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Central nervous system chemokine expression during Theiler's virus-induced demyelinating disease

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Theiler's murine encephalomyelitis virus is an endemic murine pathogen that induces a demyelinating disease of the central nervous system in susceptible mouse strains. The disease is characterized by central nervous system mononuclear cell infiltration and presents as chronic, progressive paralysis. The expression of CC and C-x-C chemokines in the central nervous system of Theiler's murine encephalomyelitis virus-infected mice was examined throughout the disease course by ELISA and RT–PCR analysis. Central nervous system expression of MCP-1 and MIP-1α protein was evident by day 11 post Theiler's murine encephalomyelitis virus infection of SJL mice and continued throughout disease progression. MIP-1α, RANTES, MCP-1, C10, IP-10, and MIP-1β mRNA was specifically expressed in the central nervous system and not the periphery following Theiler's murine encephalomyelitis virus infection. This was associated with development of clinical disease. These data suggest that the expression of multiple chemokines at particular times following viral infection is associated with demyelinating disease.

Keywords: cell migration; central nervous system; inflammation; RT–PCR; mice

Introduction

Theiler's murine encephalomyelitis virus (TMEV) is an endemic murine pathogen that induces a demyelinating disease of the central nervous system (CNS). Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) is an excellent model for human multiple sclerosis (MS) as both diseases show chronic progressive paralysis and are characterized by mononuclear cell infiltration of the CNS and subsequent axonal demyelination (Miller and Gerety, 1990). Following intracerebral (i.c.) infection, TMEV replicates in CNS cells including microglial and oligodendrocytes (Yamada *et al*, 1990; Ozden *et al*, 1991). A peripheral viremia develops and is neutralized by antibody responses during the first 2 weeks post infection, however, virus persists in CNS macrophages for the lifetime of the animal (Clatch *et al*, 1990; Lipton *et al*, 1984). Focal inflammation (predominantly T lymphocytes and macrophages) occurs only in the white matter of the spinal cord and brain (Lipton, 1975). Virus-specific $CD4^+$ T lymphocytes are believed to mediate immunopathology in the CNS (Welsh *et al*, 1987; Borrow *et al*, 1992; Gerety *et al*, 1994). Macrophages recruited and activated by Th1 cytokines phagocytose myelin (bystander demyelination) (Rossi *et al*, 1997; Cammer *et al*, 1978). From 7 to 11 weeks post disease onset, animals progress from mild clinical symptoms to more severe paralysis with total hindlimb paralysis occurring after 13 weeks. Miller *et al* (1997) have reported that early in TMEV-IDD (0–50 days post infection) viral antigen-specific T lymphocyte proliferative and delayed type hypersensitivity (DTH) responses could be detected. Approximately 50 days post infection, however, antigen-specific T lymphocyte proliferative and DTH responses to the proteolipid peptide (PLP139-151) could be measured. This phenomenon, termed epitope spreading (Lehmann *et al*, 1993), has been described as having a functional role in experimental autoimmune encephalomyelitis (EAE) disease progression (McRae *et al*, 1995).

The ability of different strains of mice to develop clinical disease is influenced by several factors. Multiple genes have been shown to be factors in susceptibility to TMEV-IDD. Examples of such

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genes include *Tmevd-1*, *Tmevd-2*, TCR (non-MHC), and H-2D (MHC) (Miller *et al*, 1998; Rodriguez *et al*, 1992; Clatch *et al*, 1987b). In addition, enhanced viral clearance (Lindsley *et al*, 1991) or down-regulation of T lymphocyte responses (Nicholson *et al*, 1996) by different populations of $CD8^+$ T lymphocytes have been implicated in TMEV resistance. The categories of disease susceptibility are defined as follows: highly susceptible exhibits clinical signs of disease in 90–100% of mice; intermediately susceptible exhibits clinical signs in approximately 50% of the mice; and resistant exhibits clinical signs in no mice by day 120 days post infection (Clatch *et al*, 1987a).

Chemoattractant cytokines (chemokines), specific for mononuclear cells and neutrophils, induce leukocyte accumulation at sites of inflammation (Oppenheim *et al*, 1991). Chemokines are divided into four highly conserved families designated by the position of the first two cysteines in the protein: C-x-C, C-C, C, and C-x_3-C. Members of the C-x-C family are predominantly chemotactic for neutrophils. The C-C chemokines are predominantly chemotactic for monocytes/macrophages, T lymphocytes, basophils, and eosinophils. C-C chemokine expression has recently been shown to play an important role in the pathogenesis of EAE. In murine EAE, it has been shown that MIP-1α expression in the CNS increases during the acute phase in animals injected with the immunodominant, encephalitogenic peptide (PLP 139-151) compared to naïve animals. Furthermore, administration of anti-MIP-1α prevents the development of both acute and relapsing disease (Karpus *et al*, 1995). In addition, MCP-1 is produced at higher levels during relapses in EAE compared to naïve animals and anti-MCP-1 can inhibit relapsing EAE (Kennedy *et al*, 1998). In Lewis rats, MCP-1 expression parallels development of disease (Hulkower *et al*, 1993). Recruitment of mononuclear cells into the CNS is a major characteristic of pathogenesis in TMEV-IDD. Therefore, the production of chemokines throughout the disease course of TMEV-IDD and their role in determining susceptibility was analyzed in the experiments described in the present report.

Results

The clinical disease course of TMEV-IDD presents as chronic, progressive. Unlike EAE, no episodes of remission or relapse occur even though the histopathology of each disease is similarly characterized by CNS mononuclear cell accumulation and demyelination. Figure 1 illustrates that SJL mice infected i.c. with the BeAn strain of TMEV begin to develop clinical signs approximately 30 days post infection and achieve maximum severity 80 days post infection. This is demonstrative of a chronic-progressive disease course. At this time there is persistent viral infection (Lipton *et al*, 1984; Clatch *et al*, 1990), extensive CNS mononuclear cell infiltration (Pope *et al*, 1996), antigen-specific delayed type hypersensitivity (Clatch *et al*, 1986), and demyelination (Dal Canto and Lipton, 1975).

In order to determine a relationship between the presence of chemokines in the CNS and development of clinical disease, chemokine-specific ELISA was utilized to analyze the expression of the C-C chemokines MCP-1 and MIP-1α. SJL mice were infected i.c. with 3×10^6 plaque forming units (PFU) of TMEV. Three mice were sacrificed on days 0 (naïve), 11, 14, 22, 28, 30, 37, 44 and 52 post TMEV infection. Days 11 and 14 post infection represent the period of time during which peripheral viremia develops accompanied by a neutralizing antibody response. The neutralizing antibody clears the virus from the periphery but not from the CNS (Lipton, 1975). Absence of the neutralizing antibody response results in a more severe and lethal disease, suggesting that initial control of virus replication is a function of the first phase of the anti-viral immune response (Rodriguez *et al*, 1990). Days 22 and 28 represent the time immediately prior to the presentation of clinical disease. The onset of clinical disease occurs in a majority of the mice at approximately 30 days post infection. Days 37, 44 and 52 represent the time during which clinical disease progresses from mild to more severe chronic paralysis. The spinal cords were harvested, homogenized in PBS, and the clarified supernatants subsequently analyzed by ELISA for the presence of MCP-1 and MIP-1α. We have previously used this strategy to identify the role of chemokines in

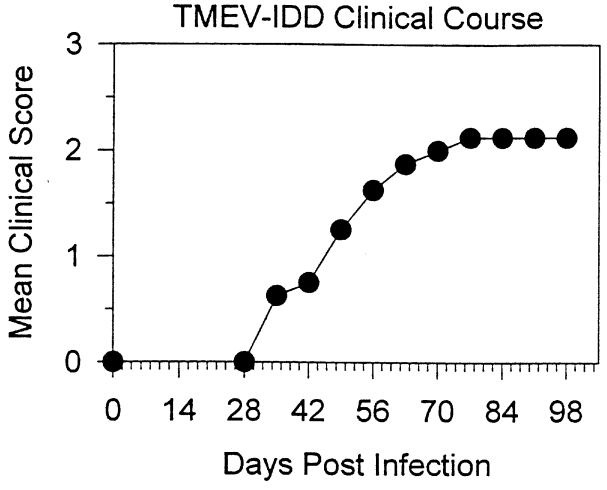

Figure 1 Clinical disease course following TMEV infection. SJL mice were infected i.c. with 3×10^6 p.f.u. of TMEV. Data are expressed as the mean clinical disease score for all mice as a function of days post TMEV infection. Disease severity was scored by the scale described in the Materials and methods.

relapsing EAE (Kennedy *et al*, 1998). Figure 2 shows the expression pattern of these chemokines in the CNS during the course of TMEV disease. Low, but detectable, levels of MCP-1 and MIP-1α were seen on day 0. By day 11 following TMEV infection, the expression of MCP-1 and MIP-1α had increased significantly. Furthermore, MIP-1α and MIP-1β expression in the CNS remained elevated throughout the disease. Chemokine protein expression as determined by specific ELISA following disease induction was limited to the CNS and not seen in peripheral lymphoid tissue such as spleen or lymph nodes from the same animals (data not shown). These data demonstrate that MIP-1α and MCP-1 expression in the CNS is associated with the development of demyelinating disease following TMEV infection.

TMEV demyelinating disease is characterized by CNS mononuclear cell infiltration. Therefore, CNS expression of additional C-C (C10, MIP-1β, and RANTES) and C-x-C (IP-10 and KC) chemokines were analyzed at the mRNA level by RT–PCR. Expression of MIP-1β and RANTES has been shown to be associated with disease development and progression in EAE (Kuchroo *et al*, 1993; Godiska *et al*, 1995; Kennedy *et al*, 1998). Furthermore, MIP-1β (Peterson *et al*, 1997) and IP-10 have been shown to be produced by astrocytes and therefore their expression was also examined in these experiments (Sun *et al*, 1997; Majumder *et al*, 1996; Vanguri *et al*, 1996). Figure 3 shows the pattern of chemokine mRNA expression in the spinal cords of TMEV-infected SJL mice. MIP-1α, MCP-1, C10, IP-10 and MIP-1β mRNA was seen in the CNS of mice 10 days following TMEV infection. It is interesting to note that only RANTES and IP10 mRNA was seen in the CNS of mice 21 days following infection. This is a timepoint following peripheral viremia clearance (Lipton, 1975) and prior to extensive mononuclear cell infiltration and development of clinical disease (Pope *et al*, 1996). At day 37 post infection, when all mice in the experiment were showing signs of clinical disease development, MIP-1α , RANTES, MCP-1, C10, IP-10, and MIP-1β mRNA expression was seen in the CNS. Expression of these chemokines was also seen at days 52 and 73 post infection. During this phase of disease mononuclear cell infiltration is extensive and clinical disease progressed to a more severe level. These data demonstrate that chemokine mRNA was expressed in the CNS prior to clinical disease development and throughout disease progression. Chemokine mRNA expression following disease induction was limited to the CNS and not

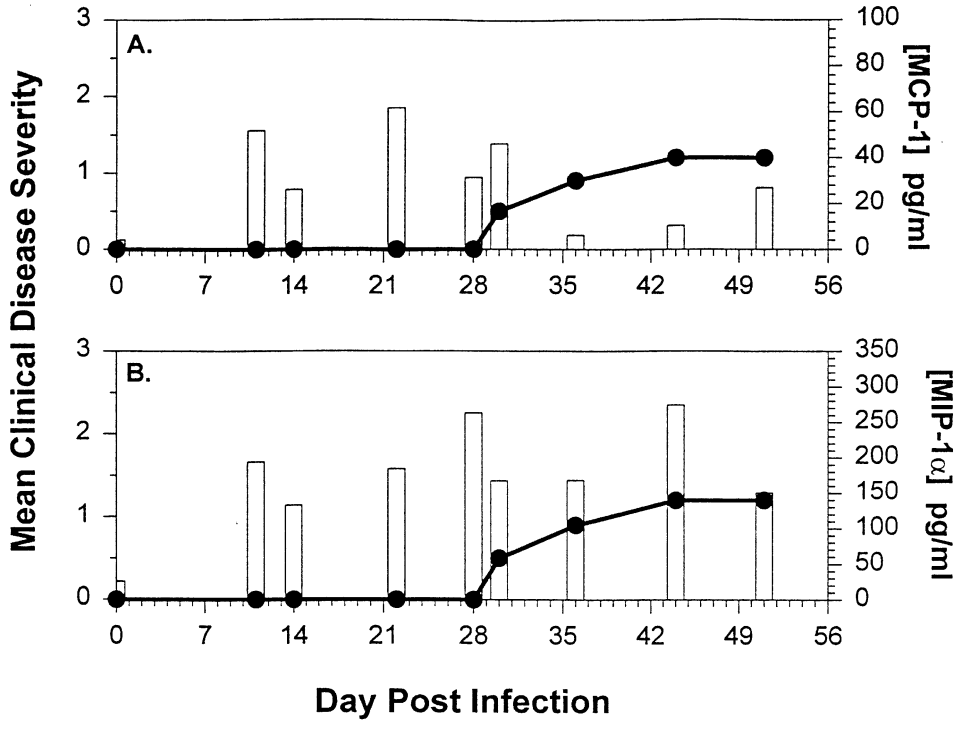

Figure 2 CNS MIP-1α and MCP-1 protein expression during the course of TMEV-IDD in SJL mice. Mice were infected i.c. with TMEV and spinal cords were harvested from three mice at each indicated time point (filled dots). Each spinal cord was individually assayed by ELISA for the presence of MCP-1 (**A**) and MIP-1α (**B**). The curve represents the mean clinical disease score for all mice while the open bars represent the average chemokine concentration in the spinal cord lysates of three mice expressed as pg/ml in the clarified lysate when compared to the recombinant standards.

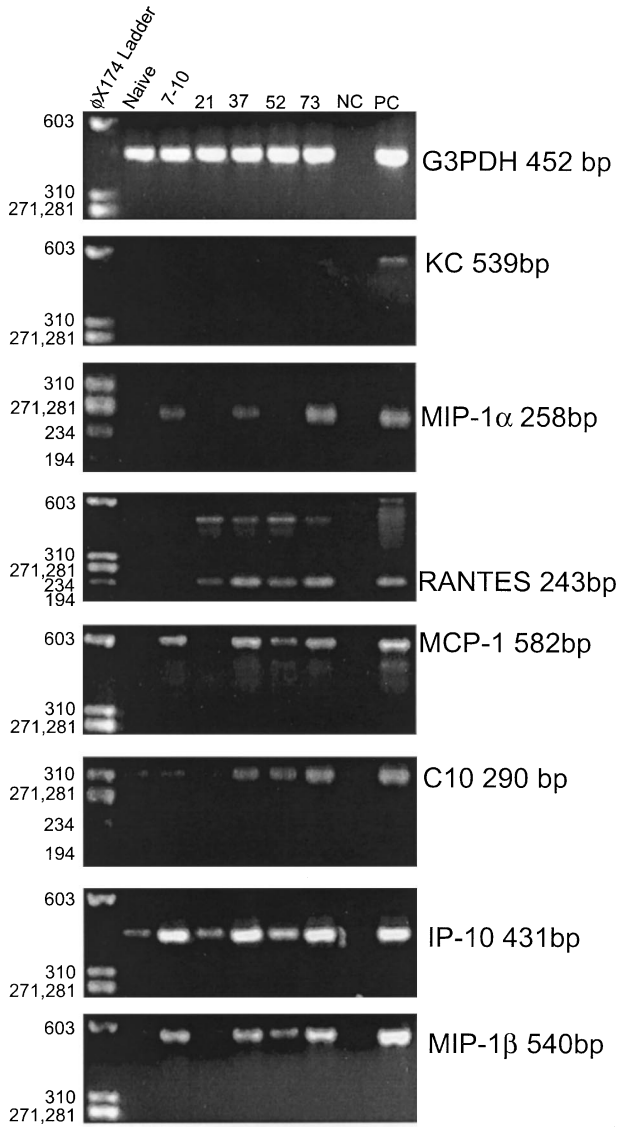

Figure 3 CNS chemokine mRNA expression during the course of TMEV-IDD in mice. Mice were infected i.c. with TMEV. Spinal cords were harvested from SJL mice at the indicated time points and total RNA was isolated. Sequences of KC, MIP-1α, RANTES, MCP-1, C10, IP-10, MIP-1β, and G3PDH (as an internal control) were amplified by PCR using 5′ and 3′ primers specific for the particular target (see Table 1) and subsequently electrophoresed in 2% agarose gels containing ethidium bromide. The size of each amplified sequence is indicated next to the corresponding chemokine name.

seen in peripheral lymphoid tissue (data not shown).

Discussion

TMEV-IDD is a $CD4^+$ T lymphocyte-mediated demyelinating disease of the CNS that serves as a model for MS. The disease is characterized by CNS mononuclear cell infiltration and demyelination resulting in a chronic-progressive disease course. The susceptibility of different mouse strains to clinical disease is dependent upon several factors including MHC and non-MHC genes. Regulation of macrophage and T cell migration from the periphery to the CNS is important for disease pathogenesis. Chemokine production in the CNS has recently been shown to play an important role in the development of EAE, a relapsing-remitting demyelinating disease of the CNS (Karpus *et al*, 1995; Hulkower *et al*, 1993; Ransohoff *et al*, 1993; Godiska *et al*, 1995; Glabinski *et al*, 1997). Furthermore, CNS chemokine expression has been demonstrated in mice infected with lymphocytic choriomeningitis virus (Asensio and Campbell, 1997) and mouse hepatitis virus (Lane *et al*, 1998). In the present report we have demonstrated an association of a subset of chemokines with TMEV-IDD.

CNS expression of MCP-1 and MIP-1α protein was evident by day 11 post infection in SJL mice (Figure 2). This is a timepoint when there is high titer anti-TMEV antibody response (Lipton, 1975) as well as an anti-TMEV T cell response designed to clear the viral infection (Borrow *et al*, 1992; Dethlefs *et al*, 1997). Expression of these chemokines remains elevated during the course of disease (Figure 2). It is reasonable to assume that these chemotactic factors induce an initial migration and continued accumulation of T cells and monocytes into the CNS. Pope *et al* (1996) have shown that the percentage of $CD4^+$ and $CD8^+$ CNS infiltrating cells in TMEV-infected SJL mice gradually increases between days 20 and 119 post infection. The expression pattern in TMEV-IDD (Figure 2) differs from what we have noted in relapsing-remitting EAE (Kennedy *et al*, 1998). In the former we observed both MIP-1α and MCP-1 expressed in the CNS following disease induction while in EAE MIP-1α was expressed in the CNS during acute clinical disease and MCP-1 was expressed in the CNS during relapsing EAE. The differences might be attributable to disease induction. TMEV-IDD was induced by direct infection of CNS resulting in active viral replication and persistence in the CNS (Lipton *et al*, 1984). In contrast, EAE was induced by the adoptive transfer of PLP139-151-specific T cells. Chemokine expression in EAE has been shown to be associated with the initial inflammatory infiltrate (Glabinski *et al*, 1995) whereas in TMEV-IDD macrophages/microglia, astrocytes, and oligodendrocytes can become infected and might directly produce a different spectrum of chemokines.

In order to further study the role of CNS chemokine expression in TMEV-IDD we utilized an RT–PCR approach for the detection of chemokines in which we do not have an established immunoassay. This analysis showed that MIP-1α, MCP-1, C10, IP-10 and MIP-1β mRNA was ex-

pressed at day 10 following viral infection (Figure 3). This time corresponds to the presence of antiviral T cells designed to clear the initial viral infection (Borrow *et al*, 1992). Additionally, at this timepoint there is very little T cell and monocyte accumulation in the CNS (Pope *et al*, 1996). At the time of clinical disease development, approximately day 37 in this study, MIP-1α, RANTES, MCP-1, C10, IP-10 and MIP-1β mRNA expression was detected in the CNS (Figure 3). It is interesting to note that at this phase of initial clinical disease development there is substantially more T cell infiltration into the CNS than at earlier times following viral infection (Pope *et al*., 1996). The difference in chemokine expression between these two time points appears to be restricted to RANTES thereby raising the possibility that RANTES is a critical chemokine for the regulation of mononuclear cell infiltration just prior to clinical disease development. We are currently testing this idea using anti-chemokine treatment approaches, similar to what we have reported in the EAE system (Karpus *et al*, 1995, 1998; Karpus and Kennedy, 1997; Kennedy *et al*, 1998). Neither KC (Figure 3) nor MIP-2 (data not shown), two C-x-C chemokines known to be neutrophil attractants, were found to be expressed in the CNS following TMEV infection. This is consistent with the idea that neutrophils are rarely seen in the CNS inflammatory infiltrate (Dal Canto *et al*, 1995). The apparent discrepancy in chemokine expression between the RT–PCR and ELISA analysis can be explained in part by the transcriptional regulation of chemokine production and the ability of extracellular matrix proteins to bind and immobilize chemokines. In the case of the former, MIP-1α and MCP-1 expression is not constitutive, but rather transcriptionally regulated and our analysis may be detecting waves of chemokine mRNA expression. Furthermore, the ability of extracellular matrix proteins to bind chemokines has been suggested to be important in the stabilization of these molecules for recognition by receptor-bearing cells during migration (Bacon and Schall, 1996) and may be the reason why protein can be detected in the tissue over long time periods. Overall, these data suggest that chemokines are associated with mononuclear cell infiltration during the course of TMEV-IDD development. Further examination of specific chemokine activity during disease development and progression will hopefully reveal novel and specific targets for anti-inflammatory intervention of the demyelinating disease process.

Materials and methods

Mice

Five to six-week-old SJL/J female mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA). Mice were 6–7 weeks old at the initiation of the experiments. Animal care was provided according to Northwestern University and National Institutes of Health guidelines.

Induction and clinical evaluation of TMEV-IDD

Mice were anesthetized by methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL, USA) inhalation and injected in the right cerebral hemisphere with 3×10^6 PFU of TMEV (BeAn 8386 strain) in 30 μl of sterile DMEM. Mice were examined 2–3 times per week for the first 3 weeks then daily until all infected animals were exhibiting neurological signs of TMEV-IDD. After signs of clinical disease, mice were examined biweekly. Clinical symptoms were scored as (1) waddling gait, (2) severe waddling gait and difficulty with righting itself, and (3) hind limb paralysis with incontinence. Clinical data have been expressed as the mean clinical score at a particular timepoint.

Table 1 PCR primer sequences

Chemokine		*Sequence*	*Size*
G3PDH	Sense	5′-ACCACAGTCCATGCCATCAC-3′	452
	Anti-sense	5′-TCCACCACCCTGTTGCTGTA-3′	
C10	Sense	5′-ATAACGCGTATGCAGGCCTCATACAAGAAATGG-3′	312
	Anti-sense	5′-TACTGCAGTCAAGCAATGACCTTGTTC-3′	
IP-10	Sense	5′-CCTATCCTGCCCACGTGTTGAG-3′	341
	Anti-sense	5′-CGCACCTCCACATAGCTTACAG-3′	
KC	Sense	5′-TCGCTTCTCTGTGCAGCGCT-3′	539
	Anti-sense	5′-GTGGTTGACACTTAGTGGTCTC-3′	
MCP-1	Sense	5′-TCTCTTCCTCCACCACCATGCAG-3′	582
	Anti-sense	5′-GGAAAAATGGATCCACACCTTGC-3′	
MIP-1α	Sense	5′-GCCCTTGCTGTTCTTCTCTGT-3′	258
	Anti-sense	5′-GGCAATCAGTTCCAGGTCAGT-3′	
MIP-1β	Sense	5′-AACCCCGAGCAACACCATGAAG-3′	540
	Anti-sense	5′-CCACAATAGCAGAAACAGCAAT-3′	
RANTES	Sense	5′-AAGATCTCTGCAGCTGCCCTC-3′	243
	Anti-sense	5′-TTGAACCCACTTCTTCTGTGG-3′	

Antibodies
Goat anti-murine MIP-1α, anti-murine MIP-1β, biotinylated anti-murine MIP-1β and rat anti-murine CRG-2/IP-10 purified antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti-murine MIP-1α and anti-murine MCP-1 antibodies were generously supplied by Dr Steven Kunkel (University of Michigan, MI, USA).

ELISA analysis
The spinal cords from three mice were harvested at each timepoint and each sample was homogenized in 1 ml of PBS. The homogenate supernatant was collected after centrifugation ($400 \times g$) for 10 min. Expression of MCP-1 was determined by immunoassay kit (Endogen, Cambridge, MA, USA) according to the manufacturer's instructions. ELISA analysis was performed for expression of MIP-1α, MIP-1β, and IP-10 as previously described (Kennedy *et al*, 1998). Briefly, flat bottom microtiter plates were incubated with 3.2 μg/ml of capture antibody in borate-buffered saline overnight. Nonspecific binding sites were blocked with 2% BSA in PBS for 1 h. The plates were washed and samples added in triplicate. Biotinylated goat anti-rabbit detection antibody (Zymed, South San Francisco, CA, USA) was then added for 1 h. The wells were developed using streptavidin-peroxidase and TMB one-step substrate (Dako Corporation, Carpinteria, CA, USA) and absorbence measured at 450 nm. All incubations were performed at room temperature. A series of dilutions of purified recombinant protein (R&D Systems) was used to generate standard curves for each chemokine. Chemokine expression levels were quantitated by comparison to the standard curves.

Isolation of RNA
Spinal cords from TMEV-infected mice were harvested and RNA isolated as described previously (Begolka *et al*, 1998). Briefly, three mice from each time point following TMEV infection were anesthetized with methoxyflurane and sacrificed by total body perfusion through the left ventricle with PBS. After harvesting the spinal cords, a single cell suspension was made from the tissue and pelleted by centrifugation. Pellets were resuspended in 4 M guanidinium isothiocyanate/50 mM Tris-Cl (pH 7.5)/25 mM EDTA (Life Technologies, Gaithersburg, MD, USA) and 1% 2-ME and 0.5% *N*-lauroylsarcosine (Sigma-Aldrich, St. Louis, MO, USA). The suspension was forced through a 23-gauge needle to aid in shearing the DNA. Total RNA was isolated by high-speed gradiant centrifugation of the suspension through 5.7 M CsCl for 20 h at 4°C. RNA was resuspended in diethylpyrocarbonate-treated water.

RT–PCR
Reverse transcription of poly A mRNA into cDNA was accomplished using advantage-RT kit (Clontech, Palo Alto, CA, USA) oligo (dT) primers according to the manufacturer's instructions. Sequences of C10, IP-10, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and G3PDH (as an internal control) were amplified by PCR using 5′ and 3′ primers specific for the particular chemokine (Table 1). Primer sets were designed to span multiple introns to eliminate genomic DNA contamination. The samples were amplified for 30 cycles of 94°C, 62°C, and 72°C (MCP-1, MIP-1α, and MIP-1β) or 94°C, 65°C and 72°C (C10, IP-10, KC, and RANTES) and then electrophoresed in 2% agarose gels containing ethidium bromide. G3PDH can be amplified at either temperature set.

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