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Chemokines and the inflammatory response to viral infection in the central nervous system with a focus on lymphocytic choriomeningitis virus

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Leukocyte migration to the central nervous system (CNS) is a common process with often devastating consequences that follows infection of this tissue compartment with a variety of viruses. The mechanisms underlying this process are poorly defined but, it is hypothesized that chemokines may be important regulatory signals for the cerebral recruitment and extravasation of leukocytes. Here we discuss this hypothesis in the context of different viral infections of the CNS with emphasis on lymphocytic choriomeningitis virus (LCMV). In general, the pattern of chemokine gene expression in these CNS viral infections is dynamic and complex with often overlapping expression of a number of different subclasses of chemokine genes. In the case of CNS infection with LCMV, cerebral chemokine gene expression was observed in euthymic and to a lesser extent athymic mice and preceded increases in cytokine gene expression and in euthymic mice, CNS leukocyte recruitment. These observations together with the finding that CRG-2/IP-10, a prominently expressed chemokine gene in many different CNS viral infections, was expressed by cells intrinsic to the CNS e.g. astrocytes, suggest that activation of chemokine gene expression may be a direct, early and localized host response to viral infection. These findings are consistent with the proposed involvement of chemokines as key signaling molecules for the migration of leukocytes to the CNS following virus infection.

Keywords: chemokine; virus; central nervous system; neuroinflammation

Introduction

The CNS of healthy adult mammals is characterized by a relative absence of cellular elements of the immune system. The CNS presents the immune system with some unique problems with how immune cells traffic in and out and recognize foreign antigen when it is present (see Campbell and Mucke, 1993; Hart and Fabry, 1995; Lassman *et al*, 1991; Williams and Hickey, 1995 for reviews). First, the presence of the blood-brain barrier (BBB) restricts the entry of cells and macromolecules from the circulation into the CNS. Second, there is deficient expression of MHC class I and MHC class II molecules and an absence of professional antigen presenting cells such as dendritic cells required for the presentation of antigen and activation of the adaptive immune response. Third, there is evidence of yet unidentified brain-derived factors that may suppress or counter-regulate the actions of proinflammatory mediators in the CNS (Anderson *et al*, 1992a,b). In spite of these impediments to the development of inflammation, activated T cells can enter the CNS parenchyma after first migrating through the BBB (Hickey *et al*, 1991). Significantly, in numerous pathological conditions immune cells are readily recruited and migrate into the CNS. For example, cellular immune responses in the CNS are evoked following infection with a variety of pathogenic agents (e.g. bacterial and viral meningoencephalitis) as well as in autoimmune disorders exemplified by the human demyelinating disease MS and its experimental counterpart EAE. Although having evolved primarily to clear invading infectious particles and thus protect the host, accumulating evidence suggest an undesirable consequence of such responses is tissue injury which, in the CNS, may account for the considerable morbidity and mortality that accompanies neuroinflammatory dis-

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ease states. Therefore, studying the specific mechanisms underlying the localization, extravasation and activation of immune cells in the CNS and the subsequent interactions between these cells which contribute to CNS damage is an important objective.

Pertinent to this objective is the issue of what regulates the recruitment and trafficking of leukocytes to the CNS. In this regard, much recent interest has focused on the possible involvement of chemoattractant cytokines also called 'chemokines'. The chemokines (see Baggiolini, 1998; Haelens *et al*, 1996; Howard *et al*, 1996; Prieschl *et al*, 1995; Rollins, 1997; Taub, 1996 for recent reviews) are a large and growing family of small proteins which can be separated into four distinct subfamilies. Members of each subfamily possess a variation of a conserved amino terminal cysteine structural motif being CXC (the α-subfamily), CC (the β-subfamily), C (the γ family) or CX_3C (the δ subfamily) where X is an intervening amino acid residue. In general, within each chemokine subfamily the members show considerable homology in their amino acid sequence and often possess overlapping chemoattractant specificity. The α-chemokines include MIP-2, CRG-2/IP-10, IL-8 and GRO-α-γ, are known to be mainly chemotactic for polymorphonuclear cells. The β-chemokines include C10, MCP-3, MIP-1β, TCA-3, MCP-1, MIP-1α and RANTES are principally chemotactic for monocytes and lymphocytes. The γ and δ chemokine subfamilies are each currently represented by only a single member being lymphotactin (Kelner *et al*, 1994) and human fractalkine (Bazan *et al*, 1997) or mouse neurotactin (Pan *et al*, 1997), respectively.

Chemokines mediate their activities through G protein coupled cell surface receptors, which have a characteristic seven transmembrane structure (Premack and Schall, 1996). It is apparent that many chemokine receptors are promiscuous meaning that chemokine receptors bind several different chemokines and conversely, individual chemokines can often bind to several different receptors. The chemokine receptors comprise, three groups, the CC receptors 1–8 (CCR1–8) that bind CC chemokines, and the CXC receptors 1–4 (CXCR1–4) which bind CXC chemokines (Baggiolini *et al*, 1997; Mackay, 1997; Moser *et al*, 1998). Recently, Imai *et al*. identified the fractalkine receptor (CX3CR1) which is expressed mainly in NK cells and monocytes and to a lesser extent in $CD3^+CD8^+$ T-cells (Imai *et al*, 1997). The receptor for lymphotactin remains to be identified. The CC and CXC receptors are expressed on a number of different cell types and their expression is often regulated by exogenous and endogenous stimuli. The discovery that chemokine receptors serve as co-receptors for the fusion and entry of HIV virus into $CD4^+$ T-cells (via CXCR4 chemokine receptor) and macrophages (via CCR5 chemokine receptor) has opened new directions in our understanding of mechanisms of viral entry and pathogenesis (Alkhatib *et al*, 1996; Choe *et al*, 1996; Deng *et al*, 1996; Dragic *et al*, 1996; He *et al*, 1997). The finding that expression of many of the chemokine receptors can be found on glial and neuronal cells suggests there may be direct interaction between chemokines and these neural cells. For example, CCR5 and CCR3 have been shown to be expressed on microglial cells (He *et al*, 1997). CXCR4 is expressed by a broader range of tissues and cell types, including astrocytes (Heesen *et al*, 1996), microglial cells and T cells (Tanabe *et al*, 1997b). Tanabe *et al* reported that resident cells (astrocytes) of the CNS express MIP-1α receptor CCR1 (Tanabe *et al*, 1997a). Other studies have shown that chemokine receptors CCR3, CCR5, CXCR3 and CXCR4 are expressed in perivascular infiltrates in the brain as well as on neurons and glial cells of SIV-infected Macaque monkeys (Westmoreland *et al*, 1998). The Duffy antigen receptor for chemokines (DARC) is expressed by subsets of endothelial cells and Purkinje cells in the cerebellum suggesting that this enigmatic receptor may have multiple roles in normal and pathological physiology (Horuk *et al*, 1996).

As alluded to above, CNS infection with a number of different classes of viruses can provoke vigorous inflammatory responses with subsequent recruitment of large numbers of leukocytes. Intracranial infection of mice with lymphocytic choriomeningitis virus (LCMV) is a well-characterized experimental model that exemplifies this point (Buchmeier *et al*, 1980; Doherty *et al*, 1990). Thus, immunocompetent adult mice infected with LCMV develop an acute monophasic disease characterized by infiltrating mononuclear cells in the meninges, choroid plexus and ependymal membranes leading to convulsive seizures and death of the animals 6–8 days later (Buchmeier *et al*, 1980). The infiltrating cells consist of predominantly T-lymphocytes as well as macrophages. Of particular prominence in the immune response to LCMV is the presence of MHC class I restricted anti-LCMV $CD8^+$ cytotoxic lymphocytes (CTL) which in addition to clearance of the virus are also the primary effectors of LCM (Buchmeier *et al*, 1980; Doherty *et al*, 1990). Similar to LCMV, CNS infection with other viruses e.g. murine hepatitis virus (MHV) (Lane and Buchmeier, 1997; Weiner, 1973) and herpes simplex virus (HSV) (Sobel *et al*, 1986), can produce robust recruitment of leukocytes to the brain with resultant tissue injury, often leading to death. In general, the well defined and relatively acute nature of the immune pathological process makes these different experimental viral models excellent tools for better understanding the specific mechanisms underlying the migration of leukocytes to the CNS and the subsequent interactions between these cells that contribute to disease pathogenesis. Here we discuss these

issues, focusing on the regulation and role of chemokine gene expression in the CNS following viral infection, with special emphasis on LCMV.

Results and discussion

Chemokine gene expression patterns in viral infections of the CNS

Much interest has focused on the chemokines as potentially pivotal regulators of the CNS recruitment and trafficking of leukocytes (see Glabinski *et al*, 1995a; Ransohoff, 1997; Tani and Ransohoff, 1994 for reviews). Many reports have focused on the experimental autoimmune encephalomyelitis (EAE) model where various chemokines were found to be expressed in the brain (Berman *et al*, 1996; Eng *et al*, 1996; Glabinski *et al*, 1995b; Godiska *et al*, 1995a; Hulkower *et al*, 1993; Karpus *et al*, 1995; Miyagishi *et al*, 1997; Ransohoff *et al*, 1993), and where some of which correlated with the onset and progression of the disease process (Glabinski *et al*, 1997; Godiska *et al*, 1995). The central role of chemokines in the pathogenesis of EAE was further clarified with the demonstration that neutralization of different chemokines such as MIP-1α (Karpus *et al*, 1995) or CRG-2 (Wojcik *et al*, 1996) produced a marked attenuation in disease.

Recent reports have focused attention on the possible involvement of chemokines in the immunopathogenesis of different viral diseases of the CNS including HIV encephalitis (Schmidtmayerova *et al*, 1996), SIV encephalitis (Sasseville *et al*, 1996), LCM (Asensio and Campbell, 1997) and MHV encephalomyelitis (Lane *et al*, 1998). Similar to EAE, the findings from these different viral diseases highlight a marked promiscuity in the cerebral chemokine expression patterns with simultaneous expression of multiple chemokines being the general rule. This is illustrated in the case of LCMV infection shown in Figure 1A. In brain from sham infected mice little detectable chemokine gene expression was observed however, in euthymic and athymic mice at day 3 postinfection the expression of a number of chemokine genes was evident including, C10, MCP-3, MIP-1β, MCP-1, CRG-2/IP-10 and RANTES. By day 6 postinfection the expression of all these chemokine genes increased markedly and the expression of the lymphotactin gene was also evident in the brain of euthymic but not athymic mice. In both euthymic and athymic mice expression of CRG-2/IP-10 was predominant at both time points after infection. It should be emphasized that these data are representative of chemokine gene expression at the RNA level which may not necessarily correlate with bioactive protein. Ongoing studies by us seek to resolve this important issue.

Similar patterns of cerebral chemokine gene expression (Table 1) were recently reported for MHV encephalomyelitis (Lane *et al*, 1998) and SIV encephalitis (Sasseville *et al*, 1996). In particular, a common feature associated with all these CNS viral infections was the prominent expression of the α-chemokine CRG-2/IP-10. Thus, CRG-2/IP-10 gene expression appears to be intimately coupled to the antiviral host response.

To determine whether these chemokine responses to i.c. viral infection were restricted to the brain we examined the chemokine gene expression profiles in the spleen. As shown in Figure 1A, compared with brain from the same infected host, the spleen exhibited a qualitatively matched but more exaggerated early chemokine gene response which was maximal by day 3 postinfection and was similar for both euthymic and athymic animals. Interestingly, by day 6 postinfection the expression of MCP-1 and MCP-3 RNA was preferentially reduced in spleen from euthymic but not athymic mice. Thus, these findings indicate that while the temporal profile of the individual responses may differ somewhat, the overall chemokine gene expression patterns are similar between brain and spleen following LCMV infection. In any case, the activation of chemokine gene expression is not restricted to the brain in LCM pointing to a more global significance of chemokines in the development of leukocytosis following LCMV infection.

Chemokine gene expression by cells intrinsic to the brain during viral infection

The expression of CRG-2/IP-10 was examined in more detail because it is a chemokine that, as noted earlier, is prominently expressed in the brain following LCMV infection and the virus-induced demyelinating disease associated with MHV (Lane *et al*, 1998). In addition CRG-2/IP-10 is also expressed in SIV encephalitis (Sasseville *et al*, 1996) as well as in other inflammatory disorders of the CNS, including EAE (Glabinski *et al*, 1995b, 1997; Godiska *et al*, 1995). Consistent with these findings the CNS localization of CRG-2/IP-10 as well as LCMV-NP gene expression was analyzed by *in situ* hybridization and revealed some overlap between the expression of the chemokine and viral transcripts (Figure 2). At day 3 after infection expression of CRG-2/IP-10 and LCMV-NP RNA was particularly prominent in the meninges, choroid plexus and ependyma (Figure 2A). Expression of both these transcripts at these sites increased dramatically at day 6 postinfection (Figure 2B). In addition, more widespread expression of the CRG-2/IP-10 RNA was also apparent, associated with infiltrating mononuclear cells as well as by cells located in parenchymal locations including the cerebellum and sub-cortical regions of the brain. Further analysis to identify these parenchymal brain cells expressing CRG-2/IP-10 identified astrocytes as the major cellular source for the expression of this chemokine following LCMV infection (Figure 2C). The expression of CRG-2/IP-10 was

also found to be mainly localized to astrocytes in MHV encephalomyelitis (Lane *et al*, 1998). Studies *in vitro* demonstrate the induction of CRG-2/IP-10 gene expression by astrocytes as well as microglia infected with MHV (Lane *et al*, 1998) or NDV (Fisher *et al*, 1995), as well as exposed to cytokines such as IFN-γ (Ransohoff *et al*, 1993; Vanguri, 1995). These *in vivo* and *in vitro* findings indicate that cells intrinsic to the CNS can provide a source for chemokine gene expression following viral infec-

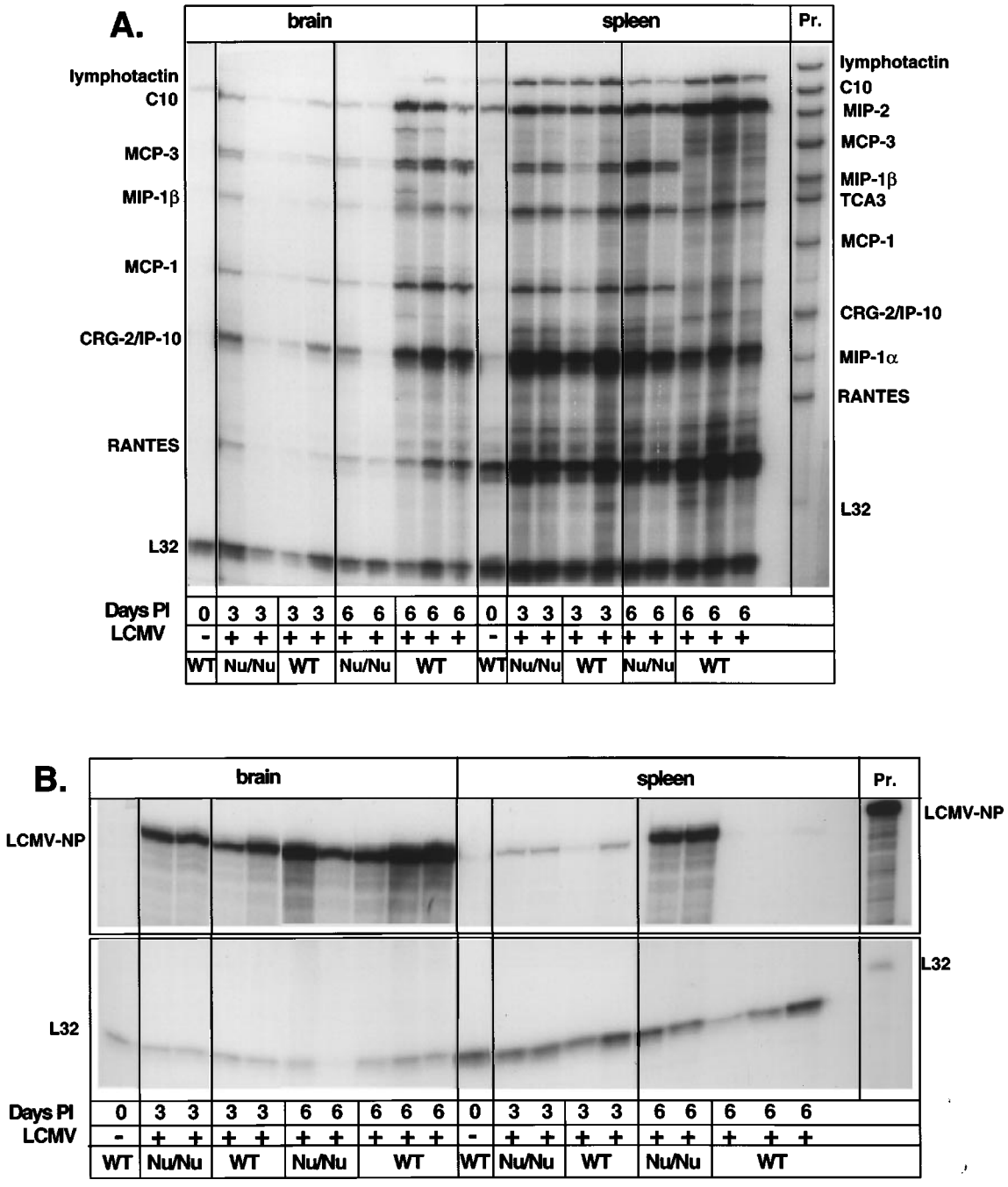

Figure 1 (**A**) Chemokine gene expression in the brain and spleen of mice following infection with LCMV. C57BL6 X SJL F1 (WT) or athymic nude (Nu/Nu) mice were injected i.c. with saline or LCMV (200 pfu of Armstrong strain 53b). At the times indicated the mice were killed and the organs removed for poly(A^+) RNA isolation. Analysis of chemokine RNA levels was performed by RPA as described previously (Asensio and Campbell, 1997) using 2 μg of poly(A^+) RNA. (**B**) LCMV-NP gene expression in the brain and spleen. Total RNA was prepared as described in the Materials and methods section. Analysis of LCMV-NP RNA levels was performed by RPA as described previously (Sandberg *et al*, 1994b) using 5 μg of total RNA.

Table 1 Chemokine gene expression reported in different viral infections of the central nervous system

Virus	*Chemokines produced* *α-chemokine*	*β-chemokine*	*Cell sources*	*References*
HIV		RANTES, MIP-1α, MIP-1β	$CD8^+$ T-cells perivascular cells	(Schmidtmayerova *et al*, 1996)
SIV	IP-10; IL-8	MIP-1α, MIP-1β, MCP-3, RANTES	Perivascular mononuclear cells, vascular endothelium	(Sasseville *et al*, 1996)
MHV	crg-2/IP-10[a]	MIP-1β, MCP-1, MCP-3 RANTES	Astrocytes, Mac-1+cells	(Lane *et al*, 1998)
LCMV	crg-2/IP-10[a]	C10, MCP-1, MCP-3 RANTES, MIP-1β	Astrocytes, ependymal and meningeal cells	(Asensio and Campbell, 1997)
NDV[b]	crg-2/IP-10[a]	RANTES[a]	Astrocytes, microglial cells	(Fisher *et al*, 1995)

[a]Chemokines that the cell source was defined for. [b]*In vitro* cell culture studies.

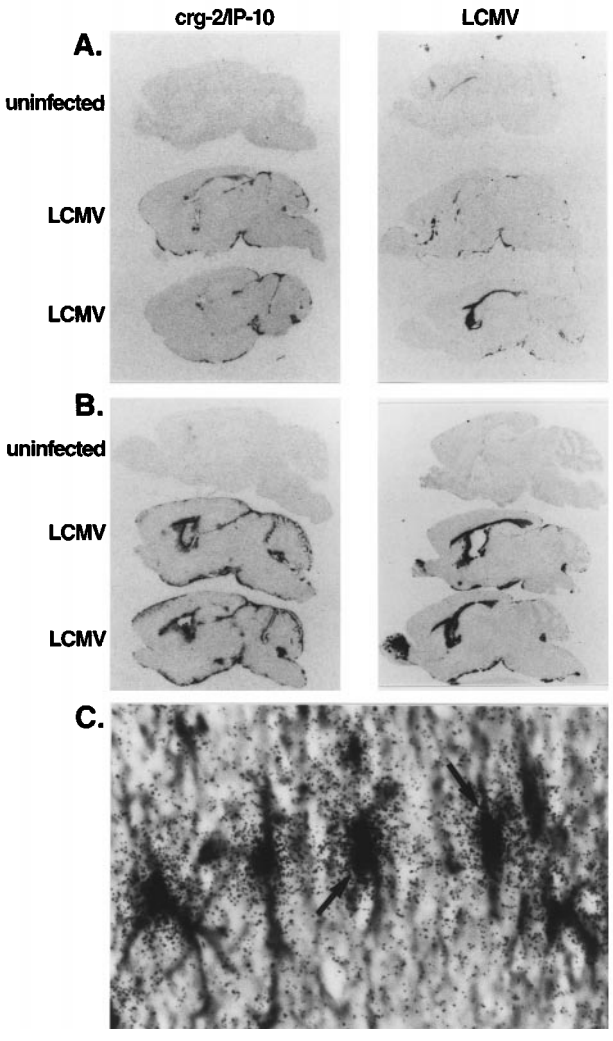

Figure 2 Localization of LCMV-NP and CRG-2/IP-10 RNA expression in the brain of euthymic mice following infection with LCMV. Mice were infected as described for Figure 1. At day 3 (**A**) or day 6 (**B**) postinfection the brains were removed and processed and analyzed by *in situ* hybridization as described previously (Asensio and Campbell, 1997). Some sections were further subject to dual label analysis to identify the cellular localization for CRG-2/IP-10 RNA expression. Combined *in situ* hybridization for CRG-2/IP-10 and immunostaining for GFAP revealed numerous double positive cells (**C**; arrows).

tion and importantly, may serve as a key component of a localized host response involved in a signaling pathway to the periphery that facilitates the recruitment of immunocytes to the brain.

Chemokine gene expression and its relationship to viral replication and other parameters of the antiviral host response in the CNS

Our studies with LCMV (Asensio and Campbell, 1997) and those with MHV (Lane *et al*, 1998) infection highlight a complex regulation of the cerebral chemokine gene response throughout the evolution of the immunopathogenic disease process. Critical factors likely to be involved in the modulation of this response include the viral load and distribution in the CNS as well as other elements of the host response such as local cytokine expression and the extent of infiltration by activated leukocytes. Here we examined the dynamics of these various parameters in parallel with the chemokine gene expression analysis discussed above and also shown in Figure 1A.

As noted above, a feature of the cerebral chemokine response is its early activation following LCMV infection. Examination of the CNS viral load (Figure 1B) revealed that in the brain from LCMV-infected mice at day 3 postinfection, expression of the LCMV-NP RNA was present at moderately high levels in both euthymic and athymic mice. In infected euthymic mice, the distribution of LCMV-NP RNA overlapped somewhat but not totally with CRG-2/IP-10 expression (Figure 2A). In contrast, expression of the antiviral type I IFNs (Figure 3A) or the proinflammatory (Figure 3B) cytokine genes at this time point showed no detectable alterations from non-infected control brain. Moreover, expression of the major lymphocyte subset genes CD8, CD4 and CD3 (Figure 3C) were also not detectable in the CNS of the LCMV-infected mice at this time. This latter finding is consistent with the absence in euthymic mice of significant T-lymphocyte infiltration of the CNS at day 3 postinfection. In all, these observations suggest as one possibility, that early following infection, LCMV may directly activate

chemokine gene expression locally in the CNS. While this possibility remains to be demonstrated formally, it should be noted that direct viral activation of chemokine gene expression has been documented *in vitro*, in the case of MHV infection of astrocytes (Lane *et al*, 1998) and NDV infection of

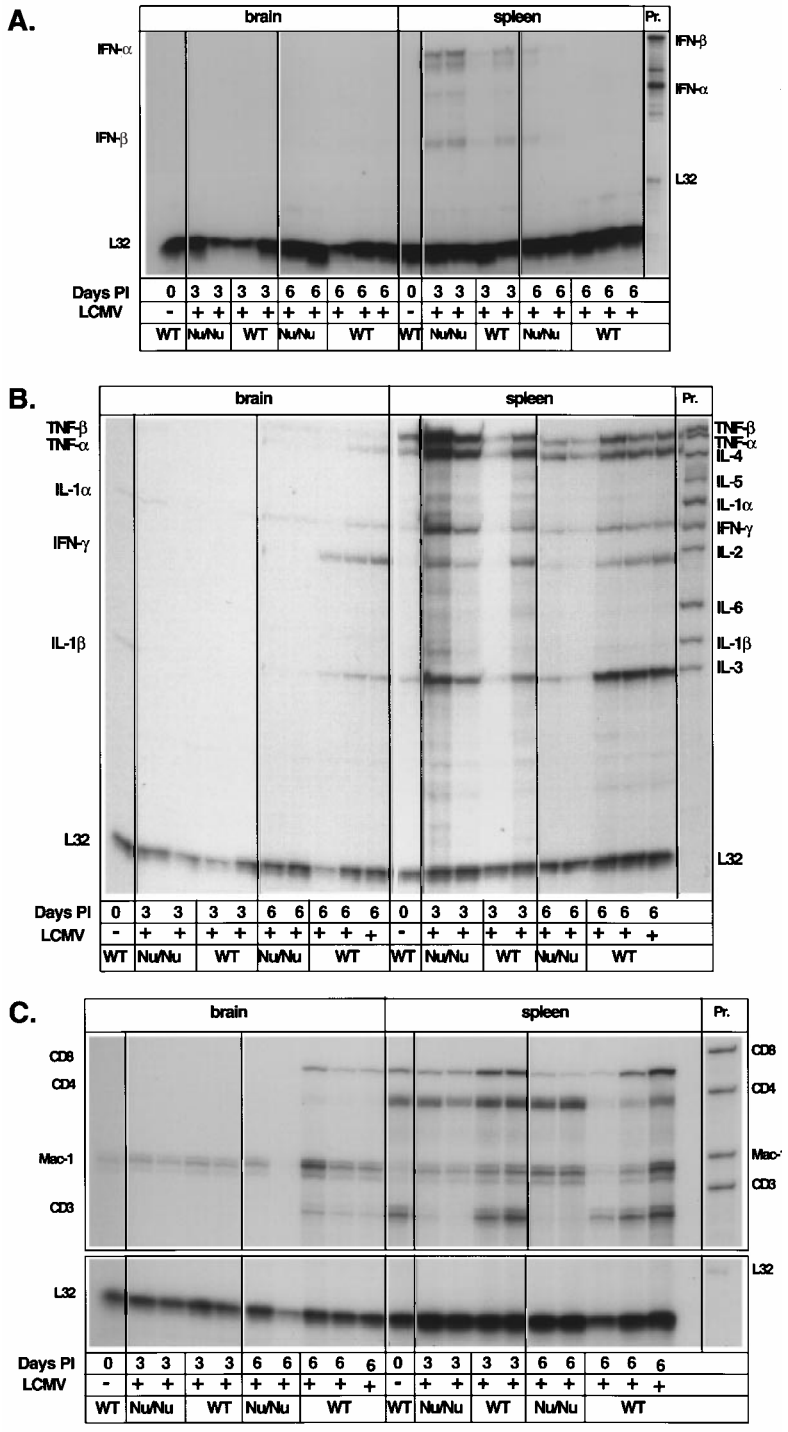

Figure 3 Type I IFN, cytokine and leukocyte phenotypic marker gene expression in the brain and spleen of mice following infection with LCMV. Poly(A$^+$) RNA (2 μg) derived as indicated in the legend to Figure 1 was analyzed by RPA as described previously (Asensio and Campbell, 1997) using multi-prone sets (see Material and methods section for details) that permitted the detection of type I IFNs (**A**), cytokine (**B**) and leukocyte marker (**C**) genes.

astrocytes and microglia (Fisher *et al*, 1995). In any event, direct modulation of cerebral chemokine gene expression by LCMV infection can only account for part of this response. First, in infected athymic mice despite increased viral loads at day 6, the levels of chemokine gene transcripts did not increase above those seen at day 3. Second, as noted above while *in situ* hybridization studies showed some overlap in the CNS sites of CRG-2/IP-10 and LCMV-NP RNA expression, expression of the chemokine gene was also observed at sites not showing detectable viral gene expression. It is therefore likely that regulation of the chemokine gene response to virus infection in the CNS is multifactorial, involving products derived from the virus as well as the host.

What the host factors are that may activate chemokine gene expression in the early stages of CNS infection remain to be determined. We were not able to detect significant alterations in either of the type I IFNs, IFN-α and IFN-β (Figure 3A) or in proinflammatory cytokine (Figure 3B) gene expression early following LCMV infection. It is possible there may have been small localized increases in the expression of these different cytokines-below the detection range of the RPAs. An alternative possibility is that peripherally derived signals may contribute to the early rise in chemokine gene expression. As shown in Figure 3A and B, expression of the type I IFNs as well as the proinflammatory cytokines was markedly increased in the spleen at day 3 postinfection. We have also previously noted significantly elevated circulating levels of IFN-α at this time point in LCMV infected mice (Sandberg *et al*, 1994a) consistent with systemic activation of the antiviral immunoinflammatory response. Support for the notion that peripheral immune challenge can act centrally to activate cerebral chemokine gene expression comes from a recent report in which it was shown that peripheral administration of lipopolysaccharide in rats can induce MCP-1 expression throughout the brain, including astrocytes (Gourmala *et al*, 1997).

The marked increase in cerebral chemokine gene expression at day 6 following LCMV infection in euthymic mice was paralleled by the expression of a number of proinflammatory cytokine genes in the brain including TNF-α, IL-1α, IFN-γ and IL-1β (Figure 3B). Consistent with the development of the LCM immune pathology, this time point was also marked by the appearance of the expression of the CD8, CD4 and CD3 T-lymphocyte transcripts as well as increased macrophage/microglial Mac-1 RNA expression. Previous studies by us (also refer to Figure 2B) focusing on CRG-2/IP-10, have shown that while infiltrating mononuclear cells may account for some of this chemokine gene expressed at day 6 postinfection, significant increases in locally expressed CRG-2/IP-10 gene expression also occurs (Asensio and Campbell, 1997). Studies of LCM in mice made genetically deficient for IFN-γ gene expression (termed GKO) (Dalton *et al*, 1993) revealed that cerebral chemokine expression was suppressed to about 50% of the levels found in wild type animals (Asensio and Campbell, 1997). Therefore, IFN-γ represents one major stimulus for the upregulation of cerebral chemokine expression at this later phase of infection with LCMV. In the case of CRG-2/IP-10, IFN-γ is a potent activation stimulus for this gene (Luster *et al*, 1985; Vanguri and Farber, 1994). In addition, to IFN-γ, IFN-α (Wu *et al*, 1994) and TNF-α (Ohmori and Hamilton, 1994, 1995) may also upregulate expression of the CRG-2/IP-10 gene. In conclusion, the findings indicate that with disease progression, proinflammatory cytokines become increasingly important candidates in the regulation of the cerebral chemokine gene expression following LCMV infection.

Role of chemokines in the inflammatory response to viral infection of the CNS

From the preceding results and discussion, it is clear that different viral infections of the CNS result in the simultaneous expression of multiple chemokine genes the patterns of which, in the case of LCMV and MHV, are qualitatively similar. In particular, expression of CRG-2/IP-10 appears to be a dominant and locally expressed chemokine in both CNS viral infections pointing to a possible key role for this chemokine in the initiation and subsequent maintenance of leukocyte recruitment to the brain. The chemotactic signature for CRG-2/IP-10 is compatible with such a function, in that it is known to be chemoattractant for monocytes and T lymphocytes but not neutrophils (Taub *et al*, 1993) – these cell populations account for the vast majority of recruited immunoinflammatory cells in both LCM (Doherty *et al*, 1990) and in MHV encephalomyelitis (Lane and Buchmeier, 1997). CRG-2/IP-10 is known to be a pleiotropic chemokine with properties (see Neville *et al*, 1997 for example) that extend beyond its chemotactic actions and include inhibition of angiogenesis (Strieter *et al*, 1995) regulation of cellular adhesion expression and activation of T-cells and monocytes. This raises the possibility that CRG-2/IP-10 may modulate the antiviral host response in other ways. Similar scenarios could be extended to other chemokines such as RANTES and MCP-1 that were also expressed at higher levels in both LCMV and MHV infection of the CNS. However, formal documentation of the precise chemotactic and possibly other functions of chemokines in different viral diseases of the CNS presently awaits verification. The advent of genetically-manipulated mice with deletion of specific chemokine receptor genes (Gao *et al*, 1997) as

well as other approaches such as the use of chemokine receptor antagonists (Wells *et al*, 1996) or neutralizing antibodies (Cook *et al*, 1995), will no doubt provide the experimental substrates to address these important issues.

Materials and methods

Mice and infection with LCMV
Male euthymic C57BL6 X SJL F1 or athymic Balbc/nu^+nu^+ mice were maintained under pathogen-free conditions in the closed breeding colony of the Scripps Research Institute and were used at 10–16 weeks of age. LCMV ARM 53b stock was obtained from a triple plaque-purified clone subsequently passaged twice in BHK cells (Dutko and Oldstone, 1983). For the induction of LCM, mice were inoculated i.c. with either 25 μl of PBS alone (control, non-infected) or PBS containing 200 plaque-forming units of LCMV. According to this infection protocol, infected euthymic mice died between days 6 and 7, while infected athymic nude mice displayed no signs of illness.

RNA preparation
Mice were killed at day 3 or day 6 postinoculation and the spleen and brain were removed and immediately snap frozen in liquid nitrogen and stored at -80°C until RNA preparation. Poly(A^+) RNA was prepared as described previously (Asensio and Campbell, 1997; Badley *et al*, 1988) with the minor modification that total RNA was prepared in parallel. Briefly, following tissue homogenization, incubation in the poly(A^+) RNA lysis buffer and addition of NaCl, a 0.7 ml aliquot was removed for the extraction of total RNA. Nucleic acids were precipitated by the addition of 2 volumes of 100% ethanol. After centrifugation, the pellet was dried and resuspended in buffer containing 4 M guanidiniun thiocyanate, 0.1 M Tris,Cl pH 7.5 and 1% β-mercaptoehtanol. The mixture was acidified by the addition of 2 M sodium acetate pH 4.0 and then extracted with H_2O equilibrated phenol, followed by phenol/chloroform and chloroform. RNA was precipitated in ethanol, centrifuged and the pellet resuspended in TE pH 8.0. The concentration of RNA was determined by UV spectroscopy at 260 nm.

RNase protection assay
For the RNase protection assays (RPA) the specific target sequences used to generate probes against individual cytokine (Hobbs *et al*, 1993) chemokine (Asensio and Campbell, 1997), LCMV nucleoprotein (NP) (Sandberg *et al*, 1994), type I IFNs (Sandberg *et al*, 1994b) and immunocyte (Guidotti *et al*, 1996) gene transcripts and their characterization was described in detail previously. In all the RPA probe sets, a similarly cloned fragment (sequence nucleotides 61–139) of the ribosomal protein RPL32 cDNA (Dudov and Perry, 1984) was used as a control for RNA loading. The RPAs were performed as described previously (Campbell *et al*, 1994).

In situ *hybridization*
Anesthetized control and infected mice were perfused transcardially with ice-cold saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Brains were removed, post fixed 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 (Campbell *et al*, 1994).

For probe, a CRG-2/IP-10 cDNA fragment (726 bp) sub-cloned in the ribovector pGEM-4 (Promega, Madison, WI) was synthesized by RT–PCR as described previously (Asensio and Campbell, 1997). For detection of sites of viral replication a 2.0 kb cDNA fragment of the LCMV-Arm NP subcloned in pGEM-3Z (Promega) was used to generate an antisense NP probe (Campbell *et al*, 1994). Adjacent sections were hybridized for LCMV-NP and CRG-2/IP-10. In addition to sections from non-infected brain, the specificity of the probes was confirmed by comparison with sections hybridized to corresponding sense probes.

Following hybridization with the CRG-2/IP-10 probe some slides were co-labeled by immunostaining for GFAP to identify astrocytes. After the final PBS wash, and before dehydration in graded alcohol, the sections were immunostained for glial fibrillary acidic protein (GFAP diluted 1 : 2000; DAKO, Carpinteria, CA) to identify astrocytes. Briefly, after the final post-hybridization wash, slides were transferred to PBS containing 2% goat serum for 1 h at room temperature to block nonspecific binding. After washing, sections were incubated overnight with the primary antibody. After extensive washing, sections were incubated with anti-rabbit avidin-biotinylated horseradish peroxidase complex (ABC Kit, Vector, Burlingame, CA) used according to the manufacturer's instructions. Staining reactions were performed with 3, 3 diaminobenzidine (Sigma Chemical Co. St Louis, MO) as substrate. After dehydration through graded alcohol and air drying, slides were then dipped in Kodak NTB-2 emulsion, dried and stored in the dark for 1–2 weeks after which time the slides were developed, counter stained with Mayer's hematoxylin and examined by dark and bright field microscopy.

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