported in microglia (Spleiss et al., 1998; Boddeke et al., 1999; Inoue et al., 1999; Xia et al., 1999). In addition, a number of reports have described CC chemokine receptor expression in astrocytes. Specifically, CCR1 and CCR5 expression was shown in astrocytes (Andjelkovic et al., 1999; Mennicken et al., 1999; Dorf et al., 2000). The appearance of contradictory reports makes the expression of CCR2 in glial cells a controversial subject (Heesen et al., 1996; Andjelkovich et al., 1999). We therefore investigated the mRNA

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Received 13 December 2001; Accepted 25 July 2002
DOI 10.1002/glia.10156

expression profile of CCR1–8 and D6 in cultured mouse astrocytes and microglia using RT-PCR. In addition to expression profiling of established chemokine receptors, various orphan chemokine receptors may be present in glial cells. The presence of three orphan chemokine receptors has already been described in astrocytes (Heesen et al., 1998; Sabri et al., 1999; Dorf et al., 2000). Currently, little is known about the expression of orphan chemokine receptors in microglia. The orphan chemokine receptor lipopolysaccharide (LPS)-inducible CC chemokine receptor (L-CCR) was first characterized in the mouse macrophage cell line RAW264.7 by Shimada et al. (1998). Since brain microglia most likely are bone marrow-derived cells that show many macrophage-like properties, we investigated possible expression of L-CCR in microglia. RT-PCR and in situ hybridization studies indeed revealed L-CCR mRNA expression in cultured microglia. Surprisingly, L-CCR mRNA was also detected in mouse astrocytes. Furthermore, similar to the previously described results that Shimada et al. (1998) obtained with the RAW264.7 cell line, the expression of L-CCR mRNA in both glial cell types is strongly enhanced by in vitro and in vivo stimulation with LPS. Binding studies and functional assays indicate that glial L-CCR might be activated by CCL2, formerly known as monocyte chemoattractant protein (MCP)-1. Thus, we present here a novel CC chemokine receptor expressed in astrocytes and microglia; its expression is greatly enhanced under proinflammatory conditions. We propose that this receptor may play a role in neuroinflammatory processes.

MATERIALS AND METHODS

Chemicals

The materials used were as follows: isoflurane (Forene) from Abbott (Baar, Switzerland); Dulbeccos modified Eagle medium (DMEM) from Gibco-BRL Life Technologies (Breda, The Netherlands); Taq-polymerase from InViTek (Berlin, Germany); TA vectors pCR2.1 and pCRII from Invitrogen (Leek, The Netherlands); digoxigenin-conjugated UTP and alkaline phosphatase-conjugated sheep antidigoxigenin from Boehringer Mannheim (Mannheim, Germany); recombinant mouse chemokines from Pepro Tech (London, U.K.); biotin rmJE (CCL2) Fluorokine Kit from R&D systems (Minneapolis, MN); antibodies for GFAP, ED-1, and MAC-1 from Chemicon (Temencula, CA). Fura-2 AM and all other chemicals were from Sigma-Aldrich (Bornhem, Belgium).

Injection of LPS

For treatment with endotoxin, 5-week-old CD-1 mice were injected intraperitoneally with LPS (50 μg/25 g weight) dissolved in sterile saline solution. Control animals received injections with 0.9% NaCl. At different time points after the injection, animals were decapitated under isoflurane anesthesia (five animals per time point, three for RNA preparation and two for in situ hybridization) and brains were removed. Brains were lysed in GTC solution for RNA preparation and fixated with Zamboni's fixative by perfusion fixation for in situ hybridization experiments.

Mixed Astrocyte Cell Cultures and Cultured Microglia

Mixed astrocyte cell cultures were established as described previously (Biber et al., 1997). In brief, mouse cortex was dissected from newborn mouse pups (< 1 day). Brain tissue was gently dissociated by trituration in phosphate-buffered saline and filtered through a cell strainer (70 μm Ø, Falcon) in DMEM. After two washing steps (200 g for 10 min), cells were seeded in culture dishes (10 cm Ø; 8×10^6 cells/dish). Cultures were maintained 6 weeks in DMEM containing 10% fetal calf serum with 0,01% penicillin and 0,01% streptomycin in a humidified atmosphere (5% CO_2) at 37°C. These cultures contained 70%–75% astrocytes (GFAP-positive cells) and 20%–30% microglia (F4/80-positive cells); 1%–5% of the cells did not stain for either GFAP or F4/80 and were most likely endothelial cells and/or fibroblasts. Culture medium was changed the second day after preparation and every 6 days thereafter. Microglia cultures were established as described previously (Biber et al., 1997). In brief, floating microglia were harvested from confluent mixed glial cultures and plated on new culture dishes. Microglia cultures were pure (> 95%) as tested by cell-specific markers (ED-1, MAC-1, and F4/80). For chemotaxis assays, cultured microglia were left in suspension.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Cells and brain tissue were lysed in guanidinium isothiocyanate/mercaptoethanol (GTC) buffer and total RNA was extracted with slight modifications according to Chomczynski and Sacchi (1987).

For 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 (35 cycles) without reverse transcriptase and using GAPDH primers in subsequent PCR amplifications. Only RNA samples that showed no bands after that procedure were used for further investigation.

For PCR, 2 μl of the RT reaction was used in subsequent PCR amplification as described (Biber et al., 1997). Table 1 lists the primer sequences, cycle numbers, and annealing temperature. Cloning into PCR2.1 (Invitrogen) and subsequent sequencing checked identity of all PCR products.

TABLE 1. Primer sequences for mouse CCRs

Gene	Primer sequences (5′–3′)	PCR product (bp)
CCR1	GTGGTGGGCAATGTCCTAGT TCAGATTGTAGGGGGTCCAG	658
CCR2	GTATCCAAGAGCTTGATGAAGGG GTGTAATGGTGATCATCTTGTTTGGA	532
CCR3	GCACCACCCTGTGAAAAAGT CGAGGACTGCAGGAAAACTC	521
CCR4	AGGCAAGGACCCTGACCTAT GGACTGCGTGTAAGAGGAGC	644
CCR5	ATTCTCCACACCCTGTTTCG TCAGGCTTGTCTTGCTGGAA	350
CCR6	GTGGTGATGACCTTTGCCTT AGGAGGACCATGTTGTGAGG	656
CCR7	AACGGGCTGGTGATACTGAC ATGAAGACTACCACCACGGC	596
CCR8	TTCCTGCCTCGATGGATTAC GCTTCCACCTCAAAGACTGC	591
D6	TCTTCATCACCTGCATGAGC TATGGGAACCACAGCATGAA	400
L-CCR	CTGGCGGTGTTTATCTTGGT AACCAGCAGAGGAAAAGCAA	489
GAPDH	CATCCTGCACCACCAACTGCTTAG GCCTGCTTCACCACCTTCTTGATG	346

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry and in situ hybridization were carried out as described (Copray and Brouwer, 1994). In brief, prior to immunohistochemical processing and between the incubation steps, the sections were washed in 0.9% saline dissolved in 0.05 M Tris, pH 7.4 (TBS). All antisera were diluted in TBS containing 0.3% Triton X-100, 1% bovine serum albumin (BSA), and heparin (5 mg/ml). Sections were preincubated in 5% BSA in TBS for 30 min and incubated overnight with GFAP and ED-1. Antibody-antigen reactions were detected using the biotin-streptavidin method and the complex was visualized with diaminobenzidine (DAB)/H_2O_2. In case of fluorescence detection, FITC-conjugated streptavidin was used to visualize the antibody-antigen complex. For in situ hybridization, L-CCR PCR product was cloned into the dual promoter PCR II vector and linearized. L-CCR sense and antisense probes were synthesized by runoff transcription and the use of digoxigenin-conjugated UTP according to the manufacture's protocol (Boehringer Mannheim). Slides were rinsed in PBS and digested with 10 μg/ml proteinase K for 0.5 h at 37°C. Subsequently, sections were rinsed in 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na citrate), dehydrated in an ethanol series, and dried.

Sections were hybridized overnight at 60°C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, 1 × Denhardt's solution, and 10% dextran sulfate. Final probe concentrations in hybridization buffer were 1–5 ng/μl. After hybridization, the sections were treated with 10 μg/ml of ribonuclease A for 0.5 h at 37°C and washed in 0.1 × SSC at 65°C.

The immunological detection of the digoxigenin-labeled RNA-RNA complex was preceded by a 0.5-h preincubation at room temperature in 0.1 M Tris, 0.15 M NaCl, pH 7.5 (buffer 1), containing 5% BSA. Slides were incubated for 2 h at room temperature with the alkaline phosphatase-conjugated sheep antidigoxigenin, diluted 1:500 in buffer 1, containing 2% BSA. After thorough rinsing in buffer 1 and a 10-min preincubation in an alkaline buffer solution (ABS: 0.1 M Tris, 0.1 M NaCl. O.05 M $MgCl_2 \cdot 6H_2O$, pH 9.5), the alkaline phosphatase was revealed with a freshly prepared solution of 0.34 mg/ml nitroblue tetrazoleum and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in ABS. Endogenous nonintestinal phosphatase activity was inhibited by the addition of levamisole (0.24 mg/ml) to the staining solution. The color development was done overnight and terminated by placing the slides in a buffer solution, consisting of 0.01 M Tris, 1 mM EDTA, pH 8.5. The dark-purple precipitate indicating the presence of hybridized mRNA was revealed with bright-field microscopy. Control experiments included hybridization with digoxigenin-labeled sense probes and hybridization after treatment of the sections with RNAse.

Chemotaxis Assays

Cell migration in response to chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe). Chemokine stock solutions were prepared in PBS and further diluted in medium for use in the assay. In some cases, microglia were incubated for 16 h with LPS (100 ng/ml) at 37°C/5% CO_2 and harvested for the assay as described. Culture medium without chemokines served as a control in the assay; 27 μl of the chemoattractant solution or control medium was added to the lower wells; lower and upper well were separated by a polyvinylpyrrolidone-free polycarbonate filter (8 μm pore size) and 50,000 cells per 50 μl were used in the assay. Determinations were done in hexaplicate. The chamber was incubated at 37°C/5% CO_2 in a humidified atmosphere for 120 min. At the end of incubation, the filter was washed, fixed in methanol, and stained with toluidine blue. Migrated cells were counted with a scored eyepiece [three fields (1 mm^2) per well] and migrated cells per chamber were calculated. The data are presented as mean values ± SD and were analyzed by student's *t*-test. *P* values ≤ 0.01 were considered significant.

Determination of Intracellular Calcium

For calcium measurements, microglia were cultured on glass coverslips and calcium measurements were performed as described earlier (Biber et al., 2001). Fluorometric measurements were performed using a sensicam CCD camera supported by Axolab 2.1 imaging software. Digital images of the cells were obtained at an emission wavelength of 510 nm following paired exposures to 340 and 380 nm excitation wavelengths sampled at a frequency of 1 Hz. Fluorescence values representing spatial averages from a defined pixel area

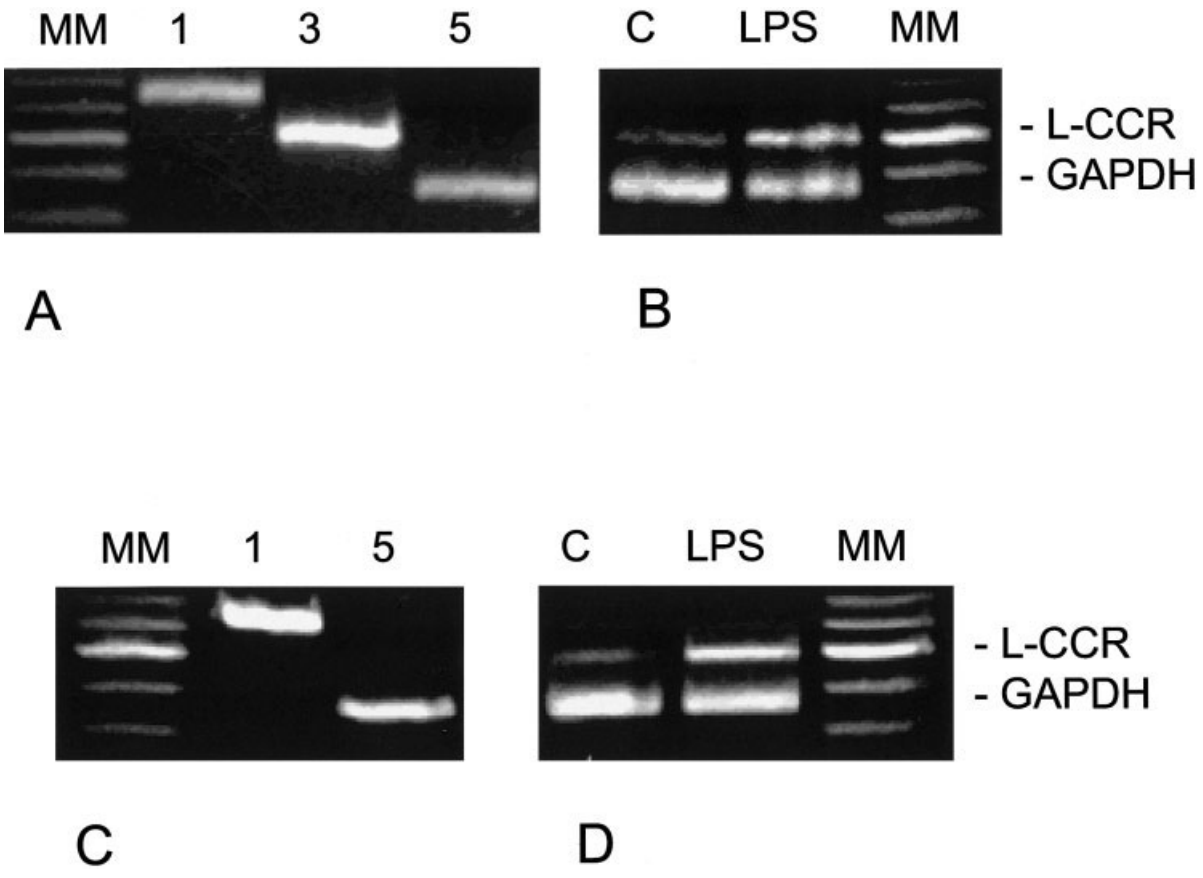

Fig. 1. RT-PCR analysis of chemokine receptor mRNA expression in cultured microglia (**A** and **B**) and cultured astrocytes (**C** and **D**). Experiments were carried out as described in text. A: CCR1, -3, and -5 mRNA was found in cultured microglia. B: Unstimulated microglia (C) did show basal L-CCR mRNA expression, which was upregulated by 2-h stimulation with 100 ng/ml LPS. C: In cultured astrocytes, mRNA expression for CCR1 and -5 was found. D: Control astrocytes (C) did show basal expression of L-CCR mRNA that was upregulated by 2-h stimulation with 100 ng/ml LPS. Number of cycles for GAPDH and L-CCR were 28 and 32, respectively. MM, molecular weight marker; highlighted band is 500 bp. B and D were both PCR products run in the same gel. Similar results were found in three independent experiments.

were recorded online. Increases in intracellular calcium concentrations were expressed as the 340/380 ratio of the emission wavelengths. Compounds were administered using a pipette positioned at a distance of 100–300 μm from the cells. Number of the cells measured: microglia (n = 86).

Binding of Biotinylated CCL2

Determination of binding of biotinylated CCL2 to cultured glial cells was determined according to the manufacturer's instructions. In brief, glial cells (5,000/well) were seeded in 6 mm wells on Teflon-coated object glasses (Cel-Line Associates, Nutacon, Leimuiden, The Netherlands) and cultivated overnight (12–16 h) in the presence or absence of LPS (100 ng/ml). After two washing steps, cells were incubated for 60 min at 4°C in a humidified atmosphere in a total volume of 30 μl with 20 nM biotinylated-recombinant mouse CCL2 (biot.-rmCCL2). This was followed by a 30-min incubation with fluorescein-conjugated avidin in order to detect the bound biot.-rmCCL2. All samples were viewed on a Zeiss Axioskop 2 with a Plan-NEOFLUAR 40 × objective. Fluorescence intensity was quantified using Zeiss KS 300 software. Background fluorescence was measured with biotinylated soybean trypsin (negative control, supplied by the manufacturer). During the measurements, conditions of aperture, pinhole, brightness, contrast, and exposure time were maintained constant. Experiments were performed in triplicate; approximately 50 cells per well were measured.

RESULTS

Expression of CCR mRNA in Cultured Mouse Astrocytes and Microglia

Using RT-PCR analysis (35 cycles), expression of chemokine receptor mRNAs (CCR1–8 and D6) was analyzed in cultured mouse glial cells. Genomic mouse DNA served as a positive control for the primers (CCR1–8 and D6) used.

In cultured mouse, microglia mRNA for CCR1, -3, and -5 was detected (Fig. 1A). No mRNA for CCR2, -4, -6, -7, -8, and D6 was found in these cells under control

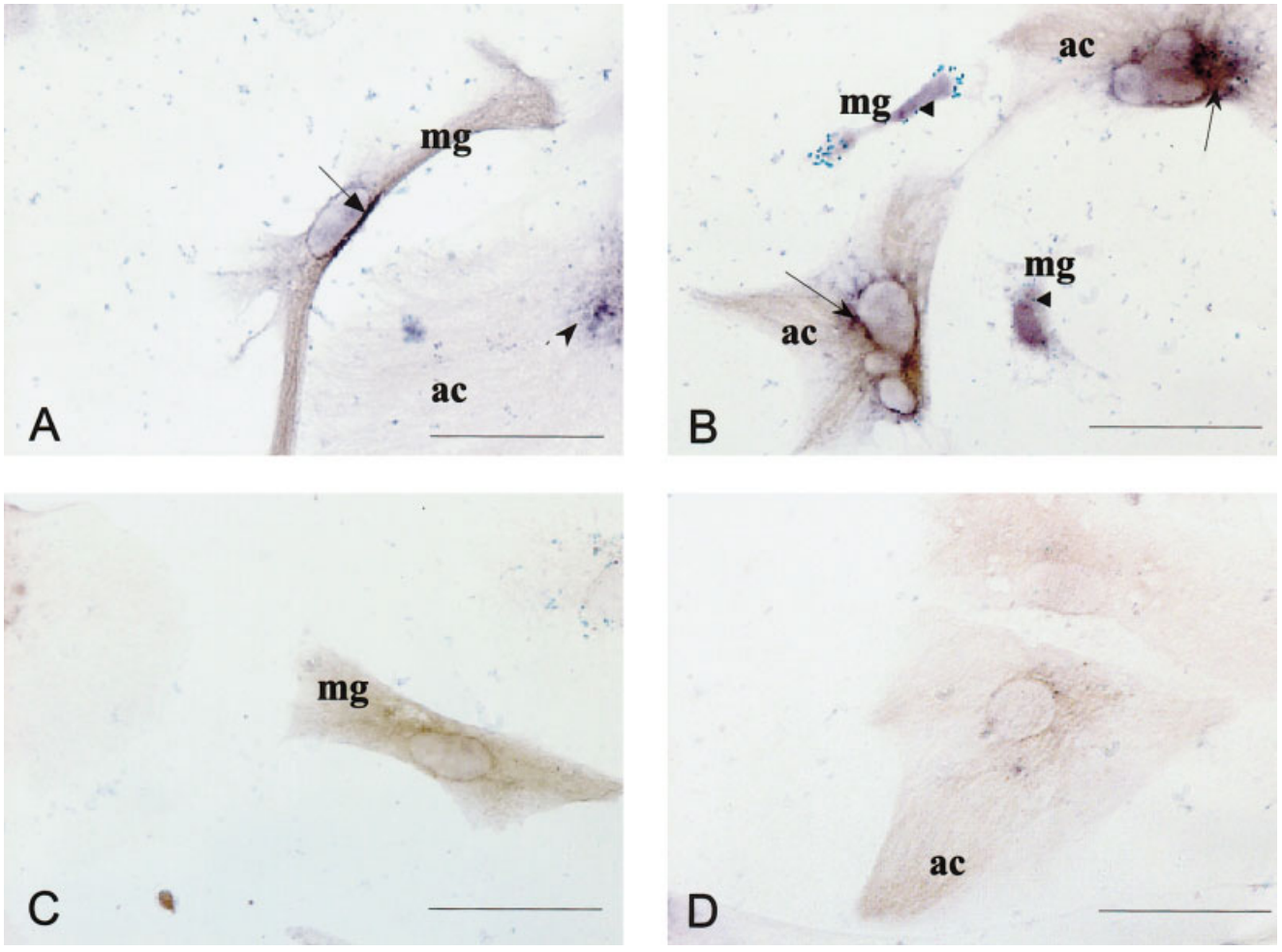

Fig. 2. In situ hybridization in combination with immunocytochemistry shows L-CCR mRNA expression in LPS-stimulated cultured microglia and astrocytes. Cultured glial cells were stimulated for 2 h with LPS (100 ng/ml) and fixed as described in text. **A:** Cells were incubated with ED-1 antibody to stain microglia (mg; brown reaction product). The combination with in situ hybridization (dark-purple reaction product) revealed that ED-1–positive microglia also express L-CCR mRNA (arrow). The ED-1–negative cell but L-CCR–positive (arrowhead) cell is most likely an astrocyte (ac). **B:** Cells were incubated with GFAP antibody to stain astrocytes (ac; brown reaction product). The combination with in situ hybridization (dark-purple reaction product) clearly showed that GFAP-positive cells also express L-CCR mRNA (arrows). The GFAP-negative but L-CCR mRNA-positive cells are most likely microglia (mg; arrowhead). **C** and **D** show cultured microglia (mg) and astrocytes (ac) positive for ED-1 and GFAP (both a brown reaction product), respectively. Hybridization with the sense probe for L-CCR showed no dark-purple staining. Bar in A and C, 10 μm; in B and D, 50 μm.

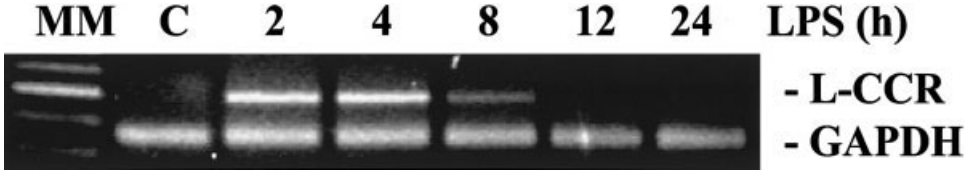

Fig. 3. Effect of LPS injection on L-CCR mRNA expression in mouse brain. RT-PCR experiments revealed that L-CCR mRNA expression in mouse brain was induced 2, 4, and 8 h after the injection of LPS (50 μg/25 g weight). Twelve hours after the injection, L-CCR mRNA expression returned to control levels. Number of cycles for GAPDH and L-CCR were 28 and 32, respectively. MM, molecular weight marker; highlighted band is 500 bp. Both L-CCR and GAPDH PCR products were run in the same gel lane per sample. Similar results were found in three independent experiments.

or LPS-stimulated conditions (35 cycles of RT-PCR; data not shown). Under control conditions, microglia did show basal expression levels for L-CCR mRNA. This expression was upregulated 2 h after stimulation with 100 ng/ml LPS (Fig. 1B). Similar but less pronounced effects were found 2 h after stimulation with 1 and 10 ng/ml LPS (data not shown). LPS induction of L-CCR mRNA expression in cultured microglia peaked at 2 h and declined to baseline expression after 8 h (data not shown).

Using RT-PCR, mRNA expression of CCR1 and -5 was detected in cultured astrocytes (Fig. 1C). No other CCR mRNA (CCR2, -3, -4, -6, -7, -8, and D6) were found in these cells under control or LPS-stimulated condi-

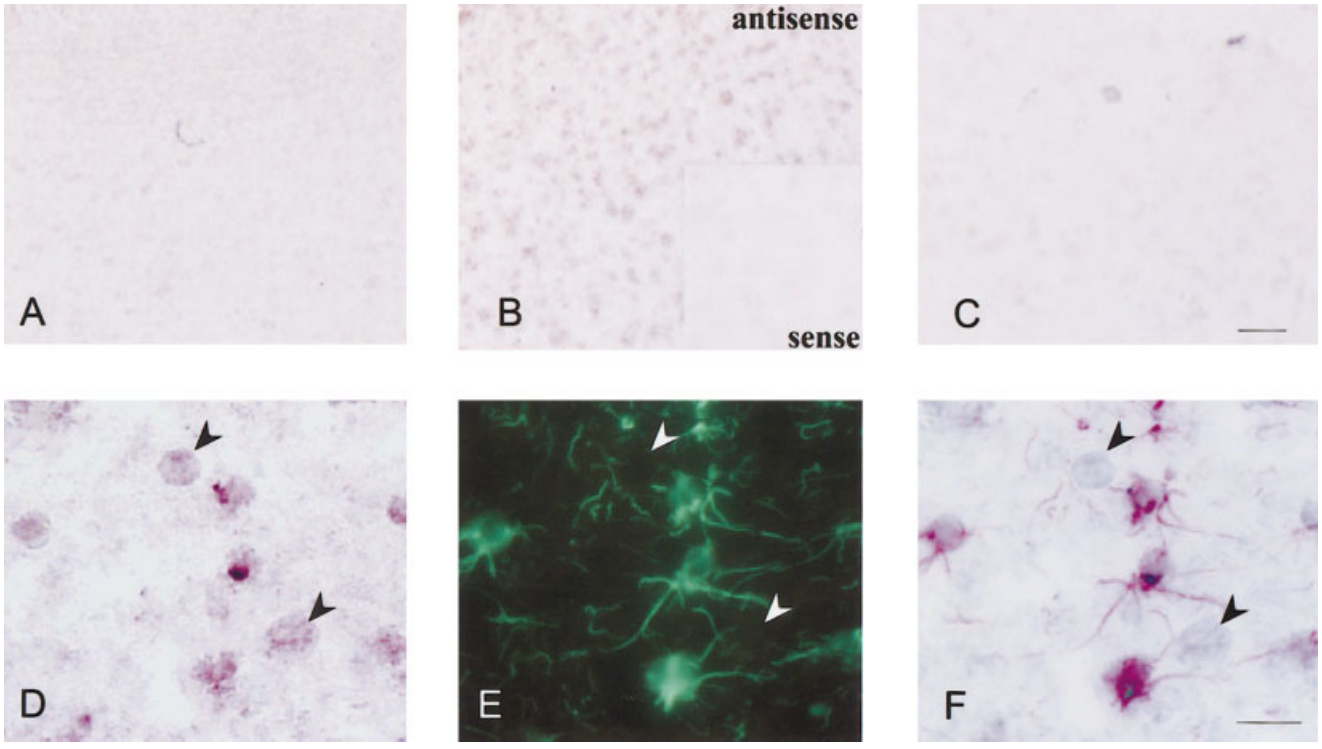

Fig. 4. L-CCR mRNA in situ hybridization in the cortex of LPS-injected mice and identification of astrocytes as L-CCR mRNA expressing cells. **A:** Lack of L-CCR mRNA expression in control brain; only nonspecific staining is visible. **B:** Two hours after the injection of LPS, L-CCR mRNA expression is induced in many cells. The insert shows tissue from the same animal hybridized with the sense probe for L-CCR mRNA. **C:** L-CCR mRNA returned to control levels 24 h after the injection of LPS. **D:** L-CCR mRNA-positive cells in higher magnification in mouse brain 2 h after LPS injection. **E:** Fluorescence micrograph of the same region as in D stained with anti-GFAP to detect GFAP-positive astrocytes. **F:** Electronic overlay of D and E to verify that some L-CCR–positive cells also stain for GFAP, indicating that astrocytes are a cellular source of L-CCR mRNA. Note that there are also L-CCR mRNA-positive cells that are negative for GFAP (arrowheads in D–F), indicating that at least one other cell type different from astrocytes expressed L-CCR mRNA. Bar in A–C, 50 μm; in D–F, 10 μm.

tions (35 cycles of RT-PCR; data not shown). Similar to microglia, untreated cultured astrocytes showed basal mRNA expression for L-CCR, which also was upregulated 2 h after stimulation with LPS (100 ng/ml; Fig. 1D). Treatment with 1 and 10 ng/ml LPS had a similar but less pronounced effect with a comparable time course as observed in microglia (data not shown). No L-CCR mRNA expression was detected in cDNA derived from cultured cortical neurons (data not shown).

In order to verify the results obtained with RT-PCR and to obtain more information on the cell type expressing L-CCR mRNA, in situ hybridization experiments were combined with immunocytochemistry staining. Mixed glial cultures were stimulated 2 h with LPS (100 ng/ml) and stained with ED-1 and GFAP to detect microglia and astrocytes, respectively. ED-1 and GFAP staining yielded a brown reaction product, whereas the positive in situ hybridization signal gave a dark-purple reaction product. Both ED-1–positive microglia (mg; Fig. 2A, arrow) as well as GFAP-positive astrocytes (Fig. 2B, arrows) showed hybridization with the L-CCR antisense probe. The in situ-positive but GFAP-negative cells (arrowheads) in Figure 2B are most likely microglia cells. Using the sense probe for L-CCR mRNA, no hybridization signal could be detected (Fig. 2C and D).

Expression of L-CCR mRNA in Brain Tissue

Mice were injected intraperitoneally with LPS (50 μg/25 g weight) or with 0.9% NaCl for controls. Brains were removed after 2, 4, 8, 12, and 24 h for RT-PCR analysis or in situ hybridization experiments. LPS induced the expression of L-CCR mRNA 2, 4, and 8 h after injection. Twelve hours after injection of LPS, L-CCR mRNA expression returned to baseline levels (Fig. 3). In contrast, control injections with NaCl solution did not affect expression of L-CCR mRNA in brain tissue (data not shown). These results were further verified by in situ hybridization experiments. In untreated control brains, no L-CCR mRNA-positive cells were found (Fig. 4A). Two hours after injection of LPS, many L-CCR–positive cells were observed in the cortex of the LPS-treated mice (Fig. 4B). The insert in Figure 4B shows a clear negative sense staining at the same time point, indicating the specificity of the antisense signal. Twenty-four hours after injection of LPS, L-CCR in situ hybridization signal returned to control levels (Fig. 4C). Combinations of in situ hybridization (Fig. 4D, purple reaction product) and immunohistochemistry (Fig. 4E, GFAP fluorescence) showed that GFAP-positive astrocytes express L-CCR mRNA in mouse cortex (Fig. 4F for overlay of D and E). For

technical reasons, it was not possible to colocalize L-CCR mRNA with microglial markers in brain tissue. Since L-CCR mRNA-positive and GFAP-negative cells were found in brain (arrowheads in Fig. 4D–F), it is suggested that there are cell types different from astrocytes expressing L-CCR mRNA, which could be microglia as observed in cell culture studies.

Effect of CCL2 Stimulation on Cultured Microglia

In several publications, physiological responses of glial cells upon CCL2 stimulation have been shown. Induction of chemotaxis of cultured mouse astrocytes by CCL2 were observed by Heesen et al., (1996). Effects of CCL2 on cultured microglia have also been shown in rat microglia (Boddeke et al., 1999) and fetal human microglia (Peterson et al., 1997). Similar experiments have not been performed yet in mouse microglia. We therefore determined the effects of CCL2 on intracellular calcium transients and chemotaxis of cultured mouse microglia. Similar to microglia from other species, CCL2 induced chemotaxis in cultured mouse microglia. Chemotaxis of unchallenged microglia was observed at 100 nM CCL2 (Fig. 5A). Preincubation of the cells with 100 nM LPS for 16 h, however, increased the chemotactic response of cultured microglia. Significant more cells migrated in the chemotaxis assay compared to unchallenged microglia and microglial chemotaxis was already found with 10 nM CCL2 (Fig. 5A). In addition to the observed chemotactic response, weak intracellular calcium transients in cultured microglia were observed upon stimulation with 100 nM CCL2 in approximately 20% of the investigated cells (19 out of 86 cells; Fig. 5B).

Binding of biot.-rmCCL2 to Cultured Glial Cells

In order to visualize binding CCL2 sites in cultured glial cells, experiments using biot.-rmCCL2 to cultured microglia (Fig. 6A) have been performed. The intensity of biot.-rmCCL2 binding to unstimulated glia cells was slightly higher than the intensity of nonspecific binding (Fig. 6C). Almost no fluorescent signal was observed when biot.-rmCCL2 was blocked by an anti-CCL2 antibody prior to the experiment (Fig. 6D). Stimulation of microglia cultures with LPS did not influence the intensity of the nonspecific signal but strongly increased the specific binding of biot.-rmCCL2 (Figs. 6B and 7). Note that LPS stimulation changed the shape of microglia from a bipolar, elongated cell into a rounded, macrophage-like cells (Fig. 6A and B).

Similar results were observed in cultured astrocytes. Unstimulated astrocytes showed specific binding of biot.-rmCCL2 (Fig. 6E), which was slightly higher than the nonspecific signal. Stimulation with LPS, however, increased the specific signal obtained with biot.-rmCCL2 (Figs. 6F and 7). Both LPS-stimulated microglia and astrocytes showed biot.-rmCCL2 binding in the membrane, indicating that biot.-rmCCL2 recognized a transmembrane receptor.

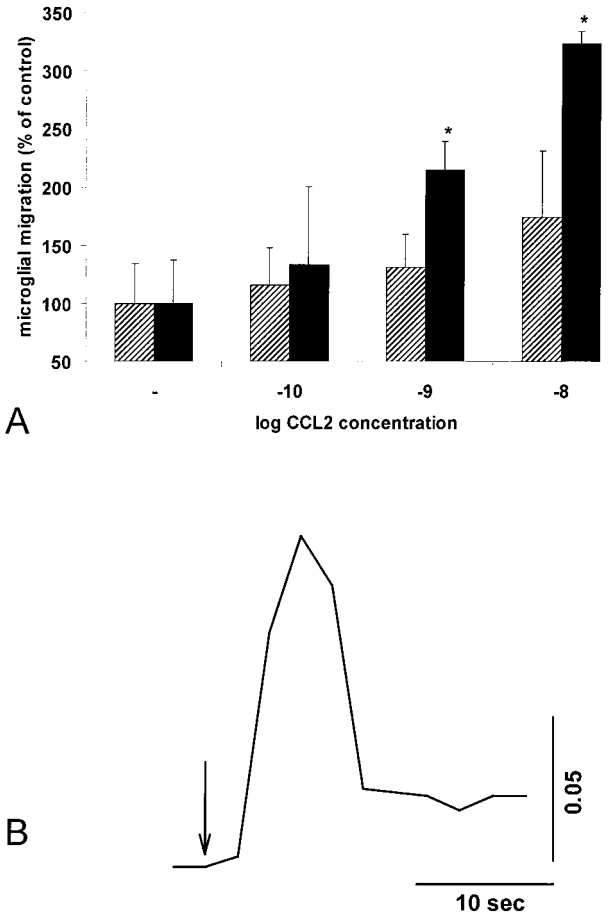

Fig. 5. **A:** Effect of LPS treatment on CCL2-induced chemotaxis of cultured murine microglia. Chemotaxis of untreated cultured microglia (dashed bars) was observed with 100 nM CCL2; lower concentrations did not induce microglial chemotaxis. Pretreatment with LPS (100 ng/ml; 16 h) significantly increased the chemotactic activity of cultured microglia compared to the untreated cells (solid bars). Number of migrated microglia (untreated): control, 184 ± 32; 100 nM CCL2, 320 ± 84. Number of migrated microglia (LPS pretreated): control, 160 ± 32; 100 nM CCL2, 520 ± 32. Numbers are given as mean ± SEM; n = 4. Asterisk, significantly different compared to untreated cells ($P < 0.05$; student's t-test). The illustration shows the results of a typical experiment; similar results were observed in three independent experiments. **B:** Induction of intracellular calcium transients in mouse cultured microglia by 100 nM CCL2. Arrow indicates time point of application. The illustration shows a typical calcium response as it was observed in 19 out of 86 cells.

DISCUSSION

Brain cells (neurons and glial cells) express various receptors for chemokines such as CCR1 and -5, CXCR2 and -4, and CX3CR (Glabinski and Ransohoff, 1999). The expression of chemokine receptors in both glial cells suggests that chemokines may contribute to an endogenous inflammatory cascade in the central nervous system, which is related to pathological conditions (Hesselgesser and Horuk, 1999; Biber et al., 2002). Effects of chemokines such as neuroprotection of hip-

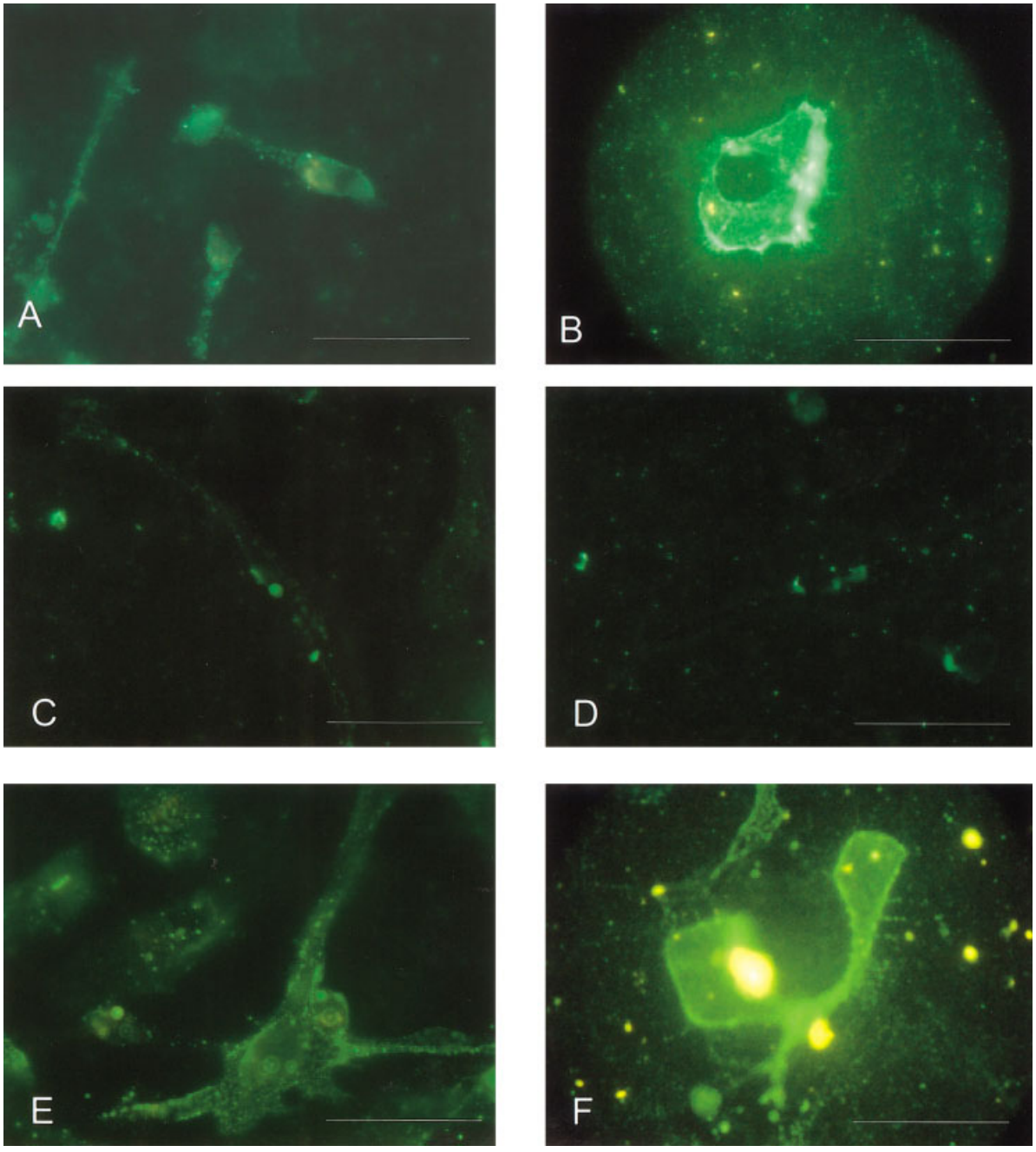

Fig. 6. LPS increased the binding of biot.-rmCCL2 to cultured glial cells. Cultured glial cells were incubated with biot.-rmCCL2 as described in text. **A:** The intensity of biot-.rmCCL2 binding to control microglia was slightly higher than the intensity of the nonspecific signal shown in **C**. **B:** Stimulation with LPS (100 ng/ml for 16 h) strongly upregulated the intensity of bound biot.-rmCCL2 to cultured microglia. **D:** Incubation of biot.-rmCCL2 with a CCL2 antibody completely inhibited the binding of the biotinylated ligand to cultured microglia. Similar results were obtained with cultured astrocytes. **E:** The binding intensity of biot.-rmCCL2 to unstimulated astrocytes was slightly higher than the signal of the nonspecific binding (not shown). **F:** Stimulation with LPS (100 ng/ml for 16 h) strongly increased the binding intensity of biot.-rmCCL2 to cultured astrocytes. colonl Bar in A–D, 20 μm; in E and F, 50 μm.[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

pocampal neurons (Meucci et al., 1998), inhibition of microglial activation (Zujovic et al., 2000), and secretion of metalloproteinases by microglia (Cross and Woodroofe, 1999) are in line with that assumption.

The mRNA expression of the CC chemokine receptors CCR1–5 in cultured glial cells has been investigated by several groups and most studies have been performed in rat or human glial cells (Table 2). Whereas in cultured rat and human astrocytes, CCR mRNA expression has not been determined yet, expression of CCR1 and CCR5 mRNA was found in mouse astrocytes (Tanabe et al., 1997; Dorf et al., 2000; Han et al., 2000). In rat and human microglia, mRNA expression of CCR1 (Tanabe et al., 1997; Boddeke et al., 1999;

McManus et al., 2000) and CCR5 (He et al., 1997; Jiang et al., 1998; Spleiss et al., 1998; Boddeke et al., 1999; McManus et al., 2000) has also been reported. Conflicting reports concerning the expression of CCR2 and CCR3 mRNA in cultured microglia cells have been published. CCR2 mRNA expression was found in cultured microglia by Boddeke et al. (1999), McManus et al. (2000), and Dorf et al. (2000), whereas others found no expression of CCR2 mRNA in cultured microglia (Heesen et al., 1996; Jiang et al., 1998). CCR3 mRNA expression in cultured microglia was found by He et al. (1997) and McManus et al. (2000), but not by Jiang et al. (1998) and Boddeke et al. (1999). The three reports investigating possible expression of CCR4 mRNA in glial cells failed to detect CCR4 mRNA expression (Tanabe et al., 1997; Boddeke et al., 1999; McManus et al., 2000). The reasons for the opposite findings concerning expression of CCR2 and -3 mRNA are currently not clear, but could be due to species differences, different culture conditions, and/or different detection techniques used (Table 2).

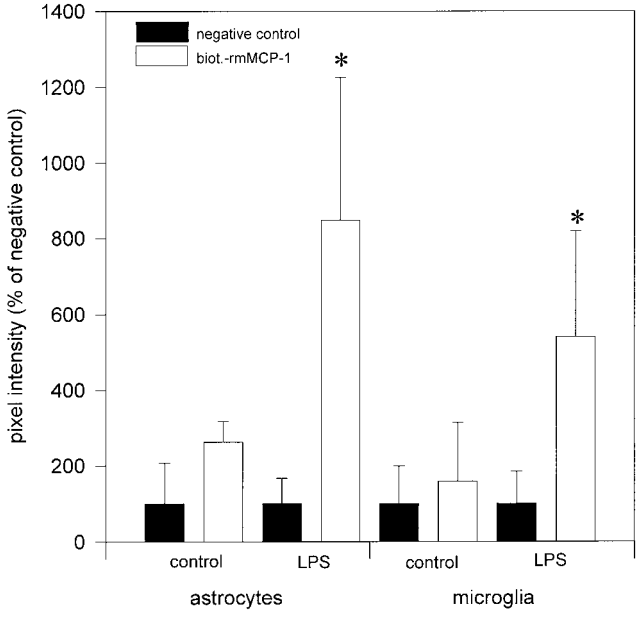

Fig. 7. Quantification of the intensity of bound biot.-rmCCL2 to cultured glial cells in the absence and presence of LPS. Binding of biot.-rmCCL2 was determined as described in text. Data shown are mean ± SD from approximately 50 cells. Asterisk, significantly different compared to untreated cells ($P < 0.01$; student's t-test). Similar results were found in two independent experiments.

In order to establish CC chemokine receptor expression profiles in mouse glial cells, we investigated CC chemokine receptor mRNA expression in cultured mouse microglia and astrocytes. Along with the known receptors CCR1–8 and D6, we examined possible expression of the orphan chemokine receptor L-CCR in murine glial cells using RT-PCR. All primers used in RT-PCR experiments were positively verified using genomic mouse DNA as a template and subsequent cloning and sequencing of the PCR product. We observed mRNA expression for CCR1, -3, -5 and CCR1, -5 in cultured microglia and astrocytes, respectively, which is in good accordance with the recent literature. No other mRNA for known CCR were found. In addition, we observed that both cultured astrocytes and microglia express L-CCR mRNA. Subsequent RT-PCR and in situ hybridization experiments showed that L-CCR expression in both cell types was strongly increased by stimulation of the cells with LPS. Similarly, L-CCR mRNA expression in mouse cortical glial cells was strongly induced after intraperitoneal injection of LPS. These results clearly indicate that mouse glial cells (in vitro and in vivo) express an additional LPS-regulated chemokine receptor mRNA that has not been described in glial cells before.

Glial chemokine receptors are functional, as it has been shown for a variety of different chemokine receptors (Dorf et al., 2000). Since mouse astrocytes respond to the CC chemokine CCL2 with chemotaxis in absence

TABLE 2. Expression profile of CCR mRNA in cultured glial cells from human, mouse, and rat

	Astrocytes			Microglia			
	Human	Mouse	Rat	Human	Mouse	Rat	Studies
CCR1		+					Tanabe et al. (1997)
						+	Boddeke et al. (1999)
	−			+			McManus et al. (2000)
		+					Han et al. (2000)
CCR2		−					Heesen et al. (1996)
						−	Jiang et al. (1998)
						+	Boddeke et al. (1999)
	−		−	+			McManus et al. (2000)
					+		Dorf et al. (2000)
CCR3			−			−	He et al. (1997)
						−	Jiang et al. (1998)
				+			Boddeke et al. (1999)
	−			+			McManus et al. (2000)
CCR4		−					Tanabe et al. (1997)
						−	Boddeke et al. (1999)
	−			−			McManus et al. (2000)
CCR5			−			+	He et al. (1997)
						+	Jiang et al. (1998)
				+			Boddeke et al. (1999)
	−			+			McManus et al. (2000)
		+					Dorf et al. (2000)

of the corresponding receptor CCR2, the existence of an additional, so far undiscovered, chemokine receptor in these cells has been suggested (Hayashi et al., 1995; Heesen et al., 1996). Indeed, in the present experiments, mouse microglia responded to CCL2 stimulation with chemotaxis and calcium in the absence of detectable CCR2 mRNA expression, leading to a similar conclusion. Since L-CCR was expressed in both microglia and astrocytes, it was investigated if L-CCR expression might explain the glial responses to CCL2 in the absence of CCR2. The expression of L-CCR mRNA in mouse glial cells was strongly enhanced by LPS treatment. We therefore investigated possible effects of LPS treatment on CCL2-induced effects in glial cells. In parallel to the L-CCR mRNA expression, LPS treatment had a pronounced effect on the binding of biot.-rmCCL2 to the membranes of cultured microglia and astrocytes. Moreover, the migratory capacity of microglia in response to CCL2 was strongly enhanced by LPS treatment. It is therefore tempting to speculate that glial L-CCR is a functional chemokine receptor, which might explain the known effects of CCL2 in glial cells.

In summary, we provide evidence for the expression of L-CCR in glial cells in vitro and in vivo. LPS treatment not only strongly upregulated L-CCR mRNA expression of glial cells in culture and in the adult mouse cortex, but it also increased a cell membrane-located biotinylated CCL2 binding signal in cultured glial cells and the chemotactic response of microglia to CCL2. It is therefore tempting to speculate that L-CCR may play a role in the chemokine/cytokine-signaling cascade during brain inflammation.

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