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Synchronous Synthesis of α- and β-Chemokines by Cells of Diverse Lineage in the Central Nervous System of Mice with Relapses of Chronic Experimental Autoimmune Encephalomyelitis

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Chemokines are secreted peptides that exhibit selective chemoattractant properties for target leukocytes. Two subfamilies, α- and β-chemokines, have been described, based on structural, genetic, and functional considerations. In acute experimental autoimmune encephalomyelitis (EAE), chemokines are up-regulated systemically and in central nervous system (CNS) tissues at disease onset. Functional significance of this expression was supported by other studies; intervention with an antichemokine antibody abrogated passive transfer of EAE, and chemokines expressed in brains of transgenic mice recruited appropriate leukocyte populations into the CNS compartment. Chemokine expression in the more relevant circumstance of chronic EAE has not been addressed. We monitored the time course and cellular sources of chemokines (monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, interferon-γ-inducible protein of 10 kd, KC, and regulated on activation, normal T-cell expressed and secreted cytokine) in CNS and peripheral tissues during spontaneous relapses of chronic EAE. We found coordinate chemokine up-regulation in brain and spinal cord during clinical relapse, with expression confined to CNS tissues. Monocyte chemoattractant protein-1, interferon-γ-inducible protein of 10 kd, and KC were synthesized by astrocytic cells, whereas macrophage inflammatory protein-1α and regulated on activation, normal T-cell expressed and secreted cytokine were elaborated by infiltrating leukocytes. The results demonstrate stringent regulation of multiple chemokines* in vivo *during a complex organ-specific autoimmune disease. We propose that chemokine expression links T-cell antigen recognition and activation to subsequent CNS inflammatory pathology in chronic relapsing EAE. (Am J Pathol 1997, 150:617–630)

Chemokines (chemoattractant cytokines) are secreted peptides that are produced by numerous cell types *in vitro* and *in vivo* during inflammatory processes. Two major subfamilies of chemokines have been proposed, based on chromosomal localization, structure, and functional characteristics. These subfamilies are the α- and β-chemokines: α-chemokines are encoded on human chromosome 4, exhibit a conserved C-*X*-C motif near the N terminus, and chemoattract primarily neutrophils, whereas β-chemokine genes are clustered on chromosome 17 and possess a C-C motif and chemoattract monocytes and lymphocytes.[1] Recently, a third γ-chemokine subfamily was characterized with biologically similar chemoattractant properties but with a unique struc-

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ture and a distinct genetic locus.[2,3] Chemokines are encoded by immediate response genes, the transcripts of which accumulate to high levels after a diversity of stimuli, and several family members were initially identified as cytokine-inducible cDNAs during differential cloning experiments.[4–11] Other chemokines were discovered through biochemical purification of chemoattractant components in cell culture. The convergence of these two lines of experimentation provided a substantial foundation of information about chemokine structure, *in vitro* function, and regulation.[9,12–14] The functional spectrum of chemokine-mediated activities has expanded to include modulation of angiogenesis, inhibition of hematopoiesis, regulation of T-cell activation, and selective mitogenic properties.[15]

The role of chemokines *in vivo* in inflammatory processes has come under intensive investigation. Chemokines are expressed in a wide variety of organ-specific inflammatory states, including those affecting the skin, lung, kidney, peripheral nervous system, and central nervous system (CNS).[16–24] Both acute and chronic processes feature chemokine expression, reflecting the requirement for leukocyte recruitment in inflammatory pathologies.[25] Chemokine expression correlates with target-specific leukocyte infiltration in acute and chronic lung injury, inflammatory and ischemic renal pathology, and cutaneous inflammation.[26,27] In model pulmonary pathologies, chemokine antibodies abrogated leukocyte entry.[20,21,28]

Chemokine function *in vivo* has been addressed elegantly by construction of mice with targeted deletion of individual chemokine genes. Mice that lacked macrophage inflammatory protein-1α (MIP-1α) were susceptible to viral infections of the heart and lung because of deficient leukocytic inflammatory responses, indicating a specific chemokine requirement for competent host defense.[29] Additional information has come from overexpression experiments in transgenic (tg) mice. Studies using KC and monocyte chemoattractant protein-1 (MCP-1) demonstrated that chemokines exert appropriate target-specific chemoattractant function *in vivo* for neutrophils and monocytes.[30] Interestingly, chronic chemokine stimulus *in vivo* by MCP-1 resulted in desensitization of monocyte target cells and host defense impairment, further supporting the concept that chemokine functions during host defense are nonredundant.[31] Chemokine expression has been reported in ischemic, traumatic, and infectious processes of the CNS, with demonstrable functional competence in the latter setting.[18,32–34] Overexpression of chemokine MCP-1 in the brains of tg mice produced a monocytic infiltrate in the perivascular space, indicating unexpected potency for recruiting target leukocytes into the subarachnoid compartment.[35] In recent experiments, we found that tg mice with targeted expression of KC in oligodendroglia exhibited remarkable neutrophil infiltration of CNS tissues, indicating that selected chemokines could mediate all functions needed to recruit leukocytes from vasculature to the CNS parenchyma.[36]

Murine experimental autoimmune encephalomyelitis (EAE) is a well-characterized example of autoimmune pathology directed against CNS myelin proteins.[37] Disease is initiated in susceptible strains by immunization with myelin components and can be modified to exhibit chronic relapsing or acute properties depending on immunogen and strain of animal. Chronic-relapsing EAE (Ch-R EAE) after active immunization is an incisive model for the human disorder multiple sclerosis.[38,39] EAE can be transferred by T-cell lines, affording both active and passive models of disease. Reagents that block inflammatory cell recruitment to the CNS, such as very late antigen-4 antibodies or antigen-peptide analogues, strongly suppress EAE, indicating a need for leukocyte migration into the CNS for demyelination to occur.[40–42]

Initial investigation of chemokine expression in EAE suggested a role for these cytokines in the inflammatory process. Berman et al[43] and Hulkower et al[44] first demonstrated chemokine up-regulation in studies of acute EAE in Lewis rats. This report was confirmed and extended by Godiska, et al,[45,46] who showed up-regulation of multiple chemokines in acute EAE of SJL mice; reimmunization produced clinical worsening accompanied by enhanced chemokine expression. Karpus and colleagues[47] showed that passive transfer of EAE was accompanied by impressive and selective up-regulation of MIP-1α in the CNS of recipient mice. Anti-MIP-1α antibodies delivered *in vivo* blocked disease, whereas treatment of T cells with MIP-1α antibodies during *in vitro* stimulation was ineffective.[47] These results indicated that a single β-chemokine could exert an essential, nonredundant function in passive transfer EAE. We previously demonstrated MCP-1 expression by astrocytes *in vivo* in acute EAE.[48–51] MCP-1 expression was also produced by astrocytes after penetrating cerebral mechanical trauma, a non-immune inflammatory stimulus.[34] Godiska et al[45,46] showed that activated encephalitogenic T cells express MIP-1α, regulated on activation, normal T-cell expressed and secreted (RANTES) and T-cell activation gene 3 *in vitro*, and RANTES was shown *in vivo* to be expressed in inflammatory foci. These results

were supported by Hayashi and coworkers,[52] who demonstrated that cultured astrocytes synthesized MCP-1, whereas MIP-1α was expressed by microglia, the CNS macrophage-like cells. Additionally, expression of T-cell activation gene 3 correlated with encephalitogenic potential for individual T-cell clones.[53]

In the present investigation, we determined the temporal profile of expression and cellular sources for five chemokines from both α- and β-subfamilies during spontaneous relapses of Ch-R EAE. We observed synchronous chemokine up-regulation in brain and spinal cord during clinical relapse, with expression confined to the CNS. MCP-1, interferon γ-inducible protein of 10 kd (IP-10), and KC were synthesized by astrocytic cells, whereas MIP-1α and RANTES were elaborated by leukocytes within inflammatory foci. The stringent, lineage-specific regulation of individual chemokines in this complex inflammatory process may provide a model system for addressing tissue-specific control of gene expression. These observations identify local chemokine production as a component of the immune-mediated inflammatory cytokine cascade that leads to symptom formation in Ch-R EAE.

Materials and Methods

Induction and Clinical and Histological Evaluation of Ch-R EAE

Female (SWR × SJL/J)F_1 mice were bred in the animal facility of the Cleveland Clinic Foundation. They were immunized between 8 and 10 weeks of age with an encephalitogenic proteolipid protein (PLP) peptide representing residues 139 to 151 (PLPp:139–151) as previously described.[39] Seven control animals were immunized with bovine serum albumin (BSA). Additionally, three unimmunized animals of the same age and sex were used as enzyme-linked immunosorbent assay (ELISA) controls for chemokine protein studies. After immunization all mice were weighed and examined daily for clinical signs of EAE. The following clinical scoring scale was used: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; and 5, moribund state.[39] Relapse onset was defined as the day when new clinical signs appeared, and onset of remission was defined as the day when clinical signs improved. Confirmatory evidence of attack onset and remission onset was loss or gain of 5 to 10% of body weight in a single day. Mice were sacrificed by cervical dislocation, as approved by the Animal Research Committee of the Cleveland Clinic Foundation, in compliance with the Public Health Service policy on humane care and use of laboratory animals. Half of the brain and spinal cord and pieces of liver, spleen, and muscle were fixed in neutral 10% phosphate-buffered formalin and stained with hematoxylin and eosin and Luxol fast blue for histological evaluation.

Table 1. *PCR Primers Used in this Study*

MCP-1	
Forward	5′-AGAGAGCCAGACGGAGGAAG-3′
Backward	5′-GTCACACTGGTCACTCCTAC-3′
IP-10	
Forward	5′-CAACCCAAGTGCTGCC-3′
Backward	5′-GGGAATTCACCATGGCTTGACCA-3′
KC	
Forward	5′-TCGCTTCTCTGTGCAGCGCT-3′
Backward	5′-GTGGTTGACACTTAGTGGTCTC-3′
RANTES	
Forward	5′-TTTGCCTACCTCTCCCTAGAGCTG-3′
Backward	5′-ATGCCGATTTTCCCAGGACC-3′
MIP-1α	
Forward	5′-TTCTGCTGACAAGCTCACCCTC-3′
Backward	5′-GAGGAACGTGTCCTGAAGTCTTTC-3′

RNA Extraction

Animals (two to four per each time point) were sacrificed at days 1 to 4 relative to onset of the first relapse (second attack of the disease) and at days 1, 2, 4, 6, and 8 relative to onset of the following remission (second remission). Two animals were sacrificed during remission preceding the first relapse (first remission). BSA-immunized animals (n = 7) did not develop any abnormal clinical signs and were sacrificed at similar time points after immunization as PLP-immunized animals. Half of each brain and spinal cord and pieces of liver, spleen, and muscle were homogenized in guanidinium isothiocyanate for preparation of total cellular RNA by centrifugation through a cesium chloride cushion, as described.[49] Chemokine mRNA expression in two CNS sites (spinal cord and brain) was determined. Systemic immune activation was monitored by assay of hepatic, splenic, and muscle chemokine mRNA expression.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

First-strand cDNA was synthesized using 1 μg of total cellular RNA. RT with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) was performed as previously described[49] for IP-10, MCP-1, and KC using gene-

specific backward primers (Table 1). For MIP-1α and RANTES, oligo(dT) primers were used (Life Technologies, Inc., Gaithersburg, MD). The product of this reaction was amplified by PCR using *Taq* DNA polymerase (Boehringer Mannheim). Before amplification, cDNA templates were denatured at 94°C for 3 minutes (MCP-1, IP-10, and KC) or 30 seconds (MIP-1α and RANTES) and then amplified. PCR conditions were: MCP-1, IP-10, and KC, 20 cycles (94°C for 2 minutes, 60°C for 2 minutes, and 72°C for 2 minutes); for MIP-1α, 35 cycles (94°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute); and for RANTES, 25 cycles (94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1 minute). PCR cycle numbers that provided a linear relation between PCR products and cDNA input were determined empirically as previously described.[49] Negative technical controls (without the RT product) were used for each set of reactions. Amplification of tubulin transcripts confirmed intact RNA in all samples.

Dot-Blot Hybridization Analysis of Chemokine cDNA

PCR reaction products were denatured (2 mol/L NaOH, 1 mol/L Tris-HCl, pH 8.0, 80°C, 15 minutes), diluted in 6 × standard saline citrate (three fourfold dilutions), transferred to a nylon membrane (GeneScreen; DuPont, Boston, MA) using a vacuum blotter (V&P Scientific, San Diego, CA), ultraviolet cross-linked (Stratalinker 1800; Stratagene, La Jolla, CA), and air dried. Membranes were subjected to DNA hybridization analysis by prehybridization for 3 hours at 42°C, followed by hybridization with cDNA probes in hybridization solution for 16 hours at 42°C and washing at high stringency.[50] Hybridization probes were radiolabeled with [^{32}P]dCTP (DuPont, Wilmington, DE) by nick translation. The hybridization signal was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). We previously showed that in our assay hybridization, signal intensities for individual samples obtained by Southern blotting and dot-blot hybridizations were identical.[49] Data were analyzed using the Kruskal-Wallis test for nonparametric data.

ELISA Analysis of Chemokine Expression

Chemokine mRNA and protein were assessed in separate groups of mice that were immunized and monitored in parallel. Tissues for chemokine protein analysis were obtained from animals during different stages of Ch-R EAE. Four mice were sacrificed during first remission, eight mice during the first relapse, and four mice during the second remission. Tissues from three nonimmunized animals of the same age served as controls. The posterior half of each right brain hemisphere, including the cerebellum and brainstem, the lumbar half of each spinal cord, and a piece of liver were snap frozen in liquid nitrogen. Specimens were homogenized in antiproteinase buffer (500 μl/sample), containing 1 × PBS, 2 mmol/L phenylmethylsufonyl fluoride (Sigma Chemical Co., St. Louis, MO), and 1 μg/ml each antipain (Sigma), aprotinin (Sigma), leupeptin (Sigma), and pepstatin A (Sigma), using a tissue tearer, and sonicated on ice. Debris was removed by centrifugation, and the aqueous extract was decanted and stored at −70°C until chemokine ELISA and total protein determination. ELISA was performed by a modification of the procedure described previously.[54] Using the total protein determination, results for chemokine ELISA were normalized and expressed as nanograms per milligram of tissue. The analyses were performed on coded samples.

In Situ *Hybridization (ISH) Analysis of Chemokine mRNA Expression*

IP-10 and MCP-1 cDNA transcription templates were previously described.[50,55] For the other probes, cDNA for KC,[56] MIP-1α[57], and RANTES[58] served as transcription templates. *In vitro* transcription to generate sense and antisense hybridization probes incorporating tritiated UTP and CTP were performed as described.[48,50] For ISH, probe concentrations were normalized for probe length, with the mass of the probe per tissue section held constant. Hybridizations were performed at the predicted melting temperature −25°C, washes at melting temperature −8°C, and emulsion autoradiography for 4 weeks; after development, sections were lightly counterstained with hematoxylin. Sense strand probes were used for each tissue section to establish baseline hybridization to genomic DNA and nonspecific background. To avoid selection bias, slides were initially read in a blinded fashion regarding hybridization probe, hybridization probe polarity, and day of sacrifice relative to immunization or disease onset. Initial ISH using a β-actin probe was used to confirm the presence of detectable mRNA in all cell types and to verify that the quality of tissues and technical aspects of ISH were uniform.

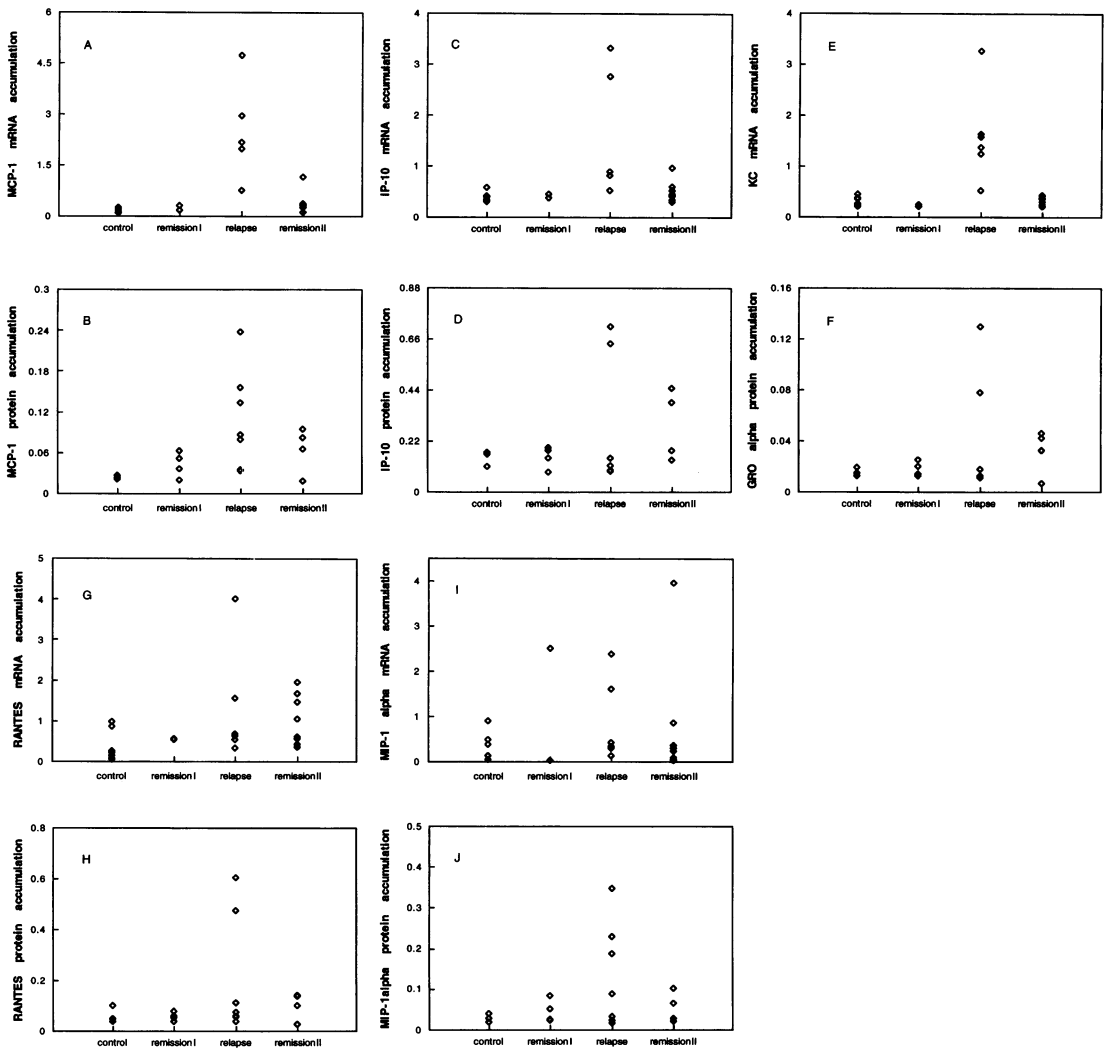


Figure 1. *Elevated chemokine mRNA and protein expression in the brain during relapses of Ch-R EAE. Parallel cohorts of mice were evaluated for brain content of chemokine mRNA and protein during the course of Ch-R EAE. Mice were sacrificed at the following stages of disease: remission I (clinical improvement after the acute first attack), first relapse (spontaneous, second disease attack), and remission II (improvement after the first relapse). Mice sacrificed at the time of peak severity of symptoms, days 2 and 3 after symptom onset, were characterized for chemokine expression during relapse. For mRNA analysis, sample sizes were: remission I, n = 2; relapse, n = 5; and remission II, n = 8. Tissues from seven mice immunized with BSA served as controls for mRNA analysis. Chemokine mRNA accumulation was evaluated by semiquantitative RT-PCR dot-blot hybridization assay with subsequent PhosphorImager analysis, with results shown in arbitrary densitometric units* $\times 10^{-5}$. *For protein analysis, a separate cohort of mice was studied, and sample sizes were: remission I, n = 4; relapse, n = 7; and remission II, n = 4; homogenates from three nonimmunized mice provided controls for protein assays. The chemokine protein content was assessed by ELISA, and results are presented as nanograms of chemokine per milligram of brain homogenate.* A *and* B: *show individual data points for brain MCP-1 mRNA and protein content, respectively.* C *and* D: *IP-10;* E *and* F: *KC (KC protein was assayed by ELISA with antibodies to human GRO-α);* G *and* H: *RANTES;* I *and* J: *MIP-1α.*

Colocalization of ISH Signal with Immunohistochemistry for Glial Fibrillary Acidic Protein

To confirm that cells expressing mRNA for selected chemokines were astrocytes, we combined ISH analysis with immunohistochemistry for the astrocytic marker glial fibrillary acidic protein as described previously.[48]

Results

CNS Chemokine Expression during Relapses of EAE

Mice were sacrificed at varying stages of Ch-R EAE, based on neurological symptoms. Chemokine expression was monitored at the mRNA level with semiquantitative RT-PCR dot-blot hybridization assays that were optimized for each tran-

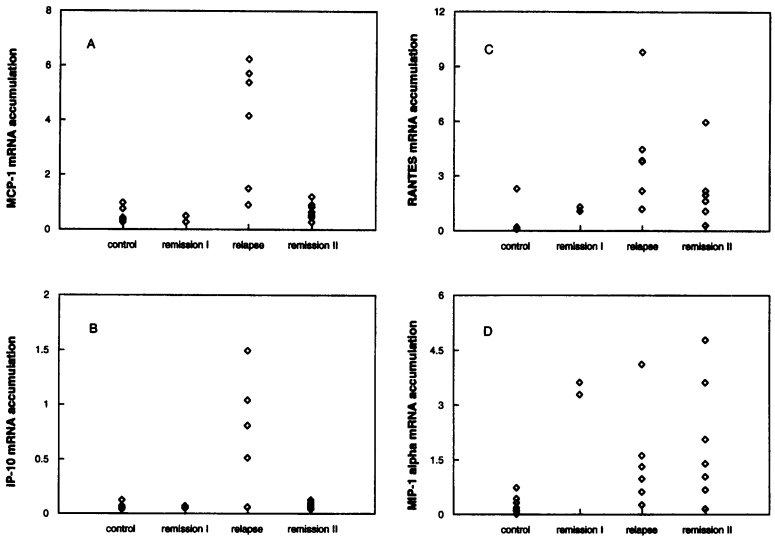


Figure 2. *Elevated chemokine mRNA expression in spinal cord during relapses of Ch-R EAE. RNA prepared from spinal cord was examined in a subset of mice described in Figure 1 at the following stages of disease: remission I (n = 2), relapse (n = 6), and remission II (n = 7). Spinal cord RNA from seven mice immunized with BSA served as controls. Chemokine mRNA accumulation was evaluated by semiquantitative RT-PCR dot-blot hybridization assay with subsequent PhosphorImager analysis, with results shown in arbitrary densitometric units* $\times 10^{-5}$. A: *MCP-1 mRNA expression;* B: *IP-10;* C: *RANTES;* D: *MIP-1α.*

script. Chemokine protein was assayed by ELISA and normalized to the protein content of the total brain homogenate. By the time that mice had recovered from the acute first attack of EAE, brain chemokine protein levels returned to baseline. This regulation was mirrored at the mRNA level (Figure 1, compare control with remission I). Results were highly uniform for all chemokine mRNAs except MIP-1α, which showed increased intersample variability, as individual mice occasionally exhibited very high levels of MIP-1α mRNA in the brain or spinal cord during remission (Figures 1I and 2D, remission I and remission II).

During spontaneous disease relapse, brain levels of chemokines rose in coordinate fashion (Figure 1, compare remission I with relapse). Comparing relapse with control, the total brain content of mRNAs encoding MCP-1 (P = 0.006), IP-10 (P = 0.012), and KC (P = 0.003) rose significantly. RANTES mRNA showed a tendency (P = 0.086) to significance. The brain content of chemokine protein increased twofold to threefold during EAE relapse (Figure 1, B, D, F, and H). This magnitude of increase in whole-brain homogenate was notable, because CNS chemokine expression is highly focal (see ISH analysis below). During remission from this first relapse, chemokine mRNA levels rapidly declined (Figure 1, compare relapse with remission II). Protein levels decayed at varying rates, with persistent elevations of KC and IP-10, presumably reflecting differential stability of chemokine proteins in the CNS (Figure 1).

Four chemokine mRNAs (MCP-1, MIP-1α, RANTES, and IP-10) were monitored in the spinal cord during relapses. The patterns of chemokine mRNA expression in the spinal cord and brain (Figure 2) were highly similar, indicating coordinate chemokine regulation throughout the CNS during relapses of Ch-R EAE, as previously described for acute first attacks of EAE.

Hepatic, Spleen, and Muscle Chemokine Expression during Relapses of EAE

To determine whether chemokine expression during relapse was confined to the CNS, we monitored hepatic chemokine mRNA and protein expression as well as chemokine mRNA expression in the spleen and muscle. Our prior studies showed that early hepatic chemokine expression reflected systemic immune activation: in mice immunized with PLPp: 139–151, hepatic chemokine expression preceded clinical and histological signs of acute EAE by 2 to 7 days.[49] Moreover, intravenous injections of interferon γ or tumor necrosis factor α up-regulated hepatic chemokine expression.[55,59] We monitored hepatic chemokine mRNA and protein levels in parallel with CNS chemokines in mice with Ch-R EAE. Hepatic chemokine expression was not elevated at any time point of this study (Figure 3). Chemokine mRNA levels in spleen and muscle also failed to rise during relapses of EAE (not shown). The results indicated that clinical relapse of EAE was dissociated from systemic immune activation and was coupled to localized CNS-specific chemokine production.

Kinetics of Chemokine CNS mRNA Expression during Relapse of Ch-R EAE

Brain mRNA expression for chemokines MCP-1, IP-10, and KC on the first day of relapse was

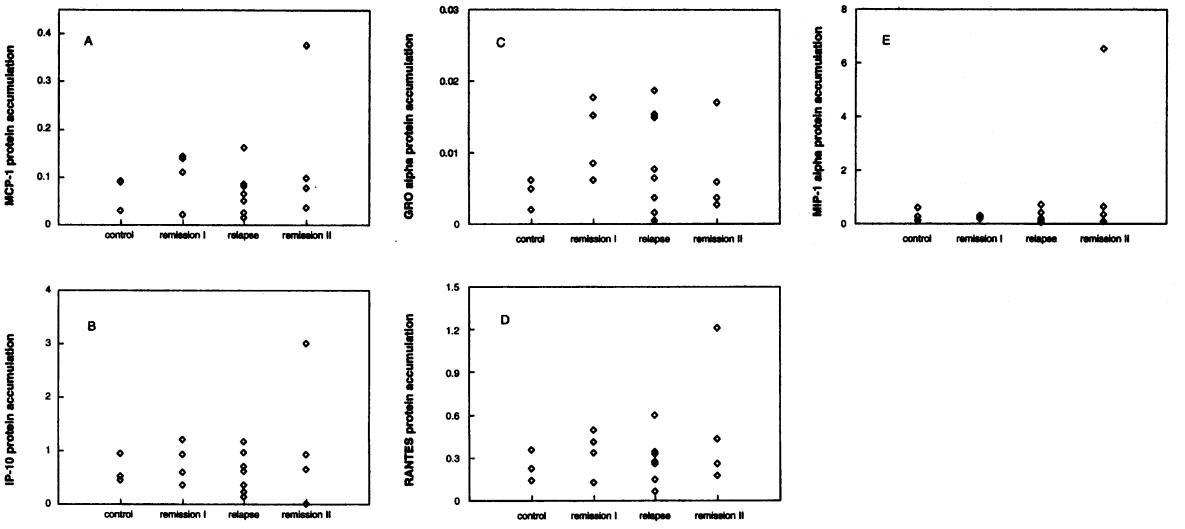


Figure 3. *Chemokine protein expression in liver during Ch-R EAE. Mice were sacrificed at the following stages of disease: remission I, relapse, and remission II. Three nonimmunized mice served as controls. Protein content was assessed by ELISA with results, presented as nanograms per milligram of liver homogenate.* A: *MCP-1;* B: *IP-10;* C: *KC (assayed with human GRO-α antibodies);* D: *RANTES;* E: *MIP-1α.*

similar to that observed during the preceding remission (day 0) (Figure 4A). On day 2 of relapse, brain levels of these three chemokines peaked and then declined by day 4 to concentrations typically seen during remission (Figure 4A). In the spinal cord the MCP-1 level was already elevated on day 1 of relapse (not shown), whereas IP-10 mRNA expression did not increase until day 2 of relapse. RANTES mRNA expression in the brain was already increased on day 1 of relapse and later declined (Figure 4B). As noted above, MIP-1α mRNA was variably elevated in both the brain and spinal cord at all times after immunization (Figure 4B). MIP-1α demonstrated increased brain protein content during relapse (Figure 1J). This difference between mRNA and protein levels for MIP-1α reflected animal-to-animal variability rather than selective translational control, because distinct cohorts of mice were assayed for protein and mRNA.

Cellular Sources of Chemokines during Relapses of Ch-R EAE

ISH studies were used to identify cellular sources of chemokines in CNS tissues of mice with relapses of Ch-R EAE. Three chemokines (MCP-1, IP-10, and KC) were expressed by parenchymal neuroepithelial cells morphologically resembling astrocytes near inflammatory infiltrates (Figures 5, A–F, and 6, E–H). Colocalization experiments with glial fibrillary acidic protein immunohistochemistry and ISH indicated that IP-10 and MCP-1 were expressed only by astro-

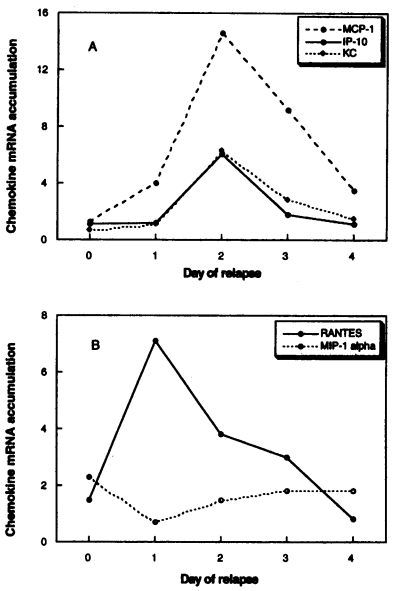
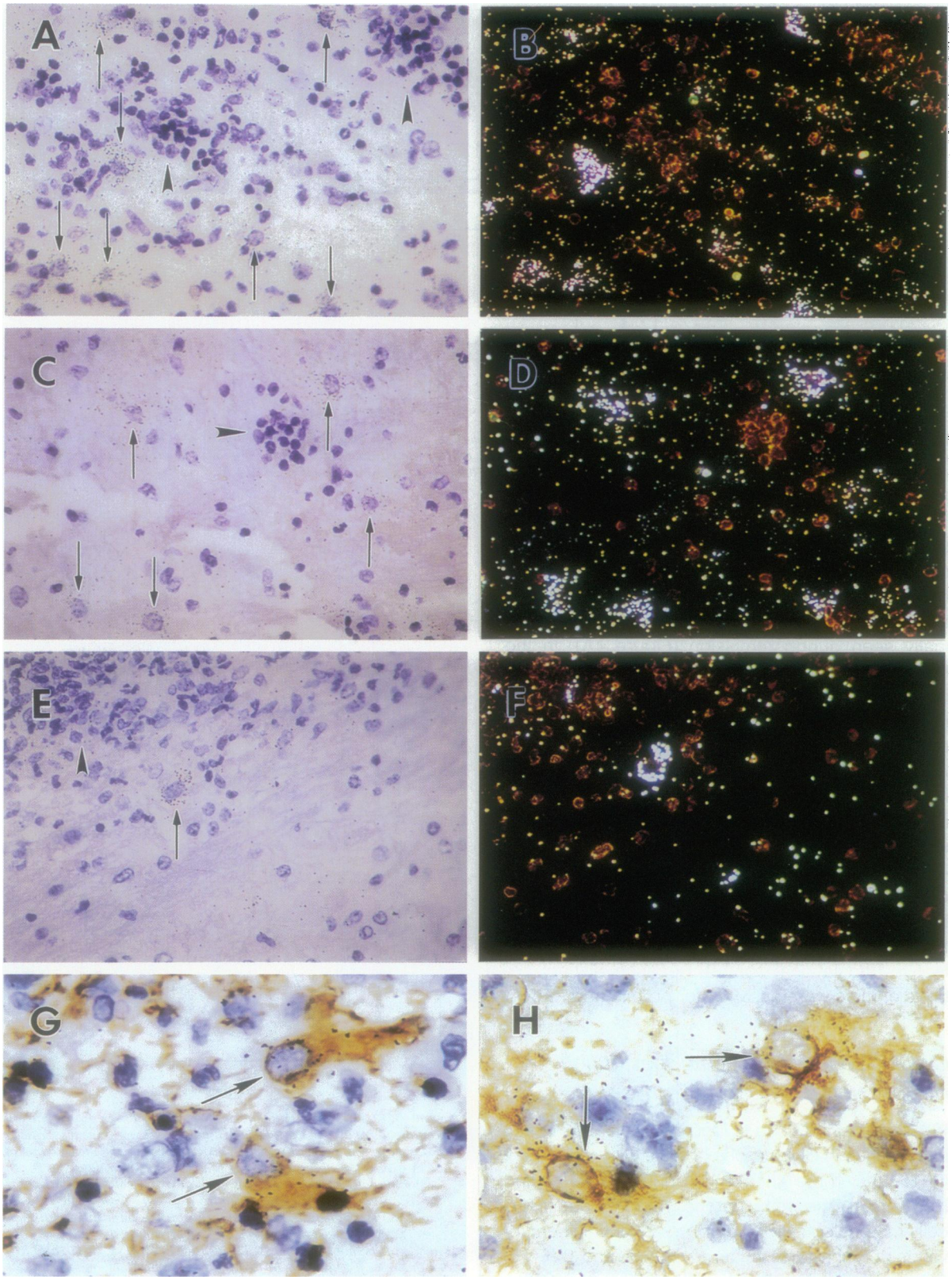


Figure 4. *Kinetics of chemokine mRNA expression in brain during spontaneous relapse of Ch-R EAE. Animals (n = 2 to 3) were sacrificed on days 1 to 4 of the first relapse. Day 0 represents the mean value of mRNA expression observed during remission before relapse (n = 2). Chemokine mRNA expression was evaluated by semiquantitative RT-PCR assay, and results were normalized to the mean value of each chemokine mRNA level detected in brains of BSA-immunized animals (n = 7). Mean levels during remission approximated a value of 1.* A: *Kinetics of chemokines expressed by astrocytes (MCP-1, IP-10, and KC) demonstrates a peak 6- to 14-fold increase in brain chemokine mRNA content during relapse.* B: *Kinetic analysis of chemokines expressed by inflammatory cells (RANTES and MIP-1α) shows a maximal sixfold elevation of RANTES content, with a negligible increase in MIP-1α over levels during remission.*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cytes (Figure 5, G and H). In contrast, two chemokines (MIP-1α and RANTES) were expressed by leukocytes within inflammatory infiltrates (Figure 6, A–D).

Discussion

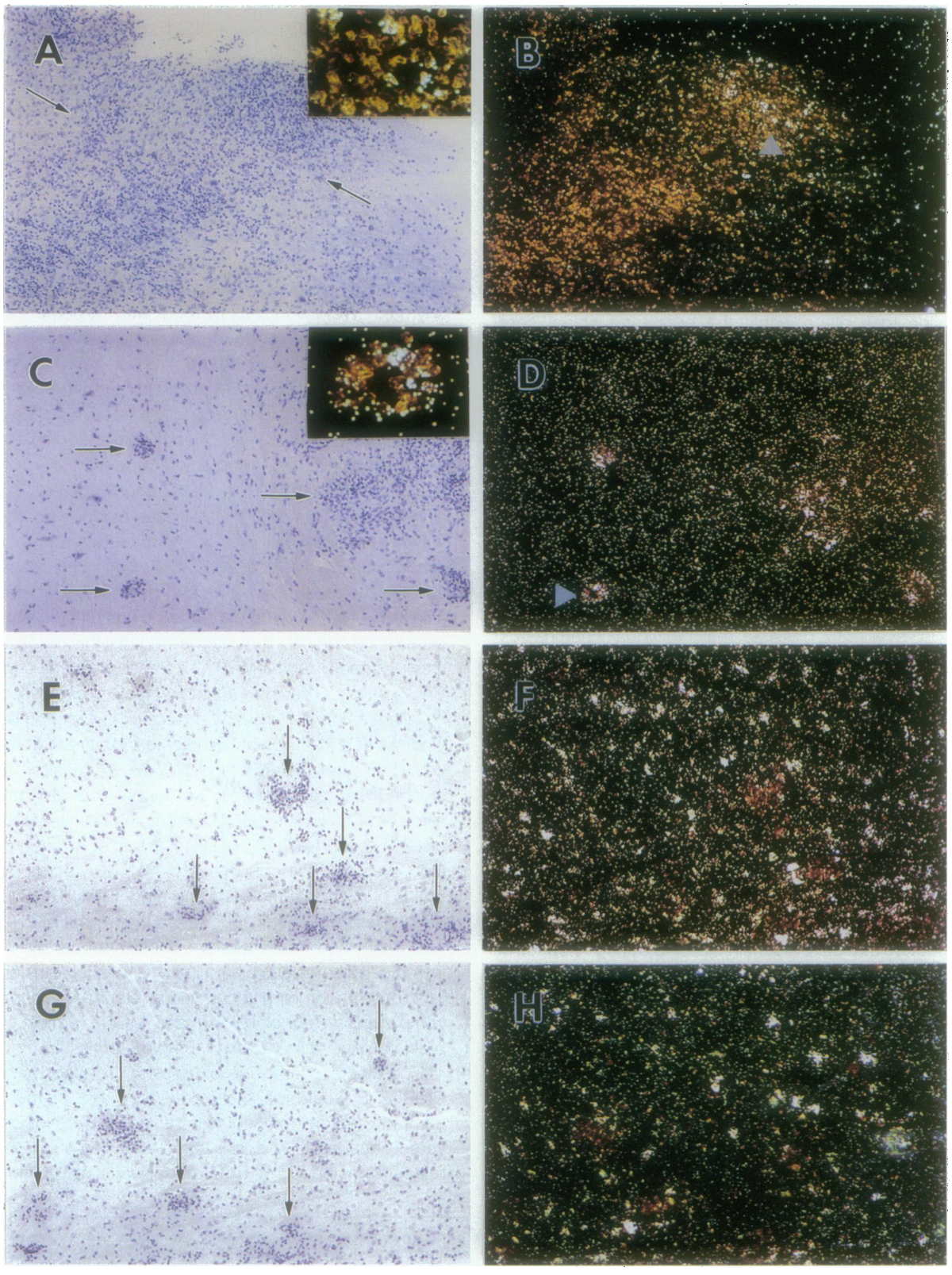
We analyzed time course and cellular sources for chemokines in mice with spontaneous relapses of Ch-R EAE. Surprisingly, five chemokines of both α- and β-chemokine subfamilies were up-regulated during relapses. This enhanced chemokine expression took place within the brain and spinal cord simultaneously and was confined to CNS tissues. Notably, the up-regulation of chemokine mRNA in spinal cords of mice with relapses was more uniform than that observed within brains of affected mice. This difference could reflect the preferential expression of EAE disease activity in the lumbar spinal cord or the sampling error imposed by diluting chemokine mRNA or protein (which was expressed focally) by assaying the total brain RNA or homogenate.[37] Chemokine mRNA and protein levels increased in concert, indicating synthesis within the CNS. By ISH analysis, there were two separate sources of chemokines within affected CNS tissues: parenchymal neuroepithelial cells produced MCP-1, IP-10, and KC; and leukocytes in inflammatory foci expressed MIP-1α and RANTES. Colocalization studies showed that MCP-1 and IP-10 were expressed by astrocytes. The nuclear morphology and location of KC-expressing cells suggested that they are astrocytes, although the formal identity of cells that express KC remains to be established. MIP-1α and RANTES were produced exclusively by mononuclear leukocytes in perivascular cuffs. The results indicate stringent regulation of chemokine expression *in vivo* by factors that are present in the CNS during relapses of Ch-R EAE and elicit a coordinate response from cells of diverse lineage. These observations support the possibility that chemokine expression may be integral to the pathogenesis of immune-mediated inflammation in the CNS.

Chemokine function in Ch-R EAE was not directly addressed in these studies. Several lines of evidence favor an important function for chemokines in CNS inflammation.[18] Karpus and colleagues[47] showed that injections of anti-MIP-1α antibodies could block passive transfer of EAE, indicating that function of MIP-1α in the intact inflammatory environment was essential for passive transfer disease. Studies in tg mice suggest unusual potency for chemokines in attracting cells from vasculature into the CNS compartment. Fuentes and coworkers[35] demonstrated that expression of MCP-1 in oligodendroglia of tg mice could recruit monocytes into the perivascular spaces of the CNS compartment, and that these cells could be activated by intraperitoneal injections of lipopolysaccharide to enter the parenchyma.[35] We found that tg mice with targeted expression of KC in oligodendroglia exhibited a striking phenotype of massive neutrophil entry into CNS parenchyma.[36] Therefore, we propose that chemokine expression in the CNS of mice with Ch-R EAE is likely to have functional consequences for leukocyte recruitment during the pathological process.

We found that MCP-1, IP-10, and KC were expressed by astrocytes in this disease model. This observation is consistent with previous reports from our laboratory in studies of acute EAE, as well as the tissue culture studies of Hayashi et al[52] and Vanguri.[60] We also found MCP-1 to be expressed by astrocytes after penetrating cortical injury, suggesting that this cell type may be specialized for MCP-1 expression after diverse CNS insults.[34] The finding that RANTES is expressed by inflammatory cells *in vivo* was previously reported by Godiska et al[45,46] in mice with acute EAE; these workers also found RANTES and MIP-1α expression by mononuclear cells *in vitro.* Our current findings may also amplify previous reports concerning relative levels of CNS β-chemokines in EAE. Karpus et al[47] found high levels of MIP-1α but not MCP-1 or MIP-2 protein in brains of mice that received activated, primed T cells during passive transfer. Our data suggest that MIP-1α was expressed in the passive transfer model by injected cells that accumulated in the CNS after transfer. In animals undergoing spontaneous relapse, we found comparable levels of MIP-1α and MCP-1 protein in brain homogenates (0.1 to 0.13 ng/mg of tissue).

Coordinate chemokine expression in relapses of Ch-R EAE would be likely driven by one or more common factors that act differently on cells of diverse lineage. It was shown recently that clinical relapses in the Ch-R EAE model we used in this

Figure 5. *Astrocytes express mRNA for chemokines MCP-1, IP-10, and KC during spontaneous relapse of Ch-R EAE. ISH analysis with radiolabeled chemokine probes was performed on CNS tissues from mice sacrificed on day 1 of disease relapse. MCP-1 ISH:* (**A**) *(brightfield) and* (**B**) *(darkfield) show the same field (magnification, ×250). IP-10 ISH:* (**C**) *(brightfield) and* (**D**) *(darkfield) (×250). KC ISH:* (**E**) *(brightfield) and* (**F**) *(darkfield) (×250).* **Arrows** *on brightfield images indicate hybridization-positive cells;* **arrowheads** *show inflammatory foci, which do not contain chemokine-expressing cells in this analysis. Darkfield images show the hybridization signal as* **white grains.** **G** *and* **H:** *Colocalization of glial fibrillary acidic protein immunohistochemistry, an astroglial marker, and ISH signal for MCP-1 and IP-10, respectively (× 640).* **Arrows** *indicate cells that exhibit colocalization.*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study are invariably associated with epitope spreading, ie, the acquisition of new myelin-specific T-cell reactivities.[38] T cells that recognize spreading epitopes express the T-helper 1 spectrum of proinflammatory products (V. K. Tuohy, unpublished observations). T-helper 1 cytokines include plausible candidates for stimulating chemokine expression: interferon γ and tumor necrosis factor α and β.[61] It will be of interest to establish whether T cells of distinct phenotype regulate CNS chemokine expression differentially.

Chemokine up-regulation occurs after T-cell activation by "spreading" myelin determinants in this model of Ch-R EAE, suggesting that chemokines participate critically in events that culminate in demyelination and clinical signs.[38] In this regard, MIP-1α, MCP-1, and RANTES are potent chemoattractants for cells responsible for demyelination, including lymphocytes, monocytes and macrophages, and microglia.[24,62,63] Macrophage activation has also been attributed to MCP-1 and RANTES and is implicated in antigen-independent T-cell stimulation.[64,65] Other proinflammatory functions of chemokines include basophil degranulation for MCP-1 and chemoattraction of neutrophils for KC.[64,66,67] However, it is possible that individual chemokines may exert opposing functions during CNS inflammation, not all of which need be proinflammatory. For example, IP-10 was recently characterized as an angiostatic agent that blocked the angiogenic effects of interleukin-8 (KC is a comparable murine product).[68] MCP-1 antibodies, but not MIP-1α antibodies, block the induction of oral tolerance to human γ-globulin.[69] Therefore, it is plausible that the varied chemokines expressed during relapses of Ch-R EAE could have distinct and even antagonistic functions during the development and resolution of inflammation. To address these issues, intervention to block function of individual chemokines will be needed, and safe, effective chemokine-directed therapy for CNS disorders will require understanding of the regulation and function of the individual chemokines.

Chemokine expression by cells of different lineages establishes spatially distinct chemokine gradients within the CNS parenchyma. Such gradient separation provides a means for orchestrating differential inflammatory cell movement patterns within the developing lesion.[70] Selective disruption of individual components of the coordinated chemokine response holds promise of defining the roles of single chemokines in lesion formation and may ultimately provide information needed for effective therapeutic intervention in autoimmune demyelinating disease.

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Figure 6. *Diverse cellular sources of chemokines during spontaneous relapse of Ch-R EAE. ISH analysis was performed on tissue sections from the CNS of animals sacrificed on day 1 of relapse.* **A** *(brightfield) and* **B** *(darkfield) represent ISH analysis of MIP-1α expression (magnification, ×64).* **Arrows** *indicate a large inflammatory infiltrate in which MIP-1α is expressed.* **Inset** *in* **A**: *High-power view of the ISH-positive cells in the region of the inflammatory focus indicated by the* **arrowhead** *in* **(B)** *(×250).* **(C)** *(brightfield) and* **(D)** *(darkfield) show ISH analysis for RANTES (×64).* **Arrows** *indicate inflammatory foci that are sites of RANTES expression.* **Inset** *in* **C**: *Higher magnification (×250) of one inflammatory focus indicated by the* **arrowhead** *in* **(D)**. *For comparison,* **(E)** *(brightfield) and* **(F)** *(darkfield) represent ISH analysis of MCP-1 expression (×64);* **(G)** *(brightfield) and* **(H)** *(darkfield) show ISH detection of IP-10 (×64).* **Arrows** *indicate inflammatory foci, from which the chemokine hybridization signal is excluded.*

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