Chemokine Expression by Glial Cells Directs Leukocytes to Sites of Axonal Injury in the CNS

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Innate responses in the CNS are critical to first line defense against infection and injury. Leukocytes migrate to inflammatory sites in response to chemokines. We studied leukocyte migration and glial chemokine expression within the denervated hippocampus in response to axonal injury caused by entorhinodentate lesions. A population of Mac1/CD11b+ CD45^{high} macrophages (distinct from CD45^{low} microglia) was specifically detected within the lesion-reactive hippocampus by 12 hr after injury. Significant infiltration by CD3+ T cells did not occur in the denervated hippocampus until 24 hr after axotomy. A broad spectrum of chemokines [RANTES/CCL5, monocyte chemoattractant protein (MCP)-1/CCL2, interferon γ inducible protein (IP)-10/CXCL10, macrophage inflammatory protein (MIP)-1 α /CCL3, MIP-1 β /CCL4, and MIP-2/CXCL2] was induced at this time. RANTES/CCL5 was not significantly elevated until 24 hr after axotomy, whereas MCP-1/CCL2 was significantly induced before leukocyte infiltration occurred. Neither T cells nor macrophages infiltrated the denervated hippocampus of CCR2-deficient mice, arguing for a critical role for the CCR2 ligand MCP-1/CCL2 in leukocyte migration. Both T cells and macrophages infiltrated CCR5-deficient hippocampi, showing that CCR5 ligands (including RANTES/CCL5) are not critical to this response. *In situ* hybridization combined with immunohistochemistry for ionized binding calcium adapter molecule (iba)1 or glial fibrillary acidic protein (GFAP) identified iba1+ microglia and GFAP+ astrocytes as major sources of MCP-1/CCL2 within the lesion-reactive hippocampus. We conclude that leukocyte responses to CNS axonal injury are directed via innate glial production of chemokines.

Key words: chemokines; CCR2; CCR5; hippocampus; axotomy; microglia; astrocytes; macrophages; T cells; flow cytometry; *in situ* hybridization; RT-PCR; RNase protection

Introduction

The CNS has traditionally been regarded as an immunologically privileged organ because of a relatively impermeable blood–brain barrier and an immunosuppressive microenvironment, which limit immune cell entry and function; however, glial cells may direct innate responses to CNS injury (Carson and Sutcliffe, 1999). Chemokines have emerged as important mediators of glial response.

Chemokines are classified on the basis of the position of conserved cysteine residues into C, CC, CXC, and CX₃C subfamilies. The CC and CXC chemokines are implicated as mediators of both CNS development and inflammation (Meng et al., 1999; Bajetto et al., 2002; Babcock and Owens, 2003). The chemokines monocyte chemoattractant protein (MCP)-1/CCL2, RANTES/CCL5, macrophage inflammatory protein (MIP)-1 α /CCL3,

MIP-1 β /CCL4, and interferon γ inducible protein (IP)-10/CXCL10 are produced by glia and infiltrating leukocytes during multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) (Glabinski et al., 1995, 1997; Godiska et al., 1995; Berman et al., 1996; McManus et al., 1998; Simpson et al., 1998, 2000; Sorensen et al., 1999, 2002; Van Der Voorn et al., 1999; Babcock and Owens, 2003). Glial chemokine expression in the lesion-reactive facial nucleus may be affected by infiltrating T cells (Harrison et al., 1998; Raivich et al., 1998; Flugel et al., 2001b). MCP-1/CCL2 and its receptor, CCR2, have been implicated as key mediators of leukocyte entry to CNS, whereas RANTES/CCL5–CCR5 interactions do not appear to be necessary for this response (Fuentes et al., 1995; Kennedy et al., 1998; Fife et al., 2000; Izikson et al., 2000; Siebert et al., 2000; Tran et al., 2000; Huang et al., 2001, 2002; Ousman and David, 2001).

Entorhinodentate axotomy transects fiber tracts that project to the hippocampus, leading to anterograde axonal (Wallerian) and terminal degeneration within this tissue (Fagan and Gage, 1994; Jensen et al., 2000). Glial reactivity has been described at the site of axonal transection and with minimal (Bechmann et al., 2001) or no (Fagan and Gage, 1994; Jensen et al., 1997; Jensen et al., 2000) immune involvement in the denervated hippocampus. Microglial reactivity precedes astroglial activation (Fagan and Gage, 1994; Jensen et al., 1994). Transgenic CNS expression of

Received March 26, 2003; revised July 7, 2003; accepted July 10, 2003.

This research was supported by a grant from the MS Society of Canada to T.O., a grant from the Canadian Institutes of Health Research to S.R., and a Natural Sciences and Engineering Research Council of Canada studentship to A.B. We gratefully acknowledge discussions with and valuable advice from Prof. Bente Finsen (University of Southern Denmark, Odense, Denmark). We thank Maria Caruso for breeding animals and Lyne Bourbonnière for help with RPA.

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interferon (IFN) γ enhanced microglial reactivity without altering kinetics, suggesting that T cells would modify, but not induce, glial responses (Jensen et al., 2000). Activated microglia express Mac1/CD11b and CD45 (Jensen et al., 1997, 2000; Bechmann et al., 2001). Flow cytometry can discriminate parenchymal microglia (CD45^{low}) from infiltrating macrophages (CD45^{high}) in the inflamed CNS (Sedgwick et al., 1991; Renno et al., 1995; Carson et al., 1998).

We show that entorhinodentate axotomy induces chemokine expression and leukocyte infiltration in the denervated hippocampus. Relative kinetics and analysis of chemokine receptor-deficient mice suggest that the CCR2 ligand MCP-1/CCL2 is critical for leukocyte infiltration, whereas CCR5 ligands such as RANTES/CCL5 are not. Our data suggest that glial chemokines direct leukocyte infiltration after CNS injury.

Materials and Methods

Mice. Female C57BL/6 mice were purchased from Charles River (St. Constant, Quebec, Canada) and kept under pathogen-free conditions. Mice deficient in CCR2 (F2 C57BL/6 $\times$ 129/Ola) or CCR5 (eighth generation backcross to C57BL/6) were generated as described previously (Kuziel et al., 1997, 2003) and were bred under pathogen-free conditions. The gross anatomy and organization of the brain is normal in these knock-outs (Tran et al., 2000). Axotomized and control mice weighed between 20 and 30 gm. Experiments were conducted according to Canadian Council on Animal Care guidelines, as administered by the McGill University Animal Care Committee.

Surgical procedures. Mice were subjected to wire-knife lesioning of entorhinodentate projections. Under anesthesia, mice were placed in a Kopf stereotactic apparatus (Kopf, Tujunga, CA), and a burr hole was drilled in the skull 1.0 mm posterior and 2.5 mm lateral from lambda. A closed wire-knife (Kopf) was inserted at an angle of 14° anterior and 10° lateral. At 4.0 mm ventral to dura, the wire-knife was unfolded, and entorhinodentate axons were transected by retracting the knife 3.5 mm dorsally. Sham-operated mice were treated the same way as the lesioned animals except that the wire-knife was not inserted into the brain. In all experiments, the lesion-reactive hippocampus was examined independently of the injured entorhinal cortex. The unlesioned hippocampus served as a control, as did hippocampi from unmanipulated or sham-operated mice. Entorhinodentate lesions reproducibly provoked glial reactivity at the site of axonal transection (stab wound) and in the lesion-reactive hippocampus (including the fimbria). Glial activation within the fimbria is consistent with lesion-induced retrograde degeneration, as has been described previously (Bechmann et al., 2001).

Flow cytometry. Contralateral and lesion-reactive hippocampi were microdissected from PBS-perfused mice between 3 hr and 14 d after axotomy. Tissue surrounding the site of axonal transection was discarded. Single-cell suspensions of individual hippocampi were prepared using a 70 μ m mesh (VWR, Ville Mont-Royal, Quebec, Canada). Cells were centrifuged at 1200 rpm for 10 min at 4°C, washed with HBSS (Life Technologies, Burlington, Ontario, Canada), and then incubated with supernatant from the anti-FcR 24G2 hybridoma containing 2% fetal calf serum (FCS) (Sigma, Oakville, Ontario, Canada) and 50 μ g/ml hamster IgG (BIO/CAN Scientific, Mississauga, Ontario, Canada) on ice for 30 min. Cells were washed with FACS buffer (HBSS with 2% FCS + 0.1% sodium azide) and then incubated with primary antibody [anti-CD45, anti-CD3e, anti-T cell receptor β , anti-Mac1/CD11b, or anti-keyhole limpet hemocyanin (isotype control); BD, Mississauga, Ontario, Canada] on ice for 30 min. Cells stained with biotinylated antibody were washed and then incubated with streptavidin-phycoerythrin (BD). Cells were then washed and staining was analyzed using Cell Quest Pro on a FACScan (BD).

RNase protection assay. At 24 hr after axotomy, the contralateral and lesion-reactive hippocampi were microdissected from PBS-perfused mice, and the tissue surrounding the site of axonal transection was collected separately. RNA was purified using Trizol. RNase protection assay (RPA) using multiprobe DNA templates for chemokines (lymphotactin/

XCL1, RANTES/CCL5, eotaxin/CCL11, MIP-1 α /CCL3, MIP-1 β /CCL4, MIP-2/CXCL2, IP-10/CXCL10, MCP-1/CCL2 and TCA-3/CCL1) and for housekeeping genes (L32 and GAPDH) was performed according to the manufacturer's protocol (BD). Briefly, T7 polymerase was used to create antisense riboprobes from DNA templates, which were then labeled with [α -³²P]UTP (Perkin-Elmer, Markham, Ontario, Canada). Labeled probes were hybridized with 7–8 μ g of total RNA, digested with RNase, and then treated with proteinase K. RNase-protected RNA duplexes were extracted with phenol/chloroform/isoamyl alcohol (VWR) and resolved on 5% denaturing polyacrylamide gels using undigested labeled probes as size markers. After an exposure time of 4 d, dried gels were visualized and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and then subsequently analyzed using ImageQuant v. 1.1 for Apple Macintosh.

RT-PCR. At 3, 12, 24, or 48 hr after axotomy, mice were perfused transcardially with PBS. The contralateral and lesion-reactive hippocampi were microdissected; tissue surrounding the site of axonal transection was collected separately. RNA was purified using Trizol (Life Technologies) and then reverse transcribed as described previously from microdissected hippocampus (Jensen et al., 2000). PCR conditions were optimized for linear amplification to allow direct comparison between samples (Renno et al., 1994). Equal amounts of cDNA were amplified using 1 $\times$ PCR buffer (Sigma), 2 mM MgCl₂ (Sigma), 80 mM each of dNTPs (Pharmacia, Baie d'Urfé, Canada), 2 U of *Taq* polymerase (Sigma), and 50 pmol of each primer (Life Technologies). The PCR reaction was performed using an Eppendorf thermocycler (VWR) for 28 cycles with denaturation, annealing, extension conditions optimized for each primer set: MCP-1/CCL2 (94°C for 30 sec, 57°C for 30 sec, and 72°C for 45 sec), and RANTES/CCL5 and β -actin (94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min). To prevent saturation of β -actin mRNA, cDNA samples were diluted 1:15 with water. The primer sequences for RANTES/CCL5 were as follows: sense, CCC TCA CCA TCA TCC TCA CT; antisense, GGG AAG CGT ATA CAG GGT CA. The other primer sequences were described previously as follows: β -actin (Renno et al., 1998) and MCP-1/CCL2 (Maatta et al., 1998). PCR products were electrophoresed in a 2% agarose gel, visualized by ethidium bromide, and analyzed by Gene Genius (Fisher, Montreal, Canada).

In situ hybridization. Mice were perfused transcardially with PBS, followed by 0.1 M Borax-buffered 4% paraformaldehyde (PFA), pH 9.5 at 4°C, at 24 hr after axotomy. Brains were removed, postfixed for 4–6 d, and then fixed overnight in 10% sucrose in 4% PFA-Borax buffer. The brains were frozen and cut into 20 μ m coronal sections on a microtome (Reichert-Jung, Cambridge Instruments, Deerfield, IL), from the olfactory bulb to the caudal medulla. Sections were collected in a cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, containing 30% ethylene glycol and 20% glycerol) and stored at –20°C. The sections were exposed overnight at 4°C to x-ray films (Kodak, Rochester, NY), defatted in xylene, dipped into NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water), and exposed for 14 d before being developed and counterstained with thionin (0.25%). Generation of the MCP-1/CCL2 riboprobes was as described (Thibeault et al., 2001).

Combined immunohistochemistry and in situ hybridization. Immunohistochemistry was combined with *in situ* hybridization (ISH) to identify the cellular source of MCP-1/CCL2 in the lesion-reactive hippocampus. Every sixth brain section was processed by the avidin–biotin method with peroxidase as a substrate, as described previously (Thibeault et al., 2001). Microglia were labeled with an antibody against iba1, previously described to be specific for microglial cells (generously provided by Dr. Y. Imai, National Institute of Neuroscience, Kodaira, Tokyo, Japan) (Ito et al., 1998). Astrocytes were labeled with an antibody against glial fibrillary acidic protein (GFAP) (DAKO, Mississauga, Canada). Briefly, sections were rinsed with K-PBS and incubated with primary antibody. Sections were once more rinsed in K-PBS, incubated with biotinylated secondary antibodies (Vector Laboratories), and then rinsed again with K-PBS before a final incubation with an avidin–biotin–peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories). After several washes in K-PBS, the brain slices were reacted in 0.05% diaminobenzidine and 0.003% hydrogen peroxide. Thereafter, sections were rinsed in K-PBS, mounted, desiccated, fixed in 4% PFA, and digested by proteinase K.

Prehybridization, hybridization, and posthybridization steps were performed according to the above description, with shorter dehydration times (alcohol 50, 70, 95, 100%) to prevent decoloration of immunoreactive cells. The slides were dried and then exposed and developed as described above.

For proportional counts, MCP-1/CCL2+ cells were identified at high magnification and then examined for iba1 or GFAP expression by bright-field microscopy. MCP-1/CCL2+ cells located within the lesion-reactive hippocampus were considered separately from those found at the site of axonal transection.

Results

Macrophages infiltrate the lesion-reactive hippocampus

Previous studies using immunohistochemistry to document microglial activation in the denervated hippocampus did not describe infiltrating macrophages (Fagan and Gage, 1994; Jensen et al., 1997). Flow cytometry can distinguish between macrophages and microglia in cellular isolates from inflamed tissues. Although both microglia and macrophages express Mac1/CD11b, microglia express lower levels of CD45 (Sedgwick et al., 1991; Renno et al., 1995; Carson et al., 1998). We used flow cytometry to characterize macrophage and microglial responses in individual hippocampi after axonal injury. Very few Mac1/CD11b+ CD45^{high} cells were identified in unmanipulated hippocampi ($0.12 \pm 0.02\%$) (Fig. 1*A,D*). These were close to the limit of detection and were likely perivascular macrophages. A similar proportion of Mac1/CD11b+ CD45^{high} macrophages was detected in contralateral hippocampi between 3 hr and 14 d after axotomy (Fig. 1*A,D*). The percentage of macrophages in the lesion-reactive hippocampus was not significantly different from controls at 3 hr after axotomy ($0.12 \pm 0.03\%$) (Fig. 1*D*). By contrast, by 12 hr after axotomy, the percentage of macrophages in the lesion-reactive hippocampus doubled ($0.25 \pm 0.07\%$; Fig. 1*D*). The proportion of infiltrating macrophages further increased by 24 hr after axotomy ($0.66 \pm 0.14\%$), peaked at 2 d ($1.22 \pm 0.46\%$), and then declined between 5 ($0.40 \pm 0.15\%$) and 14 ($0.20 \pm 0.03\%$) d after axotomy (Fig. 1*A,D*). Macrophage cell proportions within the denervated hippocampus at 24 hr after axotomy represented ~ 250 – 300 macrophages (versus ~ 50 in unmanipulated hippocampi). These data suggest that macrophages infiltrate the lesion-reactive hippocampus within 12 hr of axonal injury.

T cells infiltrate the lesion-reactive hippocampus

Previous studies described either no T cell infiltration or expression of the Th1 cytokine, IFN γ , after entorhinal lesions (Fagan and Gage, 1994; Jensen et al., 1997, 2000), or minimal

Figure 1. Macrophages infiltrate the lesion-reactive hippocampus. Flow cytometry profiles show live cells on the basis of forward and side scatter gating. Shown are representative profiles from individual unmanipulated (U, left column), contralateral (C, middle column), or lesion-reactive (L, right column) hippocampi from C57BL/6 (*A*), CCR2-deficient (*B*), or CCR5-deficient (*C*) mice at 24 hr after axotomy. Macrophages were identified on the basis of expression of Mac1/CD11b and high levels of CD45 (top right quadrant). *D* shows proportions of macrophages within groups of hippocampi between 3 hr and 14 d after axotomy (mean $\pm$ SE). Quadrants were set on the basis of fluorescence levels using isotype-matched control antibodies. Data are representative of 3–11 animals per group. * $p < 0.05$ – 0.001 versus U and C; ** $p < 0.05$ – 0.001 versus U, C, and L of C57BL/6 at 24 hr; *** $p < 0.05$ – 0.001 versus U, C, and L.

infiltration at later times (Bechmann et al., 2001). We used flow cytometry to detect CD3, a marker for all T cells. Only cells that expressed high levels of CD45 showed increased fluorescence with anti-CD3 compared with isotype-matched control antibody. This identifies them as T cells. A very low frequency of CD3+ cells was identified in isolates from unmanipulated hippocampi (0.09 ± 0.03) (Fig. 2*A,D*). Similarly few CD3+ T cells were found in contralateral or lesion-reactive hippocampi between 3 hr and 14 d after axotomy (Fig. 2*A,D*). A significant increase in the frequency of T cells in the lesion-reactive hippocampus was first detected 24 hr after axotomy ($0.27 \pm 0.05\%$) (Fig. 2*A,D*). A similar proportion of T cells was identified within

Figure 2. T cells infiltrate the lesion-reactive hippocampus. Flow cytometry profiles show live cells on the basis of forward and side scatter gating. Shown are representative profiles from individual unmanipulated (U, left column), contralateral (C, middle column), or lesion-reactive (L, right column) hippocampi from C57BL/6 (A), CCR2-deficient (B), or CCR5-deficient (C) mice at 24 hr after axotomy. T cells were identified on the basis of expression of CD3 and CD45 (box). D shows T cell proportions within groups of hippocampi between 3 hr and 14 d after axotomy (mean $\pm$ SE). Boxes were drawn on the basis of fluorescence levels using isotype-matched control antibodies. Data are representative of three to seven animals per group. * $p < 0.05$ versus U and C; ** $p < 0.05$ – 0.001 versus U, C, and L of C57BL/6 at 3, 12, and 24 hr.

the lesion-reactive hippocampus at 24 hr after axotomy using antibodies against TCR β ($0.24 \pm 0.01\%$). Infiltration by T cells at this time was more variable than infiltration by macrophages, to the extent of being undetectable in two of seven lesioned mice. The proportion of T cells at 24 hr after axotomy represents ~ 100 – 150 T cells in the denervated hippocampus compared with ~ 50 in unmanipulated hippocampi. A greater proportion of T cells was found by 2 d ($0.54 \pm 0.09\%$) and remained elevated through 5 ($0.42 \pm 0.14\%$) and 14 ($0.70 \pm 0.44\%$) d after axotomy (Fig. 2D). These data suggest that T cell infiltration of the lesion-reactive hippocampus is variable and minor compared with macrophages at early times, whereas at later times T cells outnumber macrophages.

Axonal injury induces chemokine expression

Constitutive expression of chemokines was not detected by RPA in unmanipulated hippocampi (Fig. 3A). Message for RANTES/CCL5, MCP-1/CCL2, IP-10/CXCL10, MIP-1 α /CCL3, MIP-1 β /CCL4, and MIP-2/CXCL2 was specifically induced in the lesion-reactive hippocampus and at the site of axonal transection by 24 hr after axotomy (Fig. 3A). Lymphotactin/XCL1, eotaxin/CCL11, and TCA-3/CCL1 were not detected. Chemokine signals were normalized to L32 for comparison between treatments and sources of RNA (Fig. 3B). Levels of MCP-1/CCL2, RANTES/CCL5, IP-10/CXCL10, MIP-1 α /CCL3, and MIP-1 β /CCL4 expression were significantly elevated in the lesion-reactive hippocampus and at the site of axonal transection. MIP-2/CXCL2 was only significantly induced at the site of axonal transection. Thus, axonal injury induces expression of a wide spectrum of chemokines.

Kinetics of RANTES/CCL5 and MCP-1/CCL2 expression

We examined levels of RANTES/CCL5 and MCP-1/CCL2 expression by RT-PCR at 3, 12, 24, and 48 hr after axotomy. Normalization to β -actin allowed the level of expression to be compared with control hippocampi. MCP-1/CCL2 was significantly elevated in the denervated hippocampus and at the site of axonal transection as early as 3 hr after axotomy, compared with unmanipulated and sham controls (Fig. 4A,B). RANTES/CCL5 was only significantly elevated in the lesion-reactive hippocampus by 24 and 48 hr after axotomy and only significantly induced at the site of axonal transection between 12 and 48 hr after axotomy (Fig. 4A,C). Levels of expression before 48 hr after axotomy were generally higher at the site of axonal transection than in the lesion-reactive hippocampus (Fig. 4B,C). A slight sham effect was seen for RANTES at 3 hr and for MCP-1/CCL2 at 12 hr, but not at other times (Fig. 4B,C). Despite sharing similar kinetics with RANTES/CCL5 at later times, MCP-1/CCL2 was therefore uniquely elevated at 3 hr after axotomy. These data suggest that early expression of MCP-1/CCL2 may be responsible for leukocyte recruitment to the denervated hippocampus.

Levels of MCP-1/CCL2 protein in the lesion-reactive hippocampus were less than the limit of detection using immunostaining or Western blot (~ 1 ng recombinant MCP-1/CCL2; data not shown). This is broadly consistent with other studies that showed infiltrating leukocytes as major producers of MCP-1/CCL2 in EAE, with similar detection thresholds (Nygardas et al., 2000; Rajan et al., 2000). As shown below, we find glial cells to be major sources of MCP-1/CCL2.

Leukocyte infiltration depends on signaling through CCR2, not CCR5

The chemokine MCP-1/CCL2 signals almost exclusively through the CCR2 receptor (Gu et al., 1999). We tested directly whether signaling through CCR2 directs leukocyte infiltration in response to axonal injury by lesioning CCR2-deficient mice. We also examined injury responses in CCR5-deficient mice. Glial activation did not appear to be impaired in these mice. Upregulation of Mac1/CD11b on CD45^{low} cells in hippocampus, detected by flow cytometry 24 hr after axotomy, was equivalent to wild-type mice. Immunohistochemical staining for GFAP was also not changed. There was no evidence for a shift in chemokine profile at 24 hr (data not shown).

As in wild-type mice, very few macrophages were identified within unmanipulated or contralateral hippocampi from either knock-out (Fig. 1*B–D*). Unlike wild-type mice, in which macrophages increased ~5.5-fold in the lesion-reactive hippocampus, macrophages did not infiltrate the denervated hippocampus of CCR2-deficient mice ($0.07 \pm 0.01\%$; $p > 0.05$) (Fig. 1*B,D*). A low but significantly elevated frequency of macrophages was found in the denervated hippocampus of CCR5-deficient mice (3.5-fold ; $0.26 \pm 0.02\%$) (Fig. 1*C,D*). These data point to MCP-1, the functional ligand for CCR2, as being critical for macrophage infiltration, unlike CCR5 ligands, such as RANTES/CCL5.

Unlike wild-type mice, in which T cells increased approximately threefold in the lesion-reactive hippocampus, T cells did not infiltrate the denervated hippocampus of CCR2-deficient mice ($0.04 \pm 0.01\%$) (Fig. 2*B,D*). MCP-1/CCL2 is therefore also a critical mediator of T cell infiltration. By contrast, T cell infiltration to the denervated hippocampus of CCR5-deficient mice was indistinguishable from controls ($0.26 \pm 0.03\%$) (Fig. 2*C,D*), and so CCR5 ligands are not critical for T cell entry to sites of axonal injury.

Microglia and astrocytes express MCP-1/CCL2 transcripts

We examined MCP-1/CCL2 mRNA expression by ISH at 24 hr after axotomy. No MCP-1/CCL2+ cells were found in unmanipulated hippocampus (Fig. 5*A*). MCP-1/CCL2+ cells were almost undetectable in contralateral hippocampi at 24 hr after axotomy (Fig. 5*C*). Cells strongly positive for MCP-1/CCL2 transcripts were localized to the site of axonal transection and within the lesion-reactive hippocampus, particularly in the molecular layers and fimbria (Fig. 5*B,D*).

We combined ISH with immunohistochemistry for iba1, a microglial marker, and for GFAP, which labels astrocytes, to identify cells producing MCP-1/CCL2 after entorhinal lesions. Approximately two-thirds of MCP-1/CCL2+ cells in the denervated hippocampus colocalized with iba1 immunoreactiv-

Figure 3. Axonal injury induces chemokine expression. RPA of chemokine expression in perfused hippocampi of C57BL/6 mice at 24 hr after axotomy shows that MCP-1/CCL2, RANTES/CCL5, IP-10/CXCL3, MIP-1α/CCL3, MIP-1β/CCL4, and MIP-2/CXCL2 were specifically induced in the lesion-reactive hippocampus and at the site of axonal transection. Results are representative of four separate experiments. U, Unmanipulated hippocampus; C, contralateral unlesioned hippocampus; L, lesion-reactive hippocampus; T, site of axonal transection. * $p < 0.05$ – 0.01 versus U and C.

Figure 4. Message for MCP-1/CCL2 and RANTES/CCL5 is induced by axotomy. *A*, RT-PCR analysis of MCP-1/CCL2, RANTES/CCL5, and β -actin mRNA from perfused hippocampus of C57BL/6 mice at 3, 12, 24, or 48 hr after axotomy. Levels of MCP-1/CCL2 (*B*) and RANTES/CCL5 (*C*) amplimers were determined by fluorimager analysis and then normalized to β -actin. Data are representative of 2–14 animals per group and plotted as arbitrary fluorimager units, with bars representing SE. U, Unmanipulated hippocampus; S, sham-operated hippocampus; C, contralateral unlesioned hippocampus; L, lesion-reactive hippocampus; T, site of axonal transection. * $p < 0.01$ – 0.001 versus U; ** $p < 0.01$ versus U, S, C; *** $p < 0.01$ – 0.001 versus U, S, C, and L.

ity, revealing microglia as the predominant source of chemokine at 24 hr after axotomy (Fig. 6*D,E*, Table 1). Estimates of iba1 + MCP-1/CCL2+ cells on the basis of counts in tissue sections outnumbered the Mac1/CD11b + CD45^{high} cells identified in the denervated hippocampus by flow cytometry by approximately two orders of magnitude, arguing against infiltrating iba1 + macrophages as a prominent source of this chemokine. Not all iba1 + microglia expressed MCP-1/CCL2 transcripts. GFAP+ astrocytes within denervated regions also contributed to MCP-1/CCL2 expression (Fig. 6*A–C*, Table 1). The proportion of GFAP+ MCP-1/CCL2+ cells matched the proportion of iba1-MCP-1/CCL2+ cells, suggesting that astrocytes and microglia account for essentially all MCP-1/CCL2 production at this time. We confirmed astroglial activation in the denervated hippocampus by RT-PCR for GFAP (data not shown). Most of the MCP-1/CCL2+ cells at the site of axonal transection were astrocytes (Table 1).

Figure 5. Axotomy induces MCP-1/CCL2 expression within the denervated hippocampus. MCP-1/CCL2+ cells were not detected by *in situ* hybridization in coronal sections from unmanipulated hippocampus (A) or contralateral unlesioned hippocampus after axotomy (C). Numerous robustly positive cells were found in the lesion-reactive hippocampus (D), located distally to the site of axonal transection (B). Original magnification, 10 $\times$.

Figure 6. Microglia and astrocytes express MCP-1/CCL2 transcripts. Immunohistochemistry and *in situ* hybridization were combined to determine the cellular source of chemokine expression. Brown immunoreactivity for GFAP or iba1 identifies astrocytes (A–C) and microglia (D, E), respectively. Clusters of silver grains identify MCP-1/CCL2-positive cells. Gray arrowheads identify GFAP+ astrocytes (A, C), and black arrowheads identify iba1+ microglia (D, E) that express MCP-1/CCL2. Black arrows show GFAP+ (B, C) and gray arrows show iba1+ (D, E) MCP-1/CCL2+ cells. In D, a microglial cell and an astrocyte are juxtaposed. Original magnification, 60 $\times$; except C (40 $\times$).

Discussion

We have identified macrophages and T cells within the lesion-reactive hippocampus after entorhinodentate axotomy. Axonal injury provoked the expression of CC and CXC chemokines,

including RANTES/CCL5 and MCP-1/CCL2. Kinetic studies and lesions in CCR5-deficient mice suggest that leukocyte recruitment did not depend on RANTES/CCL5-CCR5 interactions. Early expression of MCP-1/CCL2 and abrogation of lesion-induced leukocyte infiltration in mice deficient in its receptor, CCR2, support a critical role for this chemokine. Microglia and astrocytes were prominent sources of MCP-1/CCL2. Our data suggest that innate glial responses direct leukocyte entry to the injured CNS by means of chemokine production.

Macrophage infiltration has not been described previously after entorhinodentate lesions (Fagan and Gage, 1994; Jensen et al., 1997). This may reflect the rapid decline in macrophage numbers several days after lesion or relate to difficulties in discriminating macrophages from microglia by histological analyses. When activated, these cell types become phenotypically equivalent, with the exception of CD45 levels, which have been used to discriminate resting parenchymal microglia (CD45^{low}) from infiltrating macrophages (CD45^{high}) in the inflamed CNS (Sedgwick et al., 1991; Renno et al., 1995; Carson et al., 1998). This is the first study to use flow cytometry to make this distinction after axonal injury. Perivascular macrophages, which also express a Mac1/CD11b+ CD45^{high} phenotype, are seen at very low frequencies in unmanipulated hippocampi. Studies have shown that activated microglia upregulate CD45 (Jensen et al., 1997; Sedgwick et al., 1998; Carson et al., 1999), but this does not obscure the difference between microglia and macrophages (Renno et al., 1995). Nevertheless, it remains possible that Mac1/CD11b+ CD45^{high} cells include activated microglia. Rigorous analysis requires a bone-marrow chimera approach. Such studies have in fact shown that blood-derived cells can enter the CNS and become morphologically indistinguishable from microglia (Flugel et al., 2001a; Priller et al., 2001; Vallieres and Sawchenko, 2003). We assume that the Mac1/CD11b+ CD45^{high} cells in our study are infiltrating macrophages, responding to glial production of the macrophage chemoattractant MCP-1/CCL2.

Macrophages infiltrate after CNS insult, and MCP-1/CCL2 has been implicated as a key mediator of this response. Our results show that CCR2 was required for both macrophage and T cell infiltration of the denervated hippocampus. These functional data confirm production of bioactive MCP-1/CCL2 protein. Macrophage infiltration was reduced after injury to sciatic nerve or spinal cord in mice deficient in CCR2 (Siebert et al., 2000; Ma et al., 2002), or after lysophosphatidylcholine-induced demyelination after treatment with neutralizing antibodies against MCP-1/CCL2 (Ousman and David, 2001). Transgenic overexpression of MCP-1/CCL2 caused leukocyte accumulation within cerebral vessels (Fuentes et al., 1995; Huang et al., 2002). Knock-out and antibody depletion studies have implicated MCP-1/CCL2 and CCR2 as critical mediators for induction and progression of EAE, by reducing macrophage and T cell infiltration of the CNS (Kennedy et al., 1998; Fife et al., 2000; Izikson et al., 2000; Huang et al., 2001). MCP-1 production by human brain endothelial cells directly provoked transendothelial migration by CCR2+ Th2 cells *in vitro* (Biernacki et al., 2001). By contrast, macrophage infiltration was required for T cell infiltration to initiate EAE (Martiney et al., 1998; Tran et al., 1998). Whether macrophage infiltration promotes later infiltration by T cells is an interesting possibility.

Previous studies have described either no (Fagan and Gage, 1994; Jensen et al., 2000) or minimal (Bechmann et al., 2001) infiltration by T cells after entorhinodentate lesions. Our study suggests that T cell infiltration begins at 24 hr after axotomy, after macrophages have entered the denervated hippocampus. It is

important to note that early T cell responses were less marked and more variable than macrophage responses. There were individual mice in which T cell infiltration was not detectable at these times, although macrophage infiltrates were. By contrast, more T cells than macrophages infiltrated at later times. The number of T cells that infiltrate the denervated hippocampus 1–2 d after lesion correspond to those counted in histological sections in a separate study (R. Ladeby, A. Babcock, M. Jensen, T. Owens, and B. Finssen, unpublished observations). Chemokine expression in the denervated hippocampus promotes immune cell infiltration, and peripheral immune cell dysfunction may tap into such innate glial responses. Whether this is one mechanism leading toward CNS autoimmunity, such as in MS, is of interest (Carson and Sutcliffe, 1999; Becher et al., 2000; Owens et al., 2001; Carson, 2002).

Glia produce chemokines in other models of CNS injury. RANTES/CCL5, MCP-1/CCL2, IP-10/CXCL10, MIP-1 α /CCL3, and MIP-1 β /CCL4 were elevated after cuprizone-induced demyelination (McMahon et al., 2001). Aspiration lesions induced MCP-1/CCL2 expression in the visual cortex and in the thalamic nuclei before retrograde degeneration (Muessel et al., 2000). Acute excitotoxic injury elicited by local injection of NMDA or kainic acid induced MCP-1/CCL2 expression by macrophage/microglia and astrocytes (Calvo et al., 1996; Szaflarski et al., 1998). In our study, MCP-1/CCL2+ cells colocalized with both Iba1+ microglia and GFAP+ astrocytes. Other studies of CNS inflammation have documented endothelial or neuronal expression of MCP-1/CCL2 (Flugel et al., 2001b; Thibeault et al., 2001). Although we cannot exclude the possibility that these cells contributed to MCP-1/CCL2 expression, our data suggest that most, if not all, MCP-1/CCL2 is produced by astrocytes and microglia. Proportions of microglia versus astrocytes were lower at the site of axonal transection. Similar proportions were seen at 3 hr (data not shown), arguing against this being a consequence of the kinetics of degeneration. There may be microenvironmental differences between the two sites. This could reflect tissue injury/necrosis versus apoptosis, or differences in the neurochemical milieu. The innate capacity for glia to respond to injury with chemokine expression may reflect a role in normal brain function (Mennicken et al., 1999; Bajetto et al., 2002). Our observation, that microglia and astrocytes in the denervated hippocampus express chemokines, speaks for an innate program of glial reactivity that is induced by axonal injury.

The specific stimulus for glial activation has yet to be determined. Components of pathogens may trigger microglial activation via Toll-like receptors (Nguyen et al., 2002). Glial activation might result as a consequence of disrupted neurotransmission after CNS injury. Altered levels of potassium, purines, or pyrimidines may signal neuronal damage and activate glia (Khanna et al., 2001; Chakfe et al., 2002; Inoue, 2002). β amyloid and gangliosides have also been shown to stimulate microglial activation (Pyo et al., 1999; Tan et al., 1999). Many proteins not normally found in the CNS may serve as glial activating factors, either by gaining access to sites of injury or local production by resident cells. Thus, thrombin, complement fragments, heat shock proteins, and cytokines have been implicated in glial activation (Klein et al., 1997; Moller et al., 1997; Bhat and Sharma, 1999; Moller et al., 2000; Kalla et al., 2001; Hanisch, 2002; Kakimura et al., 2002). Chemokines themselves may activate glia. Fractalkine/CX3CL1 has been proposed to mediate microglial activation via CX₃CR1 after facial motor nerve axotomy or ischemia (Harrison

Table 1. Percentage of microglia and astrocytes that make MCP-1/CCL2 in the lesion-reactive hippocampus and at the site of axonal injury at 24 hr after axotomy

	Iba1 –	Iba1 +	GFAP –	GFAP +
Lesion-reactive hippocampus	37.2 $\pm$ 3.2 ^a	62.8 $\pm$ 3.2	64.8 $\pm$ 7.8	35.2 $\pm$ 7.8
Site of axonal injury	61.6 $\pm$ 2.5	38.4 $\pm$ 2.5	31.5 $\pm$ 7.2	68.5 $\pm$ 7.2

^aNumber of cells $\pm$ SE.

et al., 1998; Tarozzo et al., 2002). MCP-1/CCL2 has been proposed to initiate microglial activation in response to retrograde degeneration after injury to facial nerve (Flugel et al., 2001b) or visual cortex (Muessel et al., 2002). In our study, however, glial reactivity was not noticeably diminished in either CCR2- or CCR5-deficient mice. Although the stimulus that triggers glial reactivity after entorhinal dentate axotomy remains undefined, our results show that glia generate a broad-spectrum chemokine response in response to CNS injury that triggers leukocyte infiltration.

There is a growing appreciation that CNS infiltration by both macrophages and T cells may be neuroprotective (Schwartz et al., 1999). Infiltrating macrophages promote regeneration or repair of injured neurons in the CNS by phagocytosing myelin debris (David et al., 1990; Schwartz et al., 1999). Whether macrophages can enter and exert this repair function may depend on MCP-1/CCL2 (Siebert et al., 2000; Ousman and David, 2001; Ma et al., 2002). After entorhinal dentate lesions, regenerative axonal sprouting responses help restore hippocampal function (Fagan and Gage, 1994). Macrophages recruited to the lesion-reactive hippocampus may contribute to the efficacy of this response by phagocytosing myelin debris. T cells not only infiltrate the facial nucleus (Raivich et al., 1998) but also promote facial motoneuron survival (Serpe et al., 1999). Myelin-specific Th2 cells down-regulate microglial activation in hippocampal slice cultures, supporting an anti-inflammatory role for these cells in neuroinflammation (Gimsa et al., 2001); however, normal cholinergic sprouting was observed in the denervated hippocampus of T cell-deficient nude rats (Fagan and Gage, 1994). Whether T cells that infiltrate the denervated hippocampus are neuroprotective can only be speculated at this point.

Taken together, our observations suggest that early glial production of MCP-1/CCL2 is an innate response to axonal injury that promotes leukocyte infiltration. This role for glial-derived MCP-1/CCL2 complements that for the CCR8 ligand TCA-3/CCL1 in T cell infiltration in EAE (Murphy et al., 2002), thereby making a case for glial-derived chemokines as critical regulators of immune–CNS interaction. The innate glial responses that direct CNS–leukocyte interactions must also fulfill intrinsic roles within the CNS, and therefore play a dual role in regulation of CNS physiology and homeostasis.

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