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Chemokine Gene Expression in the Brains of Mice with Lymphocytic Choriomeningitis†

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Chemokines are pivotal in the trafficking of leukocytes. In the present study, we examined the expression of multiple chemokine genes during the course of lymphocytic choriomeningitis (LCM) in mice. In noninfected mice, no detectable chemokine gene expression was found in the brain; however, by day 3 postinfection, the induction of a number of chemokine mRNAs was observed as follows (in order from the greatest to the least): cytokine responsive gene-2 or interferon-inducible 10-kDa protein (Crg-2/IP-10), RANTES, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1β), and MCP-3. At day 6 postinfection, the expression of these chemokine mRNAs was increased, and low expression of lymphotactin, C10, MIP-2, and MIP-1α mRNAs was detectable. Transcript for T-cell activation-3 was not detectable in the brain at any time following LCM virus (LCMV) infection. With some exceptions, a pattern of chemokine gene expression similar to that in the brain was observed in the peripheral organs of LCMV-infected mice. Mice that lacked expression of gamma interferon developed LCM and had a qualitatively similar but quantitatively reduced cerebral chemokine gene expression profile. In contrast, little or no chemokine gene expression was detectable in the brains of LCMV-infected athymic mice which did not develop LCM. Expression of Crg-2/IP-10 RNA was localized to predominantly resident cells of the central nervous system (CNS) and overlapped with sites of viral infection and immune cell infiltration. These findings demonstrate the expression of a number of chemokine genes in the brains of mice infected with LCMV. The pattern of chemokine gene expression in LCM may profoundly influence the characteristic phenotype and response of leukocytes in the brain and contribute to the immunopathogenesis of this fatal CNS infection.

Leukocyte recruitment and infiltration of the central nervous system (CNS) is a cardinal feature in the pathogenesis of diverse inflammatory neurological disorders such as bacterial and viral meningoencephalitis, multiple sclerosis, human immunodeficiency virus encephalopathy, and cerebral ischemia. Chemokines are a novel family of chemoattractant cytokines that are important in leukocyte adhesion to the endothelium and emigration into tissues during inflammation (41). Individual chemokines exhibit sequence homology and structural similarity and are members of three related gene families distinguished on the basis of four conserved cysteine residues. The α-chemokines are characterized by two cysteines separated by another amino acid (CXC), while the β-chemokines have two adjacent cysteine residues (CC). Finally, a third family of chemokines containing a single cysteine residue with only one member, named lymphotactin, has recently been identified. Synthesized by a wide variety of cell types, the members of the α-chemokine family act primarily on neutrophils, while the majority of β-chemokines are monocyte chemoattractants, although some β-chemokines, such as RANTES, are also chemotactic for T cells (34, 41). Lymphotactin is produced by activated T cells and seems to be exclusively a T-cell chemoattractant (25). These small (8 to 12 kDa) inducible chemoattractants are early-response genes to inflammatory mediators and, in concert with other cytokines and growth factors, may contribute significantly to the inflammatory response (16, 20).

Intracranial (i.c.) inoculation of immunocompetent adult mice with lymphocytic choriomeningitis virus (LCMV) is followed 6 to 8 days later by convulsive seizures culminating in death. This acute monophasic disease is characterized by infiltrating mononuclear cells in the meninges, choroid plexus, and ependymal membranes (6, 12). These infiltrating cells consist predominantly of lymphocytes as well as macrophages, with few polymorphonuclear leukocytes. Of particular prominence in the immune response to LCMV is the presence of $CD8^+$ cytotoxic lymphocytes, which in addition to removing the virus are the primary effectors of LCM (6, 12). The specific mechanisms underlying the recruitment to the CNS and extravasation of mononuclear cells and the subsequent interactions between these cells that contribute to the pathogenesis of LCM remain important but unresolved issues. In the past, studies addressing these issues have focused on the involvement of antiviral and proinflammatory cytokines in the pathogenesis of LCM. Findings indicate that a number of these cytokines are expressed during the development of LCM, particularly the type I (alpha/beta interferon [IFN-α/β]) and type II (IFN-γ) interferons (8, 17, 23, 29, 38). While these cytokines no doubt have a significant impact on the development of the inflammatory response, they are relatively ineffective in promoting the recruitment and tissue infiltration of leukocytes at sites of infection or injury.

In view of the critical part played by infiltrating the immunoinflammatory cells in the development of LCM, we hypothesized that chemokines may be an important regulatory component of the cerebral recruitment and extravasation of these cells. Therefore, the objective of this study was to examine chemokine expression profiles and their relationship to the evolution of disease in mice infected i.c. with LCMV.

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TABLE 1. Chemokine cDNA target sequences used to derive the chemokine RPA probe set

Target	Sequence	Length (bp)	GenBank accession no.	Reference
Lymphotactin	30–360	330	U15607	25
C10	710–1025	315	M58004	33
MIP-2	66–336	270	X53798	45
MCP-3	105–344	239	Z12297	26
MIP-1β	86–296	210	X12531	11
TCA-3	3985–4175	190	X52401	7
MCP-1	1892–2062	170	M19681	36
Crg-2/IP-10	221–361	140	J05576	46
MIP-1α	909–102	120	X53372	19
RANTES	2303–2413	110	U02298	10
rpl32	61–139	78	K02060	13

MATERIALS AND METHODS

Mice and infection with LCMV. Male euthymic BALB/c, athymic BALB/*nu*$^+$ *nu*$^+$ or homozygous GKO (IFN-γ gene disrupted) (9) mice were maintained under pathogen-free conditions in the closed breeding colony of The Scripps Research Institute and were used at 8 to 10 weeks of age. LCMV Armstrong (ARM) strain 53b stock was obtained from a triple plaque-purified clone subsequently passaged twice in BHK cells (14). For the induction of LCM, mice were inoculated i.c. with either 25 μl of phosphate-buffered saline (PBS) alone (control, noninfected) or PBS containing 200 PFU of LCMV. At this dose of LCMV, infected BALB/c mice and GKO mice died between days 6 and 7. In contrast, athymic nude mice displayed no signs of illness.

RNA preparation. Mice were killed at various times postinoculation, and their organs were immediately removed, snap frozen in liquid nitrogen, and stored at −80°C until RNA preparation. Poly(A)$^+$ RNA was prepared according to a previously described method (2). Briefly, frozen organs were placed in 10 ml of lysis buffer (0.2 M NaCl, 0.2 M Tris-HCl [pH 7.5], 1.5 mM $MgCl_2$, 2% sodium dodecyl sulfate, 200 μg of proteinase K per ml) and were immediately homogenized. After incubation for 90 min at 45°C, the NaCl concentration of the lysate was adjusted to 0.5 M and mixed with 30 mg of oligo(dT) cellulose (In Vitrogen, San Diego, Calif.) that had been pre-equilibrated in binding buffer (0.5 M NaCl, 0.01 M Tris-HCl [pH 7.5]). The mixture was then incubated at 25°C for 90 min, with gentle rocking. Following washings with binding buffer, poly(A)$^+$ RNA was eluted from the oligo(dT) cellulose with 0.5 ml of elution buffer (0.01 M Tris-HCl, pH 7.5) and precipitated in ethanol, dried, and resuspended in 25 μl of elution buffer. The concentration of RNA was determined by UV spectroscopy at 260 nm.

Plasmid constructs. For the RNase protection assay (RPA), the specific target sequences used to generate probes against individual chemokines are listed in Table 1. CXC chemokines, macrophage inflammatory protein-2 (MIP-2), and cytokine responsive gene-2 or interferon-inducible 10-kDa protein (Crg-2/IP-10), which are chemotactic for neutrophils, monocytes, and lymphocytes, respectively, and the CC chemokines C10, T cell activation-3 (TCA-3), monocyte chemotactic protein-1 (MCP-1), MCP-3, MIP-1α, MIP-1β, and RANTES, which are principally chemotactic for monocytes and lymphocytes, were included in the RPA probe set. Lymphotactin (25), which is chemotactic for lymphocytes but not for monocytes or neutrophils and belongs to a novel chemokine family, was also included in the set. The cDNA fragments for these different chemokines were synthesized by reverse transcription-PCR from livers and spleens of lipopolysaccharide-treated mice with specific oligonucleotide primers flanked by *Hin*dIII (antisense primer) and *Eco*RI (sense primer) sites. The genomic clone RPL32-4A (13), kindly provided by M. Hobbs (The Scripps Research Institute), served as a probe for the ribosomal protein L32 and was included as an internal control for RNA loading. The primers were designed to generate fragments of desired lengths that could conveniently be separated on a standard polyacrylamide sequencing gel. After PCR, the amplified fragments were incubated with polynucleotide kinase (Promega, Madison, Wis.), ligated with T4 ligase (Promega), subsequently digested with *Hin*dIII/*Eco*RI (Promega), and then ligated into pGEM4 (Promega). The specific identity of each chemokine clone was subsequently verified by sequencing analysis. The orientation of the fragment allows antisense and sense RNA synthesis from the flanking T7 and SP6 RNA polymerase promoters, respectively. Unlabeled sense RNA for each chemokine and for the ribosomal protein RPL32 was synthesized with 5 μg of *Hin*dIII-linearized template, 40 U of SP6 RNA polymerase (Ambion, Austin, Tex.), ribose-ATP (rATP), rCTP, rGTP, and rUTP (each at 2.5 mM), and 10 mM dithiothreitol (DTT) (all from Promega). After 2 h of incubation at 37°C, 5 U of DNase I (Ambion) was added to the reaction. The final product was phenol-chloroform extracted, ethanol precipitated, resuspended in Tris-EDTA (TE), and stored at −80°C.

RPA. The RPA was performed by a previously described method (8). For the synthesis of a radiolabeled antisense RNA probe set for the chemokines and the loading control RPL32, the final reaction mixture (10 μl) contained 120 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; Andotek, Irvine, Calif.), UTP (73 pmol), GTP, ATP, and CTP (2.5 mmol each), DTT (100 nmol), transcription buffer (1×), RNase inhibitor (20 U; Ambion), T7 polymerase (10 U; Promega), and an equimolar pool of *Eco*RI-linearized templates (15 ng each). After 1 h at 37°C, the mixture was treated with DNase I (2 U; Ambion) for 30 min at 37°C and the probe was purified by extraction with phenol-chloroform and precipitated with ethanol. Dried probe was then dissolved (2.6×10^5 dpm/μl) in hybridization buffer [80% formamide–0.4 M NaCl–1 mM EDTA–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4)], and 10 μl of this was added to the tubes containing target RNA dissolved in TE. The samples were overlaid with mineral oil, heated to 95°C, and then incubated at 56°C for 12 to 16 h. Single-stranded RNA was digested for 45 min at 30°C by the addition of a mixture of RNase A (0.2 mg/ml) and RNase T_1 (50 U/ml; Promega) in 10 mM Tris (pH 7.5)–300 mM NaCl–5 mM EDTA (pH 8). After incubation, 18 μl of a mixture containing proteinase K (1.5 mg/ml; Boehringer Mannheim, Indianapolis, Ind.), sodium dodecyl sulfate (3.5%), and yeast tRNA (200 μg/ml; Sigma) was added, and the samples were incubated for 30 more min at 37°C. The RNA duplexes were isolated by extraction and precipitation as described above, dissolved in 80% formamide and dyes, and electrophoresed in a standard 6% acrylamide–7 M urea–0.5% Tris-borate-EDTA sequencing gel. Dried gels were placed on XAR film (Kodak, Rochester, N.Y.) with intensifying screens and exposed at −70°C. For quantification, autoradiographs were scanned (Scanjet 4C/T; Hewlett-Packard, San Jose, Calif.), and band density was assessed with National Institutes of Health image 1.57 software.

In situ hybridization. Anesthetized control and infected mice were perfused transcardially with ice-cold saline followed by 4% paraformaldehyde in PBS (pH 7.4). Brains were removed, postfixed in the same fixative overnight at 4°C, divided along the midline, processed, and embedded in paraffin. Sagittal sections (10 μm) were cut onto polylysine-coated slides and used for in situ hybridization as described previously (8).

For probe, a Crg-2/IP-10 cDNA fragment (726 bp) flanked with 5′ *Xba*I and 3′ *Sal*I sites was synthesized by reverse transcription-PCR from livers of lipopolysaccharide-treated mice with oligonucleotide primers specific to the reported sequence for murine Crg-2 (46) and cloned in pGEM4. Antisense Crg-2/IP-10 probe was synthesized with T7 polymerase. To detect sites of viral replication, a 2.0-kb cDNA fragment of the LCMV-ARM nucleoprotein (NP) subcloned in pGEM-3Z was used to generate an antisense NP probe (42). For synthesis of labeled probes, the reaction mixture (25 μl) contained 250 μCi ^{35}S-CTP (~800 Ci/mmol; Amersham, Arlington Heights, Ill.), ATP, UTP, GTP (5 nmol each), DTT (50 nmol), transcription buffer (1×) (all from Promega), RNasin (20 U) (Ambion), RNA polymerase (20 U) (Promega), and appropriately linearized template (1.5 μg). The mixture was incubated for 2 h at 37°C, at which time RNase-free DNase (20 U of DNase I; Ambion) was added, and the reaction was continued for 30 more min at 37°C. ^{35}S-labeled probe was purified by phenol-chloroform extraction and fractionation through a Sephadex G-50 nick column (Pharmacia, Piscataway, N.J.). The product was concentrated by ethanol precipitation, and after drying, the probe was resuspended in 100 μl of TE solution, pH 8.0, and the specific activity was determined.

Adjacent sections were hybridized for LCMV-NP and Crg-2/IP-10. The specificity of the probes was confirmed by comparison with sections from noninfected brain and sections hybridized to corresponding sense probes.

RESULTS

Cerebral chemokine gene expression after LCMV infection in BALB/c mice. To examine chemokine gene expression after LCMV infection, we developed a multiprobe RNase protection assay for the chemokines lymphotactin, C10, MIP-2, MCP-3, MIP-1β, TCA-3, MCP-1, Crg-2/IP-10, MIP-1α, and RANTES. As shown in Fig. 1A, in the brains of noninfected mice, there was very low expression of MIP-1β, MCP-1, Crg-2/IP-10, and RANTES transcripts and no detectable expression of the other chemokine genes. In the brains of LCMV-infected mice at 3 days postinfection, expression of several of these chemokine genes, i.e., MCP-1, Crg-2/IP-10, and RANTES, was increased, while MCP-3 RNA expression was induced. In particular, Crg-2/IP-10 was found to be predominantly expressed, while levels of MCP-3, MIP-1β, MCP-1, and RANTES were lower. Expression of these chemokine transcripts further increased markedly at day 6 postinfection. In addition, the expression of lymphotactin, C10, MIP-2, and MIP-1α mRNA was also detectable at day 6 after infection. In contrast, there was no detectable expression of TCA-3 mRNA in the brain following LCMV infection. Quantitation of the signal intensities revealed that relative to background, there was a

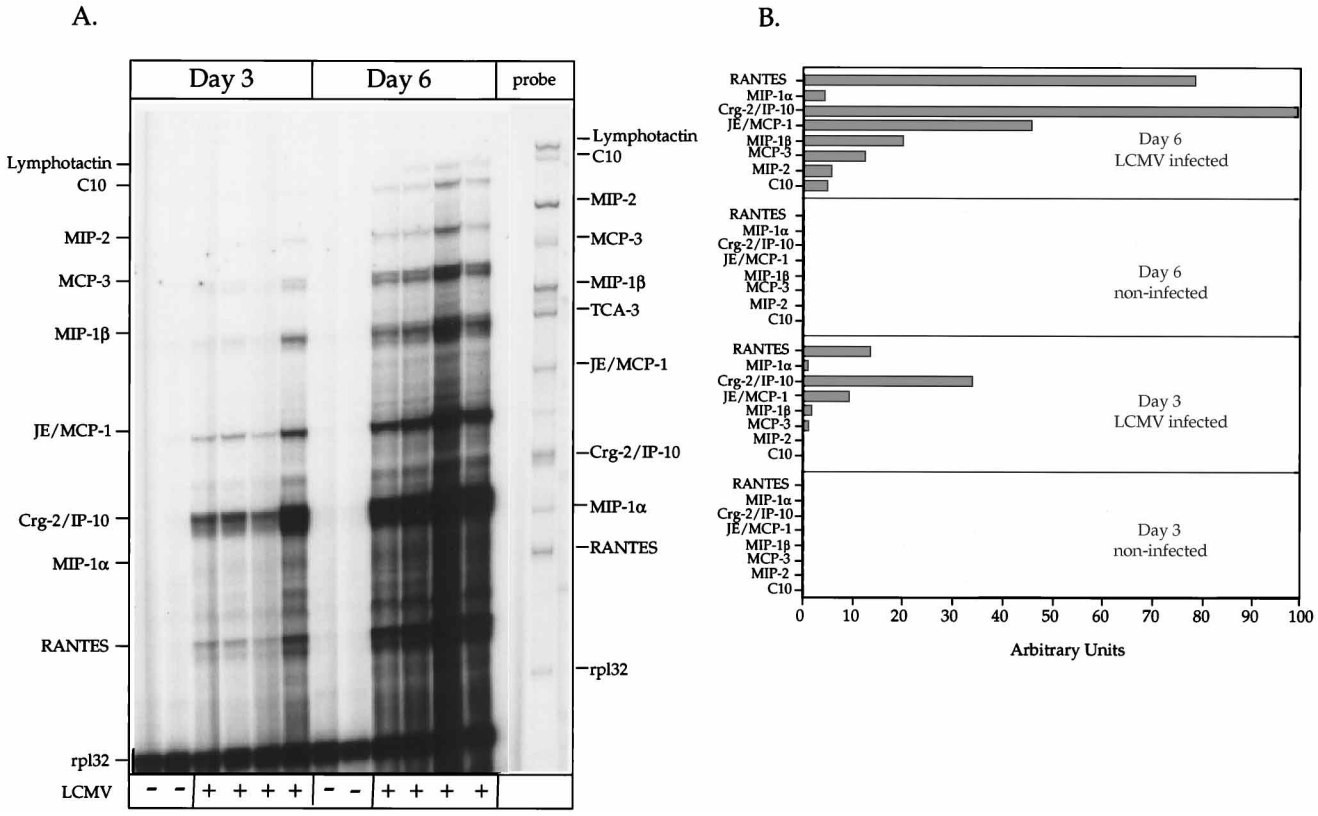

FIG. 1. (A) Chemokine mRNA expression in brain after LCMV infection. In this representative experiment, BALB/c mice were injected i.c. with saline or LCMV (200 PFU), and 2 μg of poly(A)$^+$ RNA isolated from the brain at days 3 and 6 after infection was subjected to RPA as outlined in Materials and Methods. For brain RNA, the sizes of the protected fragments are smaller than the corresponding probe band because of the additional cloning sites present in the probe which are not present in the target RNA. (B) Quantitative analysis of chemokine gene expression. Densitometric analysis of each lane was performed on scanned autoradiographs with National Institutes of Health image software.

35-fold increase in Crg-2/IP-10 and a 10-fold increase in MCP-1 and RANTES mRNA levels in the brains of mice after 3 days of infection (Fig. 1B). Compared with day 3, the level of these chemokine mRNAs as well as those of the MCP-3 and MIP-1β mRNAs increased dramatically at day 6 postinfection, being 3- to 5-fold higher for Crg-2/IP-10 and MCP-1 and 6- to 10-fold higher for RANTES, MIP-1β, and MCP-3.

Chemokine gene expression in peripheral organs during LCMV infection. To determine whether there were regional differences in the pattern of chemokine gene expression during LCM, a comparative study was performed between brain and peripheral organs. The results of representative analysis are shown in Fig. 2. In the brain, the pattern of expression in noninfected mice was similar to that described above. In the kidney, the pattern of chemokine gene expression differed in noninfected mice, and in addition to MIP-1β, MCP-1, Crg-2/IP-10, and RANTES, low levels of lymphotactin, C10, MIP-2, and MIP-1α were constitutively expressed. After LCMV infection, the pattern of chemokine gene expression in the brain at days 3 and 6 was essentially the same as described above. In kidney, lymphotactin, C10, MCP-3, MIP-1β, MCP-1, Crg-2/IP-10, and RANTES mRNAs were all increased at day 3 and remained elevated at day 6. Similar to in brain, Crg-2/IP-10 and RANTES were the predominantly expressed chemokines in kidney; however, no detectable MIP-2, TCA-3, or MIP-1α was observed in the kidneys of LCMV-infected mice. In liver, low levels of TCA-3, Crg-2/IP-10, and RANTES were constitutively expressed in noninfected mice. After LCMV infection, the levels of TCA-3, MCP-1, Crg-2/IP-10, and RANTES transcripts increased at day 3. The levels of TCA-3 and MIP-1α mRNA were further upregulated in the livers of LCMV-infected animals at day 6 postinfection, while C10 was also induced at this time. In the spleens of noninfected mice, lymphotactin, C10, Crg-2/IP-10, MIP-1α, and RANTES were expressed. Following LCMV infection, the levels of chemokine transcripts were markedly upregulated in the spleen after 3 and 6 days of infection, while expression of MIP-1β mRNA was also induced.

Cerebral chemokine gene expression in GKO and athymic mice. To delineate the role of the antiviral immune response in the regulation of cerebral chemokine expression in LCMV infection, we examined GKO-IFN-γ gene-disrupted and athymic nude mice infected with LCMV. In the noninfected GKO mice, no chemokine gene expression was detectable. Consistent with the previous experiments with infected BALB/c mice noted above (Fig. 3A), the induction of several chemokine genes, including C10, MIP-2, MCP-3, MIP-1β, MCP-1, Crg-2/IP-10, MIP-1α, and RANTES, was found in the brain, with expression of Crg-2/IP-10 and RANTES being predominant. In similarly infected GKO mice, these chemokine mRNAs were expressed at somewhat lower levels (Fig. 3A). The most striking difference was a marked decrease of Crg-2/IP-10, RANTES, and MCP-1 mRNA. In the brains from LCMV-infected nude mice, there was no significant alteration in the expression of any chemokine gene transcripts at day 3 or 6 postinfection (Fig. 3B).

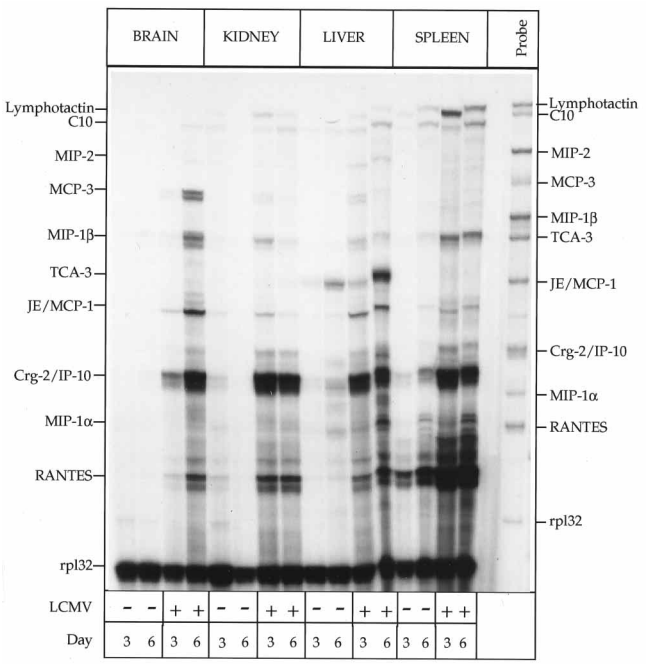

FIG. 2. Comparison of chemokine mRNA expression in brain and peripheral organs. BALB/c mice were injected i.c. with saline or LCMV (200 PFU), and poly(A)$^+$ RNA isolated from the indicated organs was analyzed by RPA as outlined in Materials and Methods.

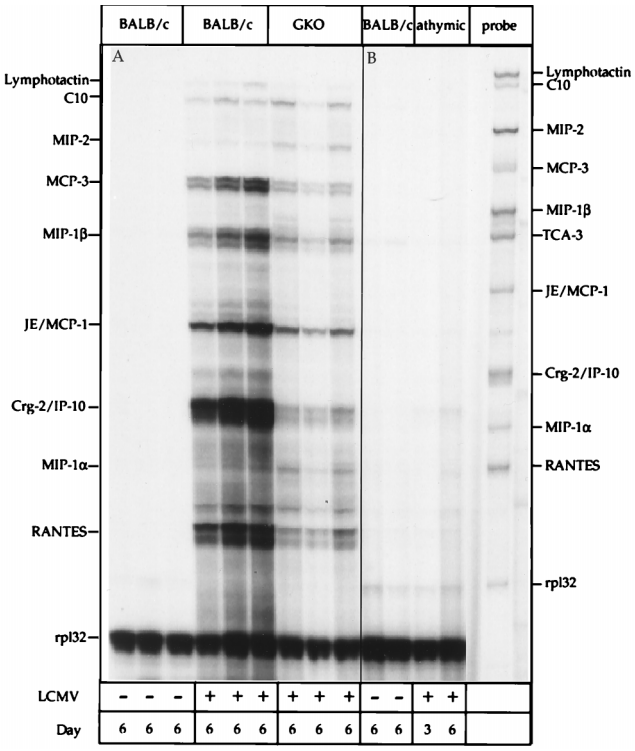

FIG. 3. Comparison of chemokine mRNA expression in brain from BALB/c, GKO (A), and athymic nude (B) mice. Mice were injected i.c. with saline or LCMV (200 PFU), and poly(A)$^+$ RNA was isolated from the brain at the indicated times and analyzed by RPA as outlined in Materials and Methods.

Cellular localization of Crg-2/IP-10 and LCMV gene expression. Our findings above indicated that expression of the Crg-2/IP-10 gene was most prominent in the brain following LCMV infection. Therefore, we next analyzed the relationship between the expression of this chemokine to the sites of LCMV infection and pathology in the brain by in situ hybridization. At day 6 postinfection, high expression of Crg-2/IP-10 RNA was observed around the ventricles, meninges, and choroid plexus and in the olfactory bulbs (Fig. 4). On adjacent sections, the expression of LCMV NP RNA was found to exhibit a regional distribution similar to that of the Crg-2/IP-10 gene. Although quantitatively less, the distribution of Crg-2/IP-10 RNA and LCMV NP RNA overlapped somewhat at day 3 postinfection (data not shown).

Sections hybridized with probes to LCMV (Fig. 5A, B, E, F, I, J, M, and N) and Crg-2/IP-10 (Fig. 5C, D, G, H, K, L, O, and P) were analyzed to determine the relationship of the cellular sources for the expression of these genes at day 6 postinfection. Microscopic examination revealed prominent expression of Crg-2/IP-10 by ependymal, choroid plexus, and meningeal cells (Fig. 5C and D). In addition, cells in the parenchyma of the brain adjacent to the subependymal plate (Fig. 5G and H) and cells in the cerebellum (Fig. 5O and P) were found to be positive for Crg-2/IP-10 expression. A small number of Crg-2/IP-10-positive cells were also found to be associated with the mononuclear cell infiltrates (Fig. 5K and L). Comparison of Crg-2/IP-10 (Fig. 5C, D, G, and H) expression with that for LCMV (Fig. 5A, B, E, and F) generally revealed an overlap between the expression of the chemokine gene and the virus. This was particularly evident in the choroid plexus (Fig. 5A through D, arrows), ependyma (Fig. 5A through D, arrowheads), parenchyma adjacent to the lateral ventricle (Fig. 5A through D, open arrowheads), and meninges (Fig. 5I through L, open arrowheads). However, Crg-2/IP-10 was also expressed in areas of the brain without detectable LCMV RNA, e.g., in parenchymal cells adjacent to the subependymal plate (Fig. 5G and H) and in the cerebellum (Fig. 5O and P, arrows).

DISCUSSION

In this study, we demonstrated that infection of mice with LCMV induced the simultaneous expression of genes encoding a number of chemokines in the brain. Since chemokines are very potent signals that coordinate leukocyte trafficking (41), their expression in LCM may be central to the recruitment and extravasation of lymphocytes and macrophages to the LCMV-

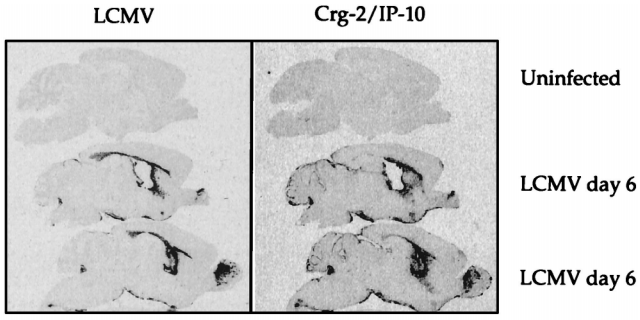

FIG. 4. Localization of LCMV and Crg-2/IP-10 RNA in brain. Mice were injected i.c. with saline or LCMV (200 PFU), and each brain was removed at day 6 for in situ hybridization. Images are from Cronex film (5-day exposure) of sagittal brain sections (10 μm) from uninfected and LCMV-infected mice. Sections were hybridized with ^{35}S-labeled antisense RNA probes as outlined in Materials and Methods.

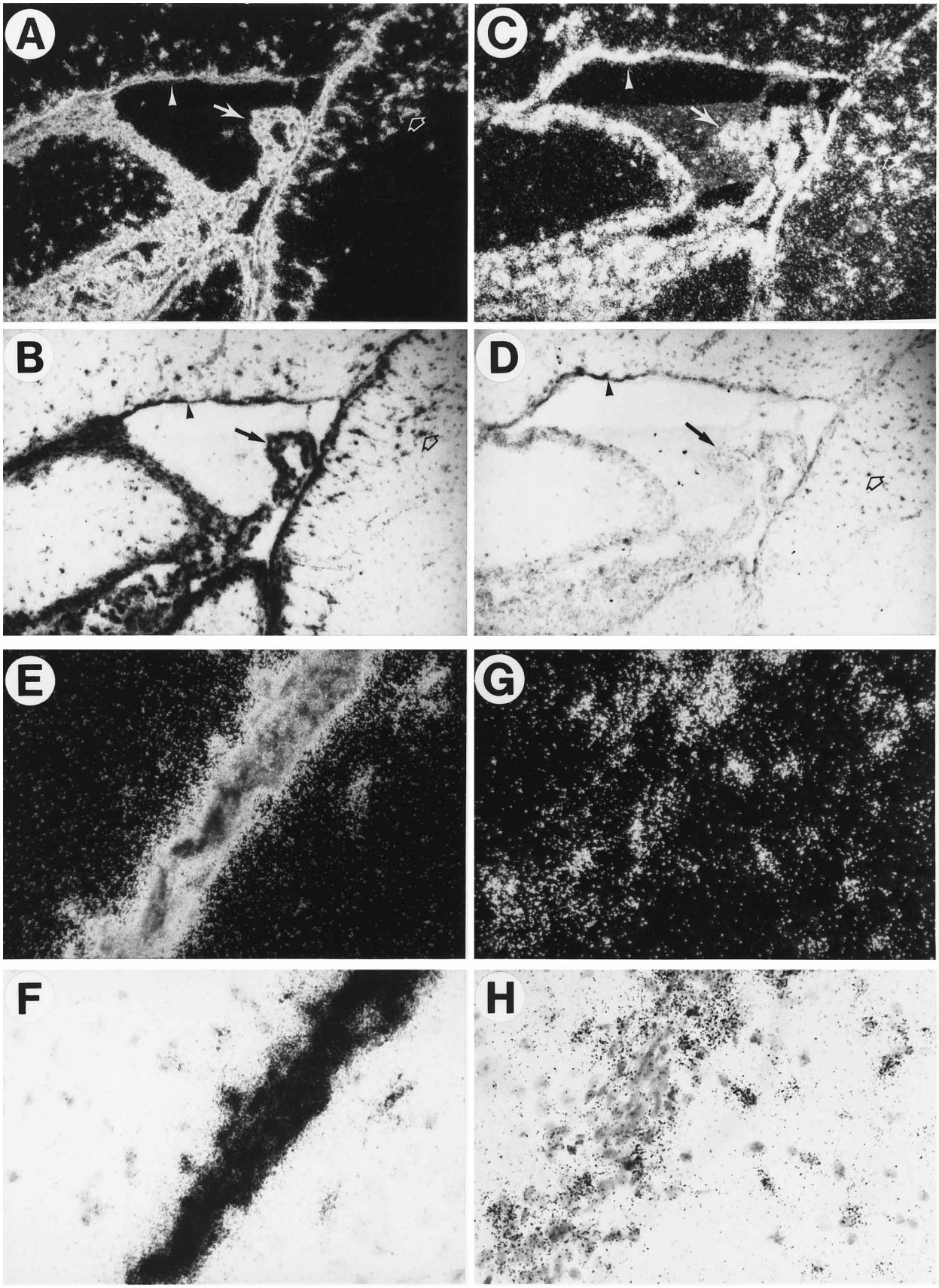

FIG. 5. Cellular localization of LCMV and chemokine RNA expression in brain in LCM. Mice were injected i.c. with saline or LCMV (200 PFU), and each brain was removed at day 6 for in situ hybridization. Sagittal brain sections (10 μm) were hybridized with ^{35}S-labeled antisense probes, coated with photographic emulsion, and developed after 1 week (Crg-2/IP-10 [C, D, G, H, K, L, O, and P]) or 2 weeks (LCMV [A, B, E, F, I, J, M, and N]), and visualized by using bright-field (B, D, F, H, J, L, N, and P) or dark-field microscopy (A, C, E, G, I, K, M, and O).

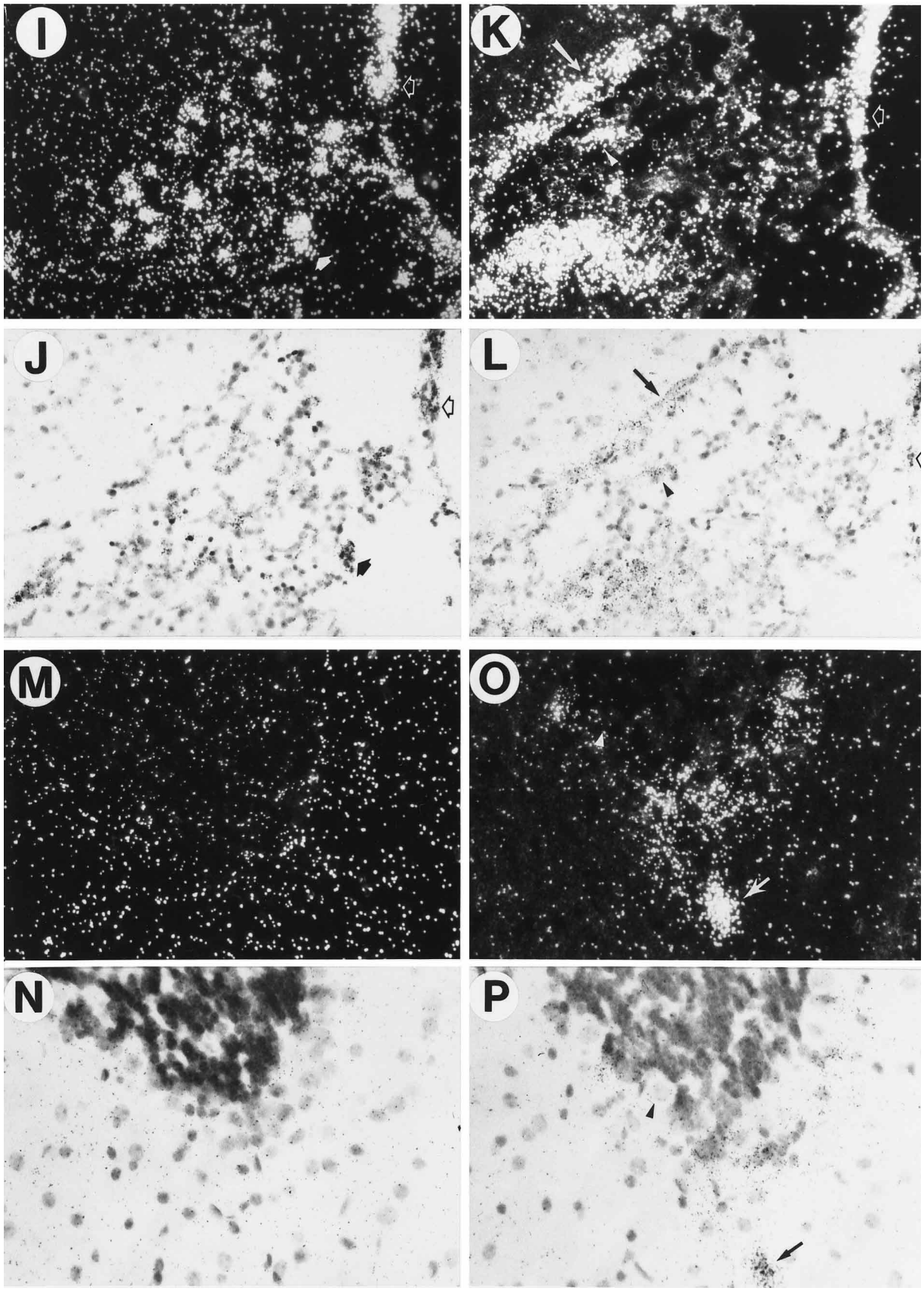

FIG. 5—*Continued.*

infected brain. Consistent with such a role, we observed an early onset of increased expression for a number of chemokines (i.e., Crg-2/IP-10, RANTES, MCP-1, and MIP-1β) in the brains of LCMV-infected mice, prior to significant infiltration by mononuclear cells. Subsequently, the expression of these chemokine genes increased in parallel with the progression of LCM. Moreover, the expression of one of these chemokine genes, Crg-2/IP-10, was found to be predominantly localized to the brain itself, close to or overlapping with sites of LCMV infection. This pattern of localization for Crg-2/IP-10 gene expression differs somewhat from that reported for the proinflammatory cytokines, e.g., IFN-γ, whose expression was found to be exclusively associated with infiltrating mononuclear cells in LCM (8). Thus, chemokine gene expression is an early local response by the CNS to LCMV infection that may facilitate the subsequent recruitment of mononuclear cells.

A number of recent studies have focused on the role of chemokines in mediating immunoinflammatory cell trafficking to the CNS (for a review, see reference 43). Of particular relevance to LCM, the injection of MIP-1 or MIP-2 into the subarachnoid space caused florid meningitis with cerebrospinal fluid leukocytosis (40). In experimental autoimmune encephalomyelitis (EAE), the expression of a number of chemokine genes in the brain and spinal cord has also been documented (5, 18, 35). A key role for MIP-1α in promoting the CNS recruitment and infiltration of mononuclear cells in this model was demonstrated when antibodies to this chemokine administered to mice were shown to prevent the histological and clinical development of EAE (24). Finally, transgenic mice with the expression of MCP-1 under the control of the myelin basic protein promoter and targeted to oligodendrocytes exhibit monocyte infiltration and accumulation at perivascular sites in the CNS (15).

The chemokine gene activation response in LCM was not limited to the CNS and, with some exceptions, showed a similar pattern in the peripheral organs. Thus, in all organs, expression of Crg-2/IP-10 and especially of RANTES was predominant and occurred early after infection, while expression of the TCA-3 gene was limited to liver, and expression of the the lymphotactin gene was limited to the brain and the spleen. TCA-3 (7) and lymphotactin (25) have been shown to be produced by activated T lymphocytes. The reason for the organ-restricted expression of these chemokines in LCM is unclear; it may indicate that tissue-resident cells (e.g., hepatocytes) also have the potential to produce these chemokines or it may reflect the presence of specific subpopulations of activated T lymphocytes in the different organs. We (37) and others (30) have previously observed that LCMV infection of the periphery occurs unavoidably following i.c. inoculation with the virus and therefore likely accounts for the activation of chemokine gene expression that we observed in the peripheral organs. The generalized nature of this activation process and the qualitatively similar pattern of the genes that are expressed highlight a more global significance of chemokines in the development of leukocytosis following LCMV infection.

Our studies emphasized the complex nature of the chemokine response in the brain and the simultaneous activation of multiple chemokine genes following LCMV infection. Members of the α-chemokine subfamily (MIP-2 and Crg-2/IP-10) and the β-subfamily (MIP-1β, MCP-1, MCP-3, and RANTES) were induced in the brain after LCMV infection. As noted above, the expression of Crg-2/IP-10, RANTES, and to a lesser degree MCP-1 was most prominent and was present by day 3 postinfection, prior to infiltration of the brain with mononuclear cells. A comparison with similarly infected athymic mice revealed that chemokine activation was almost completely absent from the brains of the immunodeficient animals. Since LCMV is known to replicate to high levels in the brains and peripheral organs of athymic mice (6), these findings suggest that infection of cells with this virus alone is not responsible for the activation of the chemokine genes in LCM. In immunocompetent mice, IFN-γ gene expression is known to be markedly elevated in the periphery and CNS during LCM (8). IFN-γ is capable of inducing or upregulating the expression of a number of chemokines by neural cells, including Crg-2/IP-10 (47), RANTES (3), and MCP-1 (21). In the present study, a comparison of chemokine gene expression in the brains of LCMV-infected BALB/c and GKO mice lacking IFN-γ (9) revealed a decrease in but not the abolition of the expression of many of the chemokine genes, including Crg-2/IP-10, RANTES, and MCP-1, in the mutant animals. Therefore, while IFN-γ appears to be responsible in part for modulating the expression of these chemokines in the brain in LCM, other factors are clearly involved. Candidates for such factors likely include other proinflammatory cytokines, e.g., IFN-α and -β (23, 29, 38) and tumor necrosis factor alpha (TNF-α) (8), since these are also expressed in LCMV infection and may be significant signals for the stimulation of chemokine gene expression (see below).

The prominent expression of Crg-2/IP-10 found in the brains of LCMV-infected mice is notable. Conspicuous expression of Crg-2/IP-10 has also been observed in other inflammatory disorders of the CNS, including EAE (18, 35) and simian immunodeficiency virus-induced AIDS encephalitis (39). This chemokine is an effective chemoattractant for human (44) and murine (27) monocytes and T lymphocytes and promotes the adherence of T lymphocytes to the endothelium (44). T lymphocytes and macrophages constitute the overwhelming majority of cells infiltrating the CNS in LCM (12), and this may reflect in part the contribution of Crg-2/IP-10. Consistent with this, we observed a general concordance between the expression of the Crg-2/IP-10 gene and the primary sites of LCMV infection and immunopathological lesions in the brain. Expression of the Crg-2/IP-10 gene appeared to be quite widespread in the brain following LCMV infection and included choroid plexus, meninges, and ependymal cells and unidentified cells in the parenchyma of the brain. These cells might include astrocytes and microglia, which have been shown to be capable of expressing this chemokine gene following exposure to IFN-γ (35, 47). The promiscuous nature of the cellular expression of Crg-2/IP-10 in the brain and its prominence in peripheral organs in LCMV infection likely underscore the pivotal role of this chemokine in the development of the immunoinflammatory response to the virus.

Studies of the regulation of Crg-2/IP-10 expression demonstrate that IFN-γ is a potent activation stimulus for this gene (28, 47) and that this chemokine may be important in modulating some of the biological actions of IFN-γ, e.g., the inhibition of angiogenesis (1). As noted above, however, our studies with GKO mice (which do not express IFN-γ due to disruption of the IFN-γ gene by homologous recombination [9]) indicated that factors other than IFN-γ may regulate the expression of the Crg-2/IP-10 gene in vivo. These factors may include the type I IFNs (IFN-α and -β) and TNF-α, whose genes are known to be expressed in the periphery and the brain in LCM (8, 38). Induction of Crg-2/IP-10 gene expression by cells exposed to IFN-α has been reported previously (48); more recently, we have observed the marked expression of this chemokine gene in the brains of transgenic mice with the astrocyte-targeted expression of IFN-α (1a). TNF-α is also known to induce Crg-2/IP-10 expression by treated cells in vitro (31) and in vivo (32).

The cellular sources of these other chemokine genes expressed in the brain in LCM were not examined in the present study. However, particularly in the case of RANTES, MCP-1, and MIP-1β, their expression at day 3 postinfection prior to infiltration of the brain with mononuclear cells suggests that, like Crg-2/IP-10, these chemokine genes may be expressed by CNS resident cells. Both activated astrocytes and/or microglia have been shown (3, 21, 22, 35) to express these chemokine genes, thereby indicating that resident neural cells might produce these inflammatory mediators. The late expression of MIP-2 and lymphotactin in the brain in LCM would be consistent with the expression of these chemokine genes by infiltrating mononuclear cells. In this regard, lymphotactin has been shown to be expressed by activated $CD8^+$ T lymphocytes (25), and cells of this phenotype are recruited to the brain and promote the development of LCM (6, 12).

In summary, our findings demonstrate the overlapping expression of a number of chemokine genes in the brains of mice infected with LCMV. The pattern of chemokine gene expression in LCM may profoundly influence the characteristic phenotype and response of leukocytes in the brain and contribute to the immunopathogenesis of this fatal CNS infection. Importantly, these findings together with those from other studies of neuroinflammatory conditions such as EAE (18, 35) and simian immunodeficiency virus-induced AIDS encephalitis (39) suggest the existence within the CNS of a complex chemokine network similar to that for the cytokines (4). Such a chemokine network may be pivotal in the regulation of inflammation within the CNS, particularly in the determination of the nature of the leukocytes that are recruited to the CNS from the periphery. The development of inhibitors of specific chemokines as well as transgenic mice with deleted or overexpressed chemokine gene expression will assist in the resolution of some of these issues.

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