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Kinetics of Cytokine mRNA Expression in the Central Nervous System Following Lethal and Nonlethal Coronavirus-Induced Acute Encephalomyelitis

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The potential role(s) of cytokines in the reduction of infectious virus and persistent viral infection in the central nervous system was examined by determining the kinetics of cytokine mRNA expression following infection with the neurotropic JHM strain of mouse hepatitis virus. Mice were infected with an antibody escape variant which produces a nonlethal encephalomyelitis and compared to a clonal virus population which produces a fulminant fatal encephalomyelitis. Infection with both viruses induced the accumulation of mRNAs associated with Th1- and Th2-type cytokines, including IFN- γ , IL-4, and IL-10. Peak mRNA accumulations were coincident with the clearance of virus and there was no obvious differences between lethally and nonlethally infected mice. TNF- α mRNA was induced more rapidly in lethally infected mice compared to mice undergoing a nonfatal encephalomyelitis. Rapid transient increases in the mRNAs encoding IL-12, iNOS, IL-1 α , IL-1 β , and IL-6 occurred following infection. Nonlethal infections were associated with increased IL-12, IL-1 β , and earlier expression of IL-6, while lethal infections were associated with increased iNOS and IL-1 α mRNA. These data suggest a rapid but differential response within the central nervous system cells to infection by different JHMV variants. However, neither the accumulation nor kinetics of induction provide evidence to distinguish lethal infections from nonlethal infections leading to a persistent infection. Accumulation of both Th1 and Th2 cytokines in the central nervous system of JHMV-infected mice is consistent with the participation of both cytokines and cell immune effectors during resolution of acute viral-induced encephalomyelitis. © 1997 Academic Press

INTRODUCTION

The goal of the immune response during viral infection is to limit replication via induction of both nonspecific and specific antiviral effectors. Acute viral infections of the central nervous system (CNS) result in vigorous, but in some instances limited, host immune responses (Sedgwick and Dorries, 1991). In contrast to responses in the periphery where limiting virus replication can generally be carried out with minimal regard to tissue damage, within the CNS checks and balances minimize inflammatory-mediated damage while limiting viral-induced cytopathology. Although a wide range of immune effectors are often induced, predominant anti-viral mechanisms appear related to the pathogenesis strategy of the individual agent. For example, infection of mice with lymphocytic choriomeningitis virus induces a predominant CD8⁺ cytotoxic T lymphocyte (CTL) response (Lehmann *et al.*, 1988). By contrast, resolution of measles virus-encephalitis in mice is mediated by CD4⁺ T cells and correlates with the local production of IFN- γ (Finke *et al.*, 1995). Finally, resolution of Sindbis virus-induced encephalitis is related to induction of neutralizing

antibody and a pattern of Th2 cytokines within the CNS (Wesselingh *et al.*, 1994).

Variations between viral infections resulting in CNS inflammation prompted an examination of the temporal induction of CNS cytokines during fatal and nonfatal CNS infections by variants of the JHM strain (JHMV) of mouse hepatitis virus (MHV). In immunocompromised hosts JHMV replicates unchecked in the CNS demonstrating the importance of immune effectors in limiting CNS virus replication (Kyuwa and Stohlman, 1990; Houtman and Fleming, 1996b; Lane and Buchmeier, 1997). Effector mechanisms implicated in protection and clearance of JHMV from the CNS include cell-mediated immunity and both neutralizing and nonneutralizing antibodies. JHMV provides an interesting paradigm of acute viral encephalitis not only because of its associated demyelination (Weiner, 1973; Lampert *et al.*, 1973) but also because some immune effector mechanisms prevent death via directly reducing CNS virus replication while other immune effectors prevent death without significantly altering virus replication (Kyuwa and Stohlman, 1990; Houtman and Fleming, 1996b; Lane and Buchmeier, 1997). A common theme appears to be prevention of neuronal infection by reducing viral load or preventing neuronal infection, most likely via cytokines.

The exact mechanisms of immune-mediated protec-

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tion and clearance of JHMV from the CNS are not yet clear. The antiviral effects of CD8⁺ T cells appear to be due to direct lysis of infected cells; however, CD8⁺ and CD4⁺ T cells may also exert antiviral activity indirectly via cytokine secretion (Biron, 1994). Neither infected neurons nor oligodendrocytes appear susceptible to major histocompatibility (MHC) class I-mediated killing *in vivo*, consistent with the inability of JHMV-specific CTL to clear virus from infected oligodendroglia (Stohlman *et al.*, 1995b). Furthermore, clearance of JHMV from the CNS is inhibited, but not abolished, in mice genetically deficient in perforin-mediated cytotoxicity (Lin *et al.*, 1997). These data suggest the possibility that cytokines contribute to either clearance or protection from JHMV infection. During JHMV infection of the CNS there is an abrupt increase in mRNA encoding interleukin-1 (α and β), IL-6, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ , at the time of maximal decrease in virus replication and mononuclear cell infiltration (Pearce *et al.*, 1994). No IFN- γ mRNA was detected in immunodeficient mice, suggesting this cytokine may be important during viral clearance (Pearce *et al.*, 1994). Consistent with this concept, mice treated with anti-IFN- γ are more susceptible to JHMV, while administration of IFN- γ provides protection (Smith *et al.*, 1991). IL-6, TNF- α , and type 2 nitric oxide synthase (iNOS) have also been detected in the CNS during acute JHMV infection (Sun *et al.*, 1995; Stohlman *et al.*, 1995a; Lane *et al.*, 1997) while IL-1 β , IL-6, TNF- α , and iNOS were detected in the CNS of chronically infected mice (Sun *et al.*, 1995). The complex interactions of multiple immune effector mechanisms during JHMV infection may reflect both the relative immune privilege of the CNS (Sedgwick and Dorries, 1991) as well as the specific tropism of the virus for CNS cell types. Neurotropic MHV isolates differ in tropism and include viruses with predominant tropisms for astrocytes, microglia, and oligodendroglia as well as neurons (Fleming *et al.*, 1986; Perlman and Reis, 1987; Kyuwa and Stohlman, 1990; Pearce *et al.*, 1994; Stohlman *et al.*, 1995a). The balance between limiting viral replication and preserving CNS function occasionally results in incomplete viral clearance and a persistent CNS infection which may or may not involve the continued presence of infectious virus (Kyuwa and Stohlman, 1990; Houtman and Fleming, 1996b). Persistence of infectious virus correlates with the presence of CTL escape variants (Pewe *et al.*, 1996). To understand the complex interrelationships between encephalitis, protection, and viral clearance leading to a persistent infection of the CNS, the expression of pro- and anti-inflammatory cytokine mRNAs in the CNS of mice undergoing either lethal or sublethal JHMV infection were compared. The two JHMV chosen for study infect either primarily microglia and astrocytes, less frequently oligodendroglia and neurons (DM) or primarily oligodendroglia, much less frequently microglia and astrocytes,

and rarely neurons (2.2v-1). These viruses contrast to the predominantly neuronotropic OBLV-60 variant previously examined (Pearce *et al.*, 1994; Lane *et al.*, 1997).

MATERIAL AND METHODS

Mice and viruses

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 6 weeks and maintained in the University of Southern California vivarium. All mice were used at 7 weeks of age. To produce a lethal infection, mice were infected by intracerebral inoculation (i.c.) with 100 PFU of the plaque-purified DM isolate of JHMV (Stohlman *et al.*, 1982) in a volume of 32 μ l. This virus has the plaque size and pathogenesis similar to the parental suckling mouse brain pool of JHMV originally described by Weiner (1973) and produces a lethal encephalomyelitis with minimal demyelination apparent at the time of death. To produce a sublethal infection, mice were infected with 25 PFU of the 2.2v-1 monoclonal antibody-derived neutralization-resistant variant of JHMV (Fleming *et al.*, 1986). This variant replicates predominantly in oligodendroglia producing a flaccid paralysis. Although viral antigen is cleared from survivors by 30 days postinfection (p.i.), viral RNA persists for at least 12 months (Adami *et al.*, 1995). Groups of at least 3 mice were sacrificed at various times p.i. Immunosuppression was induced by lethal irradiation (850R) 24 hr prior to infection. Sham-infected mice were injected i.c. with 32 μ l of sterile endotoxin-free phosphate-buffered saline (PBS).

Virus titration

Virus titers were determined by plaque assay using monolayers of DBT cells as previously described (Stohlman *et al.*, 1982). One-half of the brain was homogenized using Tenbrock tissue homogenizers in 2.0 ml of Dulbecco's PBS, pH 7.4. The remaining half was taken for histopathology or RNA extraction (see below). Following centrifugation at 1500 *g* for 7 min at 4°, supernatants were assayed immediately or frozen at -70°. Data presented are the average titer of groups of three or more mice.

Antibody titration

JHMV-specific IgM, IgG1, and IgG2a antibodies were quantitated by ELISA as previously described (Lin *et al.*, 1997) using rabbit anti-mouse IgM, IgG1, or IgG2a antibodies (Cappel, Costa Mesa, CA). Concentrations of serum antibodies were expressed as the highest dilution with O.D. values three times above background level. Neutralizing antibodies were tested in serum as previously described (Lin *et al.*, 1997).

Histology

Histopathologic analysis was performed as previously described (Stohlman *et al.*, 1995a). Briefly, tissues were

fixed for 3 hr in Clark's solution (75% ethanol, 25% glacial acetic acid) and embedded in paraffin. Sections were stained with hematoxylin and eosin or luxol fast blue. Distribution of JHMV antigen was examined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratory, Burlingame, CA) using the anti-JHMV mAb J3.3 specific for the viral nucleocapsid protein (Fleming *et al.*, 1983).

Cytokine mRNA expression

Brains were processed individually to prevent contamination. RNA was isolated from half brains by homogenization at room temperature in guanidinium isothiocyanate using Tenbrock tissue homogenizers as previously described (Cua *et al.*, 1995). Samples were sheared prior to centrifugation through 5.4 M cesium chloride at 100,000 *g* for 18 hr to isolate RNA. The cDNA were prepared using avian myeloblastosis reverse transcriptase (Promega, Madison, WI) and oligo dT primers (Promega) for 60 min at 42°. Expression of cytokine mRNA was determined by semiquantitative PCR analysis, following procedures previously described (Cua *et al.*, 1995, 1996). PCR was performed using AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ) and specific cytokine primers for IFN- γ , IL-1 α , IL-1 β , IL-4, IL-6, IL-10, TNF- α (Murphy *et al.*, 1993; Cua *et al.*, 1996), and IL-12p40. The sequences of the IL-12p40 oligonucleotides primers and probe used are as follows: 5' primer, GAC CCT GCC CAT TGA ACT GGC; 3' primer, CAA CGT TGC ATC CTA GGA TCG; oligoprobe, TGT CTG CGT GCA AGC TCA GGA. Amplification was carried out using 35 cycles of one denaturation step at 94° (45 sec), primer annealing at 59° (45 sec), extension step at 72° (1.5 min), followed by a final extension step for 7 min. For IL-4 and iNOS a nested PCR was performed by using internal primers in a second round of PCR (25 cycles) under the conditions described above. The oligonucleotide primers used in the second PCR for IL-4 were the corresponding sequences for the 5'-primer and the probe described by Cua *et al.* (1996). The nucleotide sequence for the IL-4 oligonucleotide probe was TTG AAG GAG GTC ACA GGA GAA GGA (Sideras *et al.*, 1987). The 5' and 3' outer primer sequences for iNOS were GCC TTC CGC AGC TGG GCT GT and ATG TGG TAG CCA CAT CCC GAG CC, respectively (Lyons *et al.*, 1992). Internal 5' and 3' iNOS primers were AGC TAC TGG GTC AAA GAC AAG AGG CT and the 3' outer primer, respectively. The oligonucleotide probe consisted of the sequence CTC CCT TCC GAA GTT TCT GGC AGC A.

For quantification, PCR products were diluted in denaturing solution (0.4 N NaOH, 25 mM EDTA), neutralized with Tris-HCl (1.0 M; pH 8.0), and transferred to 0.45 μ m Nytran membranes (Schleicher & Schuell, Keene, NH) using a Minifold I dot blot apparatus (Schleicher & Schuell). Membranes were hybridized (60°; overnight)

with ³²P-ATP-labeled internal oligonucleotide probes. Membranes were washed (three times; 2 \times SSC, 0.1% SDS; room temperature), exposed to Storage Phosphor Screens (Molecular Dynamics, Sunnyvale, CA), and scanned using a phosphorimaging scanner (Molecular Dynamics).

Radioactive signals of cytokine cDNA were quantified and normalized to the house-keeping enzyme hypoxanthine phosphoribosyltransferase (HPRT) values to adjust for differences in cDNA as previously described (Cua *et al.*, 1995, 1996). The sample with the highest specific activity was designated the 100% maximal response and values for the remainder were derived as percentage of the highest value. Data shown are mean values for 3–4 mice at each time point \pm 1 standard deviation.

RESULTS

Acute and subacute JHMV-induced encephalitis

Fatal encephalomyelitis induced by JHMV is associated with minimal demyelination (Kyuwa and Stohman, 1990; Houtman and Fleming, 1996b). This contrasts with infection by 2.2v-1 which produces an acute nonfatal encephalomyelitis with extensive demyelination (Fleming *et al.*, 1986; Wang *et al.*, 1990). Although both viruses replicated rapidly to high titer in the CNS (Fig. 1A), JHMV-infected mice succumbed within 8 days while 2.2v-1-infected mice underwent a subacute disease with little or no mortality (Fig. 1B). Peak 2.2v-1 replication was at Day 3 while the peak of JHMV replication was delayed until Day 5. 2.2v-1 clearance began at day 5 p.i. and by Day 7 virus was nearly undetectable. By contrast, titers in JHMV-infected mice initially decreased at Day 7 p.i. and detectable virus was still present in the CNS of moribund mice at Day 8 p.i. (Fig. 1A). During lethal JHMV infection, virus replication within the CNS is not reduced as rapidly as in mice which survive infection (Fig. 1A) consistent with the notion that rapid clearance correlates positively with protection. Consistent with these findings, immunohistologic examination of the brains of JHMV-infected mice at Day 7 showed abundant viral antigen in regions of encephalitis while only focal residual viral antigen was found in 2.2v-1-infected animals (Fig. 2). Encephalitis was prominent in mice infected with either 2.2v-1 or JHMV and no differences in the amount or distribution of mononuclear cell infiltrates were found at Day 7 (Fig. 2). No serum neutralizing antibodies were detected in either group by Day 9 postinfection, even though the virus titer in the CNS had declined over 3 log₁₀ (Lin *et al.*, 1997; data not shown). In contrast to neutralizing antibodies, IgM was first detected at Day 5 post-2.2v-1 infection (data not shown) and both IgG1 and IgG2a were detected as early as 7 days p.i. (Fig. 1C). The IgG1 and IgG2a response suggest the absence of a shift toward either a Th1- or Th2-type response reported to be in-

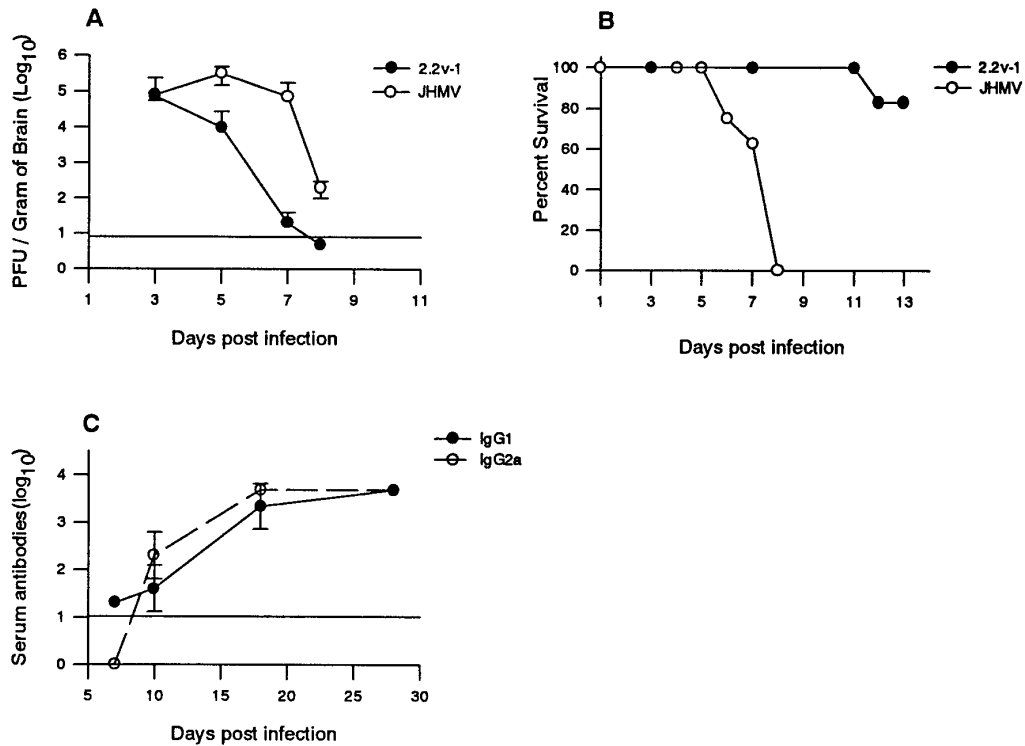


FIG. 1. Comparison of virus replication, cumulative mortality, and anti-viral antibody synthesis during a sublethal (2.2v-1) or lethal (JHMV) infection of the CNS. (A) Kinetics of virus clearance from the CNS. (B) Cumulative mortality. (C) Kinetics of JHMV-specific serum IgG1 and IgG2a in 2.2v-1-infected mice. Each point represents data for 3 or more mice per group.

involved in the response to Sindbis virus-induced encephalitis (Wesselingh *et al.*, 1994).

Proinflammatory cytokines

The mRNA encoding IFN- γ increased in both groups of mice through Day 5 postinfection, consistent with the rapid accumulation of both NK and T cells in the CNS of infected mice (Williamson *et al.*, 1991; Williamson, 1992) (Fig. 3A). No IFN- γ mRNA was detected in either sham-infected mice or in infected immunodeficient mice. During the lethal JHMV infection IFN- γ mRNA did not increase between Day 5 and Day 7. However, in mice undergoing a sublethal infection the level of IFN- γ mRNA continued to increase to Day 7 and remained elevated, suggesting the possibility that IFN- γ is important following infection with a JHMV variant tropic for oligodendroglia. Even though IFN- γ mRNA increased during the early phase of infection, a sharp transient increase in iNOS mRNA was detected at Day 5 p.i. in mice with a lethal encephalomyelitis (Fig. 3B). Only a slight increase was detected in mice undergoing subacute encephalomyelitis. Interestingly, infection of immunodeficient mice with JHMV induced the accumulation of iNOS mRNA to approximately 50% the level found in infected immunocompetent mice, suggesting a direct response to viral infection. The increase in iNOS and TNF- α mRNA in immuno-

deficient mice, which showed no evidence of IFN- γ mRNA, suggests TNF- α may also contribute to iNOS mRNA induction (Colasanti *et al.*, 1995; Gazzinelli *et al.*, 1993). Consistent with this notion, TNF- α mRNA was first detected at Day 3 in mice undergoing a lethal infection and at Day 5 in mice sublethally infected (Fig. 3C). Similar to the kinetics of IFN- γ , TNF- α mRNA increased until death of lethally infected mice. In mice undergoing a sublethal infection, TNF- α mRNA declined following the peak of virus replication and approached baseline levels by 14 days p.i.

Similar to both TNF- α and iNOS, IL-12 is secreted from macrophages during the induction of cell-mediated immunity and protects from a number of viral infections via a IFN- γ -dependent mechanism (Ozmen *et al.*, 1995; Orange and Biron, 1996). No IL-12 mRNA was found following sham infection; however, IL-12 mRNA increased rapidly and peaked at 3 days following both infections (Fig. 4A). Increased IL-12 mRNA also occurred in immunodeficient mice at 3 days p.i., suggesting a direct response to infection which may be related to the recently described IFN- γ -independent induction of IL-12 (Heinzel *et al.*, 1996). IL-12 mRNA levels decreased after Day 3 and nearly approached base line levels found in uninfected mice by 14 days p.i.

The IL-1 β mRNA level found at Day 1 p.i. declined by 3 days p.i., consistent with induction of an early transient

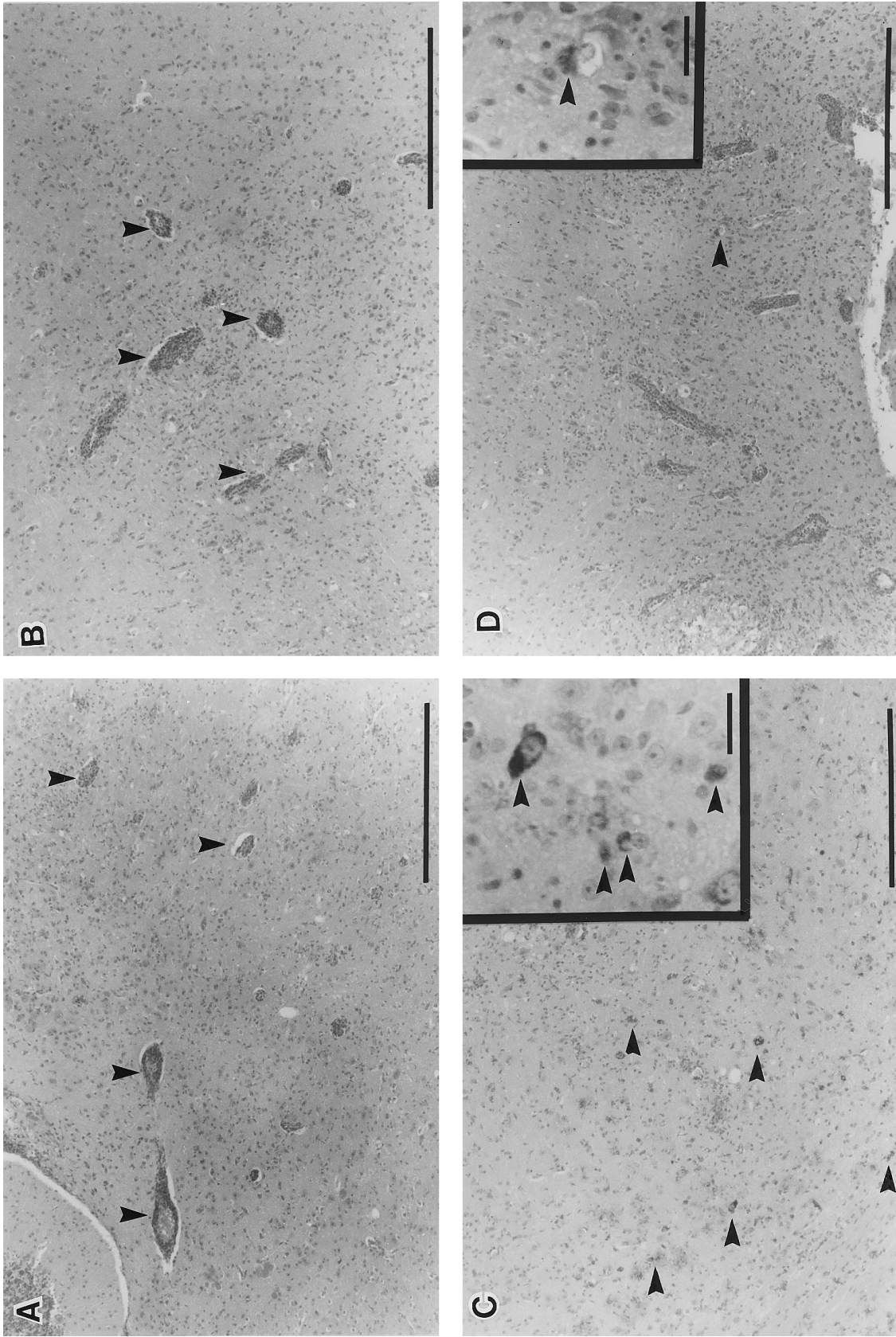


FIG. 2. Histologic sections of brains at Day 7 postinfection with JHMV or 2.2v-1. One-half of the brain was fixed in Clark's fixative, embedded in paraffin, and stained either with hematoxylin and eosin (A, B) or by immunohistochemical methods for viral antigen using mAb J3.3 (C, D). Encephalitis was widespread after infection in either JHMV (A) or 2.2v-1 (B); no differences in amount or distribution of inflammation were found. Glial cells and neurons positive for viral antigen were present throughout the areas of encephalitis at Day 7 after JHMV infection (C) after 2.2v-1 infection only very rare degenerating cells were positive for viral antigen (D). Magnification $\times 80$ (insert $\times 320$).

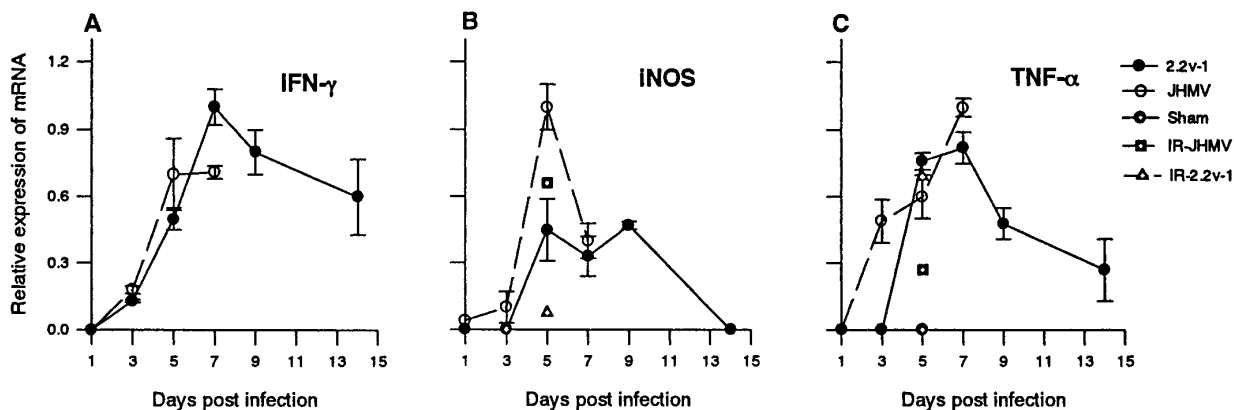


FIG. 3. Kinetics of IFN- γ (A), iNOS (B), and TNF- α (C) mRNA accumulation in the CNS of mice during a sublethal (2.2v-1) or lethal (JHMV) encephalomyelitis. RNA was extracted from the brains of groups of C57BL/6 mice infected with JHMV or the neutralization-resistant 2.2v-1 variant at various times p.i. The levels of cytokine mRNA determined by semiquantitative dot blot, normalized to the level of HPRT mRNA, and expressed as a relative amount value obtained for comparison. Sham-infected, irradiated JHMV-infected (IR-JHMV), and irradiated 2.2v-1-infected (IR-2.2v-1) mice are represented as a single point. Each point is the mean value for at least 3 mice per group.

increase in IL-1 β mRNA in sham-infected mice (Fig. 4B). IL-1 β mRNA peaked at Day 5 following sublethal infection and subsequently declined as virus was cleared from the CNS. Following a lethal infection, the quantity of IL-1 β mRNA increased from Day 3 p.i. until death. IL-1 α mRNA peaked at Day 3 p.i. in the mice undergoing a

subacute infection (Fig. 4C) and then declined but never returned to baseline. In lethally infected mice the peak of IL-1 α mRNA was delayed (Day 5 p.i.) and then declined as the animals succumbed to infection (Fig. 4C). IL-6 mRNA peaked at Day 5 postinfection in lethally infected mice and declined by Day 7 as virus was cleared from

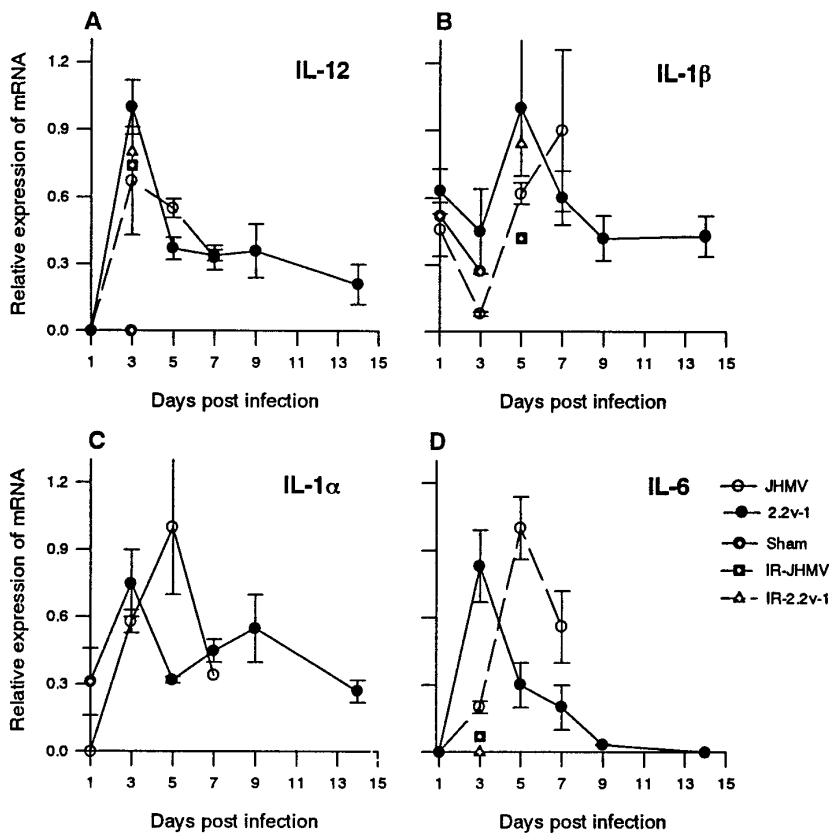


FIG. 4. Kinetic of IL-12 (A), IL-1 β (B), IL-1 α (C), and IL-6 (D) mRNA expression in the brain at various times after i.c. infection of C57BL/6 mice with JHMV or the neutralization-resistant 2.2v-1 variant. Each point is the mean value for at least three mice.

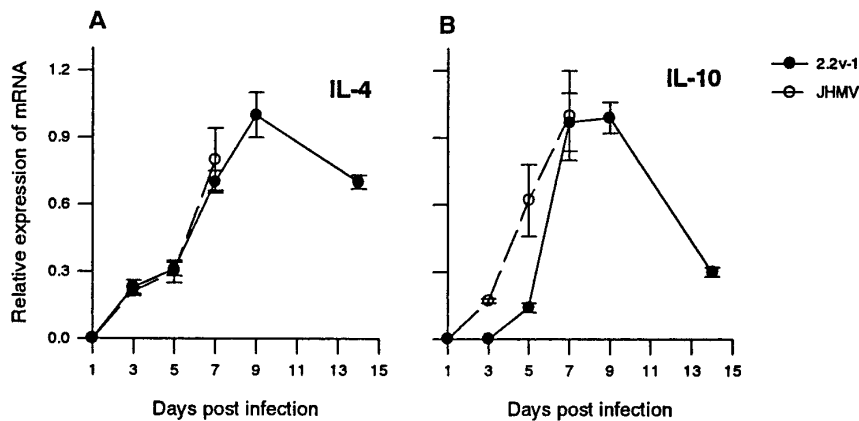


FIG. 5. Kinetic of accumulation of IL-10 (A) and IL-4 (B) mRNA in the CNS of C57BL/6 mice with a lethal or sublethal encephalomyelitis after infection with JHMV or 2.2v-1 variant, respectively. Each point is the mean value for at least three mice.

the CNS (Fig. 4D). In contrast to the lethal infection, the levels of IL-6 mRNA increased rapidly and peaked at Day 3 p.i. following subacute infection. The level then declined rapidly by Day 5 and had reached baseline levels by Day 9 p.i. No IL-6 mRNA was detected in sham-infected mice, suggesting a rapid response to virus infection. Very low levels of IL-6 mRNA were detected in immunodeficient mice infected with either virus.

Th2-related cytokines

IgG1 and IgG2a virus-specific antibodies were detected in survivors of JHMV infection; however, there appeared to be little relationship between induction of antibody and control of JHMV infection within the CNS. Induction of both isotypes suggest that Th1 and Th2 cytokines are induced by JHMV infection. The kinetics of IL-10 mRNA induction was of interest due to the association of IL-10 with reduced Th1 activity *in vitro* and with remission during experimental allergic encephalomyelitis (Kennedy *et al.*, 1992). IL-10 mRNA was first detected at Day 3 p.i. in lethally infected mice, but not until Day 5 postinfection in the CNS of the mice undergoing a subacute encephalitis. However, at the time most lethally infected mice were about to succumb to infection (Day 7), there was no difference in the peak levels of IL-10 mRNA between the two groups. The kinetics of IL-10 mRNA accumulation differed between the groups; IL-10 mRNA accumulation in mice undergoing a sublethal infection was slower and remained at peak levels until Day 9 p.i., prior to declining to near basal levels by Day 14. No IL10 mRNA was detected in the CNS of sham-infected or infected immunodeficient mice. No IL-4 mRNA was detected following a single amplification during lethal or sublethal JHMV infections. However, after a second amplification, low abundant mRNAs were detected (Fig. 5A). No IL-4 mRNA was detected in either sham-infected or infected immunosuppressed mice following two amplifications (data not shown). The kinetics

of IL-4 mRNA expression following acute and subacute infections showed that the levels increased in parallel through Day 7 p.i. (Fig. 5A). In 2.2v-1-infected mice, the level of IL-4 mRNA continued to increase until Day 9 p.i. and then declined slightly by Day 14.

DISCUSSION

JHMV produces an acute CNS infection associated with several immune effector mechanisms, including both CD4⁺ and CD8⁺ T cells (Kyuwa and Stohlman, 1990; Houtman and Fleming, 1996b). Kinetic analysis of cellular CNS infiltrations during JHMV infection of mice shows that NK cells accumulate prior to CD8⁺ T cells, which in turn precede accumulation of CD4⁺ T cells and macrophages (Williamson *et al.*, 1991; Williamson, 1992). There is no direct evidence for a role of NK cells in suppressing JHMV replication (Houtman and Fleming, 1996a); however, CD8⁺ CTL appear to be critical immune effectors (Williamson and Stohlman, 1990; Stohlman *et al.*, 1995b). Recent analysis of JHMV pathogenesis in mice deficient in perforin suggests that in addition to cytolytic effectors other immune components also contribute to sterilizing immunity (Lin *et al.*, 1997). Similarly, the adoptive transfer of virus-specific CD4⁺ T cells to JHMV-infected mice demonstrates that some clones protect via reducing viral replication (Yamaguchi *et al.*, 1991), while others protect without reducing virus replication (Stohlman *et al.*, 1986), suggesting that cytokines may play an important role in providing sterile immunity.

In general the kinetics of cytokine mRNA expression correlated with the temporal presence of CNS infiltrating mononuclear cells. Many cytokine transcripts, with the exceptions of IL-12, IL-1 α , and IL-6, were maximally expressed by 7 day p.i., near the peak inflammatory cell infiltration and during the elimination of virus from the CNS (Williamson *et al.*, 1991; Williamson, 1992). Previous data using the OBLV-60 JHMV variant which has a selec-

tive tropism for neurons suggested a correlation between IFN- γ induction, T cell accumulation, and reduction of virus replication (Pearce *et al.*, 1994). The semiquantitative kinetic analysis of IFN- γ mRNA in the CNS of mice undergoing both lethal and sublethal JHMV infections supports the positive correlation between IFN- γ and viral clearance. However, the OBLV-60 JHMV variant is cleared from the CNS of IFN- γ -deficient mice (Lane *et al.*, 1997), consistent with IFN- γ exhibiting poor *in vitro* anti-JHMV activity (Zhang *et al.*, 1997) and inability of rIFN- γ to inhibit CNS virus replication (Smith *et al.*, 1991). These data contrast with other viral-induced encephalopathies in which IFN- γ plays a significant role (Kundig *et al.*, 1993; Finke *et al.*, 1995), including some (Yu *et al.*, 1996), but not all (Wesselingh *et al.*, 1994), neuronotropic viruses. The kinetics of IFN- γ mRNA induction suggests that it may play a more prominent role in the pathogenesis of JHMV variants with predominant tropisms for microglia, astrocytes (JHMV), or oligodendroglia (2.2v-1).

The isotype diversity of the anti-JHMV antibody response suggests that both Th1 and Th2 subsets of CD4⁺ T cells are activated during infection. IL-4 mRNA accumulation in the CNS corresponds to infiltration of Th2 cells (Cua *et al.*, 1995) and kinetic analysis suggests that T cells expressing Th2 cytokine profiles are recruited into the CNS with nearly equal kinetics in both lethally and sublethally infected mice (Fig. 5A). IL-4 increases the severity of encephalitis (Ikemoto *et al.*, 1995) and could potentially play a role in JHMV persistence via inhibition of viral clearance (Moran *et al.*, 1996). In support of the recruitment of Th2 cells, IL-10 mRNA also increased with kinetics similar to those of IFN- γ and IL-4. Whether this difference in detection of Th2 cytokines is due to differences in mouse strains or the selective tropism of the virus is not known. It is interesting that although IL-10 is secreted by activated microglia *in vitro* (Lodge and Sriram, 1996), no IL-10 mRNA was detected in sham-infected or immunodeficient mice. This contrasts with other cytokine mRNA detected in either sham-infected or virus-infected immunodeficient hosts (see below).

TNF- α mRNA is induced following JHMV infection (Pearce *et al.*, 1994; Stohlman *et al.*, 1995a; Sun *et al.*, 1995) and TNF- α is present during both the acute and persistent JHMV infections. TNF- α mRNA is not translated in JHMV-infected cells (Stohlman *et al.*, 1995a), although it may be secreted by adjacent but not infected cells. In addition, inhibition of TNF- α , which prevents experimental autoimmune encephalitis (Ruddle *et al.*, 1990), has no effect on either JHMV-induced encephalitis or demyelination (Stohlman *et al.*, 1995a). As anticipated, based on the relative tropism of the two viruses analyzed, TNF- α mRNA accumulated initially in the CNS of mice infected with JHMV. However, by Day 5 p.i. there was little difference in the levels of TNF- α mRNA in the two groups. Finally, the level of TNF- α mRNA decreased with

increasing time following subacute infection, consistent with the resolution of encephalitis. It is interesting that the CNS of mice with active macrophage-mediated demyelination (Day 14 p.i.) showed little evidence of TNF- α mRNA, consistent with the inability of anti-TNF- α to prevent JHMV-mediated demyelination (Stohlman *et al.*, 1995a).

A surprising number of mRNAs peaked relatively early following JHMV infection. The mRNAs encoding iNOS, IL-12, IL-1 α , IL-1 β , and IL-6 peaked either prior to or coincident with initiation of viral clearance. In most cases (except iNOS mRNA) the levels were either higher or increased more rapidly in the mice undergoing subacute infections. Accumulation of iNOS mRNA was first detected in mice undergoing a lethal infection coincident with the initial detection of IFN- γ mRNA. However, the mRNA levels declined as virus replication declined, suggesting a direct effect of virus on iNOS induction. In contrast to lethal infections, iNOS mRNA lagged detection of IFN- γ in mice undergoing subacute infections and increased to less than 50% the level detected in mice undergoing a lethal infection. Similar to the recent data demonstrating low levels of iNOS in the CNS of both nude mice and mice deficient in IFN- γ (Lane *et al.*, 1997), iNOS mRNA in immunodeficient mice was approximately 50% the levels detected in the CNS of intact mice at Day 3 p.i. Although JHMV is susceptible to inhibition by iNOS *in vitro*, iNOS is not associated with *in vivo* protection (Lane *et al.*, 1997).

IL-12, predominantly produced by cells of the myelomonocytic lineage, is associated with the induction of Th1 CD4⁺ T cells (Brunda, 1994). IL-12 mRNA peaked early (Day 3) in mice undergoing both lethal and sublethal JHMV infections. However, no significant differences were found comparing mRNA levels in immunodeficient mice to intact mice. This may suggest that JHMV infection induces transcription of IL-12 mRNA in CNS cells. In addition, 2.2v-1 infects predominantly, but not exclusively, oligodendroglia, while JHMV infects predominantly microglia and astrocytes. The relatively higher level of IL-12 mRNA in 2.2v-1-infected mice suggests the possibility that oligodendroglia transcribe IL-12 mRNA in response to JHMV infection, similar to the induction of IL-12 mRNA following measles virus infection of oligodendroglia (Yamabe *et al.*, 1994).

During both the lethal and sublethal infections the IL-1 α mRNA peaks appear to coincide with replication and not clearance, suggesting that infection induces a rapid induction of IL-1 α mRNA. These data contrast to the association of IL-1 α mRNA and the clearance of the OBLV-60 variant of JHMV (Pearce *et al.*, 1994), suggesting an additional difference in cytokine responses depending on the tropism of the virus analyzed. IL-1 β mRNA, previously detected in the CNS of JHMV-infected mice (Pearce *et al.*, 1994), increased directly after infection at

Day 1 p.i. However, the level was approximately the same as the level detected in sham-infected mice, suggesting it was induced by trauma. In all mice the levels subsequently dropped by Day 3 p.i. The levels of IL-1 β mRNA peaked at Day 5 following 2.2v-1 infection and at Day 7 following JHMV infection, suggesting IL-1 β mRNA was also induced by infection. Analysis of the levels in immunodeficient mice were consistent with the notion that infection, and not immune infiltrates, contributed the majority of the IL-1 β mRNA levels.

IL-6, another pleiotropic cytokine with numerous effects on immune responses (van Snick, 1990), was also detected early following both lethal and sublethal infections. By contrast, IL-6 mRNA was also only detected at 6 days p.i. with the neuronotropic OBLV-60 JHMV variant (Pearce *et al.*, 1994). Kinetic analysis shows that the levels of IL-6 mRNA peaked at Day 3 post-2.2v-1 infection and at Day 5 post-JHMV infection. Interestingly, analysis of the mRNA levels in the immunodeficient mice showed virtually no induction of IL-6 mRNA, suggesting that in contrast to IL-1 α and IL-1 β an intact immune response was required for IL-6 mRNA induction. Rapid induction of IL-6 mRNA following JHMV infection is consistent with other models of viral-induced encephalitis in which it also precedes IFN- γ (Moskophidis *et al.*, 1991). Although both IL-6 and IL-10 are cofactors for CTL induction (Chen and Zlotnik, 1991; Takai *et al.*, 1988), kinetic analysis is consistent with the notion that IL-6, and not IL-10, may be involved in the induction or recruitment of JHMV-specific CTL. JHMV infection induces IL-6 secretion from both brain endothelial cells and astrocytes following *in vitro* infection with JHMV (Joseph *et al.*, 1993), consistent with data showing that it is produced by resident CNS cells following infection with lymphocytic choriomeningitis virus (Frei *et al.*, 1989). It is interesting that IL-6 mRNA peaks first in mice infected with the 2.2 v-1 variant compared to JHMV, which infects a significantly larger number of astrocytes. The rapid induction of IL-6 and IL-1 β following infection with 2.2v-1 is consistent with the induction of these mRNA in oligodendroglia infected by measles virus *in vitro* (Yamabe *et al.*, 1994).

These data demonstrate that lethal and sublethal infections of the CNS induce mRNAs associated with both Th1 and Th2 cytokines. Predominant infections of microglia and astrocytes by JHMV and of oligodendroglia by the neutralization escape variant 2.2v-1 results in the accumulation of mRNA encoding IFN- γ , IL-4, and IL-10. This is the first demonstration of the induction of IL-4 and IL-10 mRNA following JHMV infection. IL-10 is an immunosuppressive cytokine suggested to play a role in the clinical and histological remission phase of experimental autoimmune encephalitis (Kennedy *et al.*, 1992; Cua *et al.*, 1995). Although IL-10 is increased following both the lethal and sublethal infections, the elevation of IL-10 mRNA at Day 7 and Day 9 during sublethal infection

suggests it may play a positive role in reducing the extent of CNS inflammation thereby inadvertently contributing to persistent infection. Some aspects of our data, i.e., the rapid induction of IL-12 mRNA in mice infected with 2.2v-1, suggest that infection of specific cell types may influence the induction of cytokine mRNA (Yamabe *et al.*, 1994). This supports the notion that the cytokine mRNA patterns more closely reflect diversity of the immune response to an individual agent, although differential secretion of cytokines following infection of unique CNS cell types cannot be ruled out (Benveniste, 1992; Sun *et al.*, 1995). While the kinetics of IFN- γ , IL-4, and IL-10 showed little difference between the groups undergoing lethal or sublethal infections, mRNAs encoding IL-6 and IL-1 β either appeared more rapidly (IL-6) or accumulated to higher levels (IL-1 β) following infection with 2.2v-1 virus. By contrast the induction of iNOS and IL-1 α mRNAs were increased in mice undergoing a lethal infection. These data suggest that an early induction of IL-6, and possibly IL-1 β , are associated with sublethal infection or the different tropisms exhibited by these two JHMV variants. However, during both infections the mRNA levels decreased as virus was cleared. Similarly, there appears to be an inverse correlation between a rapid induction of iNOS mRNA and sublethal disease, consistent with the recent demonstration that although iNOS is protective *in vitro*, inhibition of iNOS activity *in vivo* appears to have no effect on JHMV pathogenesis (Lane *et al.*, 1997). Taken together, kinetic analyses of the induction of cytokine mRNA during the lethal and sublethal JHMV infections are consistent with the accumulation of both Th1- and Th2-associated cytokines and support the interaction of multiple cellular and soluble effector mechanisms whose balance may be critical in providing protection and sterilizing immunity.

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