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

VALÉRIE C. ASENSIO AND IAIN L. CAMPBELL*

Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California

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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 observed in the peripheral organs of LCMV-infected mice. Mouse 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 chemoattractants 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 by a 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 low 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 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.
MATERIALS AND METHODS

**Mice and infection with LCMV.** Male euthymic BALB/c, athymic BALB/c nu/nu or homozygous GKO (IFN-γ gene disrupted) (9) mice were maintained under pathogen-free conditions in the clean 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 LCMV, 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, 0.1 M Tris-HCl [pH 7.5], 1.5 mM MgCl2, 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 50 μl 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.

**Chemokine gene expression in LCMV-infected mice.** To examine chemokine gene expression after LCMV infection, we developed a multiprobe RNase protection assay (RPA) (8). The RPA was performed by a previously described method (8). For synthesis of labeled probes, the reaction mixture (25 μl) contained 250 μCi 35S-CTP (3,000 Ci/mmol; Andotek, Irvine, Calif.), UTP (73 pmol), GTP, ATP, and CTP (2.5 nmol each), DTT (50 nmol), and 10 mM dithiothreitol (DTT) (all from Promega). After 2 h of incubation at 37°C, 5 μl 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.

**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-1, MIP-1β, C-10, and RANTES. As shown in Fig. 1A, in the brains of noninfected mice, there was very low expression of MIP-1β, MCP-1, C-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, C-10, and RANTES, was increased, while MCP-3 RNA expression was induced. In particular, C-10 and RANTES were found to be predominantly expressed, while levels of MCP-3, MIP-1β, MIP-1α, and RANTES were lower. Expression of these chemokine transcripts further increased markedly at day 6 postinfection. 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 C-10-32P mRNA in the brain following LCMV infection. Quantitation of the signal intensities revealed that relative to background, there was a

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

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>GenBank accession no.</th>
<th>Reference</th>
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</thead>
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<tr>
<td>Lyphotactin</td>
<td>30–360</td>
<td>330</td>
<td>U15607</td>
<td>25</td>
</tr>
<tr>
<td>C10</td>
<td>710–1025</td>
<td>315</td>
<td>M58004</td>
<td>33</td>
</tr>
<tr>
<td>MCP-2</td>
<td>66–336</td>
<td>271</td>
<td>X53708</td>
<td>45</td>
</tr>
<tr>
<td>MCP-3</td>
<td>105–344</td>
<td>239</td>
<td>Z12297</td>
<td>26</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>86–296</td>
<td>210</td>
<td>X12531</td>
<td>11</td>
</tr>
<tr>
<td>TCA-3</td>
<td>3985–4175</td>
<td>190</td>
<td>X52401</td>
<td>7</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1892–2062</td>
<td>170</td>
<td>M19681</td>
<td>36</td>
</tr>
<tr>
<td>Crg-2/IP-10</td>
<td>221–361</td>
<td>140</td>
<td>J05576</td>
<td>46</td>
</tr>
<tr>
<td>MCP-3</td>
<td>909–1020</td>
<td>120</td>
<td>X33372</td>
<td>19</td>
</tr>
<tr>
<td>RANTES</td>
<td>2303–2413</td>
<td>110</td>
<td>L02780</td>
<td>13</td>
</tr>
<tr>
<td>rpl32</td>
<td>61–139</td>
<td>78</td>
<td>K02060</td>
<td>13</td>
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</table>
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, MCP-3, and MIP-2 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).
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-infected areas.
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 35S-labeled antisense probes, coated with photographic emulsion, and developed after 1 week (Crg-2/IP-10 [C, D, G, 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).
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 subarachnoidal 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 exhibited 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 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 LCMV 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 to be expressed by 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 LCMV 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 similar 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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