

Basis of rabies virus neurovirulence in mice: expression of major histocompatibility complex class I and class II mRNAs

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Expression of major histocompatibility complex (MHC) molecules on cells of the central nervous system (CNS) plays an important role in the pathogenesis of acute viral encephalitis. We have compared the induction of MHC class I and II mRNA transcripts in mice upon infection with the virulent challenge virus standard (CVS) strain of rabies virus and avirulent rabies virus variant RV194-2. Rabies virus antigen was detected with immunoperoxidase staining and ³⁵S-labeled RNA probes were used to detect MHC class I and class II mRNA transcripts by *in situ* hybridization in infected brains. In CVS and RV194-2 infected animals, MHC class I mRNA expression occurred in the brain in neurons, glia, choroid plexus epithelial cells, ependymal cells, and inflammatory cells; expression was moderately higher in CVS-infected mice. In contrast, MHC class II mRNA expression was minimal in CVS-infected mice and it was markedly upregulated in CNS inflammatory cells upon RV194-2 infection. Both viruses induced an acute inflammatory reaction in the cerebrospinal fluid (CSF), which was more pronounced in CVS-infected mice. Both viruses also induced an antigen specific T and B cell response detectable in lymph nodes and spleen. These studies, which show a correlation between greater expression of MHC class II mRNA in the brain following intracerebral RV194-2 infection and protection against RV194-2 infection in the brain, suggest that recovery from avirulent rabies virus infection of neural cells involves T helper cells produced and/or retained in the brain for reasons that are not entirely clear.

Keywords: gene expression; *in situ* hybridization; pathogenesis; rabies; virulence

Introduction

The molecular and biologic bases for rabies virus neurovirulence have been studied to date by comparing the effects of infections with the virulent challenge virus standard (CVS) strain of rabies virus and avirulent variants of the CVS strain (Jackson, 1997). The molecular difference between the virulent and avirulent rabies viruses is generally represented by a single amino acid substitution in the rabies virus glycoprotein that is thought to affect either pH-independent virus-induced cell fusion (formation of syncytia) or virus spread in neuronal cells *in vitro* and *in vivo* (Dietzschold *et al*, 1985; Seif *et al*, 1985; Morimoto *et al*, 1992; Jackson,

1994). Comparisons of avirulent rabies variants with the virulent parent virus in animal models using a variety of routes of inoculation have shown that the avirulent rabies virus variants spread less efficiently in the CNS than the virulent parent virus (Torres-Anjel *et al*, 1984; Dietzschold *et al*, 1985; Kucera *et al*, 1985; Coulon *et al*, 1989; Jackson, 1991; Lafay *et al*, 1991). It is clear that an antigen-specific immune response limits the spread of the avirulent virus because the virus is also virulent in immunodeficient mice (Xiang *et al*, 1995; Hooper *et al*, 1998).

Humoral immunity plays a pivotal role in neutralizing free virus and limiting the spread of rabies virus in vaccinated animals, even in protection against an intracerebral challenge (Wiktor *et al*, 1984; Xiang *et al*, 1995). Mice that lack B cells, or B cells and T cells, fail to clear virus from the CNS and develop progressive disease and die (Hooper *et al*, 1998). On the contrary, mice lacking CD8⁺ (cytoly-

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Received 14 January 1999; revised 14 May 1999; accepted 3 June 1999

tic) T cells only, interferon (IFN)-receptors, or complement components C3 and C4 showed no significant differences in the development of clinical signs by comparison with mice of the same genetic background having these components. T cells, because they produce soluble factors such as type 1 IFNs that control immune responses or have antiviral activity, play a role in viral clearance in naive (i.e., unvaccinated) animals (Miller *et al*, 1978; Wiktor, 1978; Mifune *et al*, 1981; Smith, 1981; Xiang *et al*, 1995; Hooper *et al*, 1998). T cells also recognize antigen in association with major histocompatibility complex (MHC) determinants; cytolytic T cells require expression of MHC class I and T helper cells respond to antigen associated with MHC class II. Neurons are one of the few cell types that lack expression of either MHC class I or II antigen or at best produce low levels of MHC class I determinants upon induction by cytokines such as IFN- γ that are commonly released by activated lymphocytes during the course of a viral infection (Olsson *et al*, 1988; Gogate *et al*, 1991; Gombold and Weiss, 1992; Duguid and Trzepacz, 1993; Bilzer and Stitz, 1994; Pearce *et al*, 1994; Neumann *et al*, 1995). It is possible that viruses may evade the immune system in the brain and become persistent by failing to induce expression of MHC class I molecules in neurons (Joly *et al*, 1991; Oldstone, 1997).

Expression of MHC class II molecules in the brain is limited to microglia/macrophages and possibly endothelial cells during CNS inflammatory processes (Tyor and Johnson, 1992; Carson *et al*, 1998). Microglial cells in the CNS, being similar to immature antigen-presenting cells, display weak MHC class II expression (Carson *et al*, 1998). Antigen associated with MHC class II molecules can interact with T-helper cells, which release cytokines that signal and amplify the activity of B cells and cytolytic T cells, and initiate inflammatory reactions. In the absence of any significant levels of MHC class II antigen in brain, the burden of viral clearance may fall upon MHC class I-restricted CD8⁺ (cytolytic) T cells for production of cytokines at the site of infection (Hooper *et al*, 1998).

Avirulent rabies virus variant RV194-2 (also referred to as CVS-F3), which was selected from the virulent CVS strain of fixed rabies virus *in vitro* by its resistance to neutralization with a monoclonal antibody directed against the rabies virus glycoprotein, causes a mild encephalomyelitis with recovery after intracerebral or footpad inoculation of adult mice (Dietzschold *et al*, 1983, 1985; Xiang *et al*, 1995). While specific immune responses appear to control the spread of RV194-2 following intracerebral and peripheral inoculations, and virus can be cleared from the CNS, the mechanisms of recovery from infected neural cells are not entirely clear (Xiang *et al*, 1995; Hooper *et al*, 1998). In order to better understand whether MHC class I and/or MHC class II molecules and the respective T cell

subsets play a role in limiting the spread of avirulent *versus* virulent rabies virus in the CNS and whether the levels of these molecules correlate with an antigen-specific immune response and virus clearance, we examined their expression in virus infected brains. In this report, we have compared the expression of both MHC class I and class II mRNAs in the brains of CVS- and RV194-2-infected mice using *in situ* hybridization. In addition, we compared the rabies virus specific T and B cell mediated immune responses elicited by both viruses.

Results

Clinical disease, rabies virus antigen distribution, and T lymphocyte infiltration in the CNS

Mice infected intracerebrally with CVS-11 developed typical neurologic signs of rabies, including ataxia and paralysis, and became moribund 7–9 days post-inoculation (p.i.). On the other hand, mice infected intracerebrally with RV194-2 failed to demonstrate neurologic signs of rabies. In CVS-11-infected mice the quantity of rabies virus antigen gradually increased in the brain until it reached a maximum 5 days p.i. At this time viral antigen was distributed widely in brain neurons. In RV194-2-infected mice, rabies virus antigen was

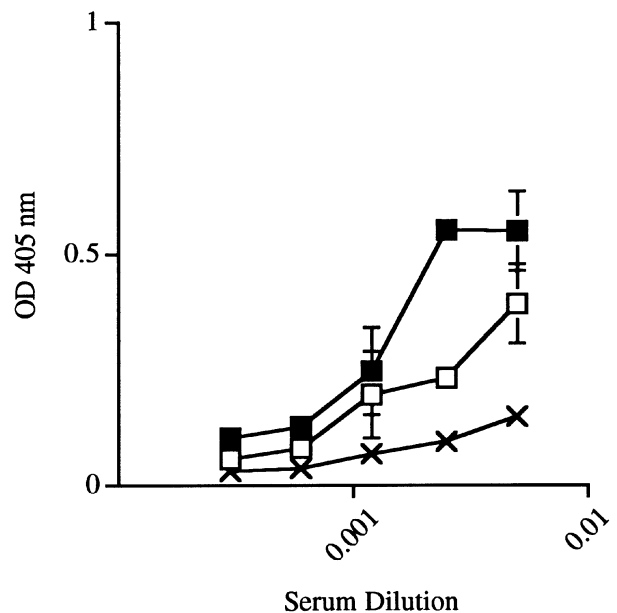


Figure 1 Antibody titers in mice infected with neurovirulent CVS-24 or avirulent RV194-2 virus. Groups of mice were injected into the footpad with comparable doses of CVS-24 (open squares) or RV-194-2 (closed squares) virus. Sera were harvested 7 days later and antibody titers were determined by ELISA using serum from naive mice (X) for comparison. The vertical bars represent ± 1 standard deviation (s.d.).

detected in a fewer number of neurons compared to CVS-11 infection (data not shown). Observed viral antigen was maximal 5 days p.i. in RV194-2-infected mouse brains and little viral antigen was observed after day 7.

Both CVS-24 and RV194-2 viruses induced antibody responses measurable 1 week after infection via footpad inoculation. CVS-24 was used instead of CVS-11 for peripheral route (footpad) inoculations since the mouse neuro-adapted virus spreads better to the brain. The antibody response was higher in RV194-2 infected mice compared to mice infected with CVS-24 virus (Figure 1). In contrast, CVS-24 virus caused between 10 and 100 times more lymphocytic infiltration into the CSF than RV194-2 virus (data not shown). Both viruses upon peripheral inoculation induced cytolytic T cells and cytokine secreting T helper cells to rabies virus that could be detected within 7 days p.i. in the popliteal lymph nodes as well as in spleens (Figures 2 and 3). The magnitude of the T cell responses to either virus was similar in the popliteal lymph nodes draining the inoculation site. A small T cell response in cervical lymph nodes draining the CNS upon CVS-24 infection could be observed,

whereas, no response was detected upon RV194-2 virus infection. This presumably reflects the higher replication rate of the CVS-24 virus within the CNS. Cytokine secreting T cells were not detected in cervical lymph nodes after infection with either virus. They could readily be found in the spleens of RV194-2-infected, but not of CVS-24-infected mice, supporting the difference observed in the B cell response (Figures 1 and 3).

MHC class I mRNA expression

In order to evaluate whether MHC class I expression occurred in rabies virus-infected brain cells, tissues were probed for class I mRNA expression. Expression of MHC class I mRNA was not detected in intracerebrally inoculated control mice (Figure 4A and B) and *in situ* hybridization with probes of the same sense as mRNA showed very low background. In CVS-11 infection, signals were observed as early as 1 day p.i. in mononuclear inflammatory cells in the leptomeninges and in choroid plexus epithelial cells. By 3 days p.i., MHC class I signals were increased and widespread in neurons in the brain, glia, choroid plexis epithelial cells, and inflammatory cells in brain parenchyma, perivascular cuffs, and leptomeninges. Signals gradually increased as

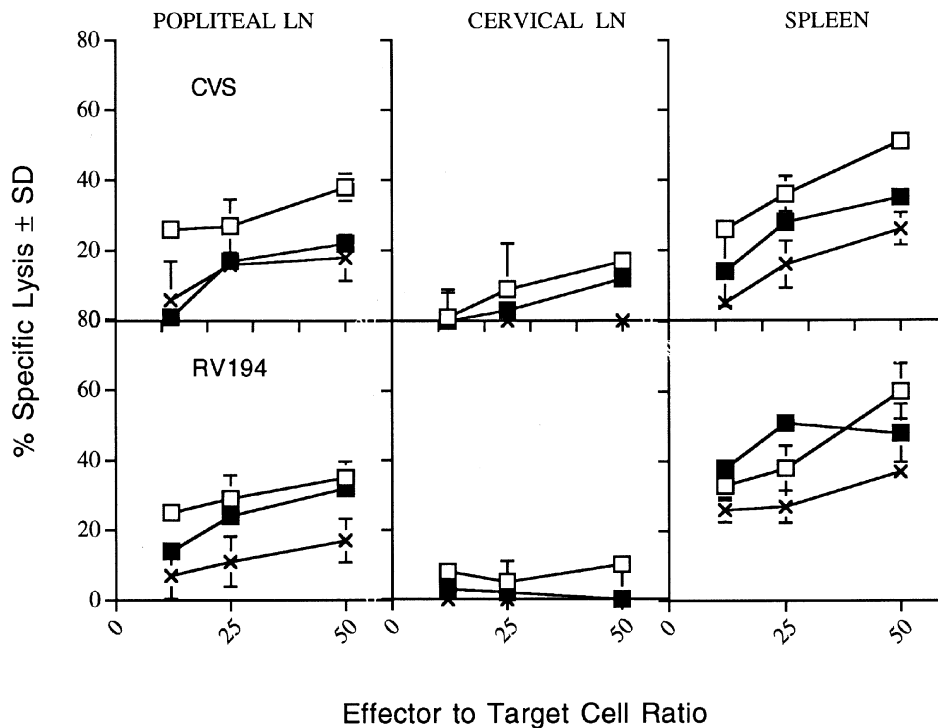


Figure 2 Response of cytolytic T lymphocytes in mice infected with neurovirulent CVS-24 or avirulent RV194-2 virus. Groups of mice were infected with CVS-24 (upper panels) or RV194-2 (lower panels) as described in the legend to Figure 1 and euthanized 7 days after infection. Lymphocytes from lymph nodes or spleens were taken and restimulated *in vitro*. Effector cells were then tested at various effector to target cell ratios on L929 fibroblasts infected with a recombinant vaccinia virus expressing the rabies virus glycoprotein (VRG, closed squares), a recombinant vaccinia virus expressing the rabies virus P/NS protein (VRNS, open squares), or vaccinia virus Copenhagen strain (X). Data are expressed as the mean percentage specific lysis of triplicate wells. The vertical bars represent ± 1 standard deviation (s.d.).

the infection progressed and they became maximal at the last time point examined on day 8 p.i. (Figure 4C and E).

In RV194-2 infection MHC class I signals were also noted on 1 day p.i. and they progressively increased and became maximal 8 days p.i. Expression was noted in the same neural cell types and the signals were noted to be moderately less than in CVS infection (Figure 4D and F). After day 8 there was a gradual reduction in the signals and at day 14 p.i. levels were still mildly increased above background levels.

MHC class II mRNA expression

MHC class II mRNA expression was not observed in uninfected control mice and probes of the same sense as mRNA showed low background. MHC class II expression was first noted in RV194-2 infection 3 days p.i. in mononuclear inflammatory cells in a perivascular location and in the leptomeninges. Signals increased to a maximal level 8 days p.i. in inflammatory cells in the brain parenchyma, perivascular inflammatory cells, and in mononuclear cells that infiltrated the leptomeninges

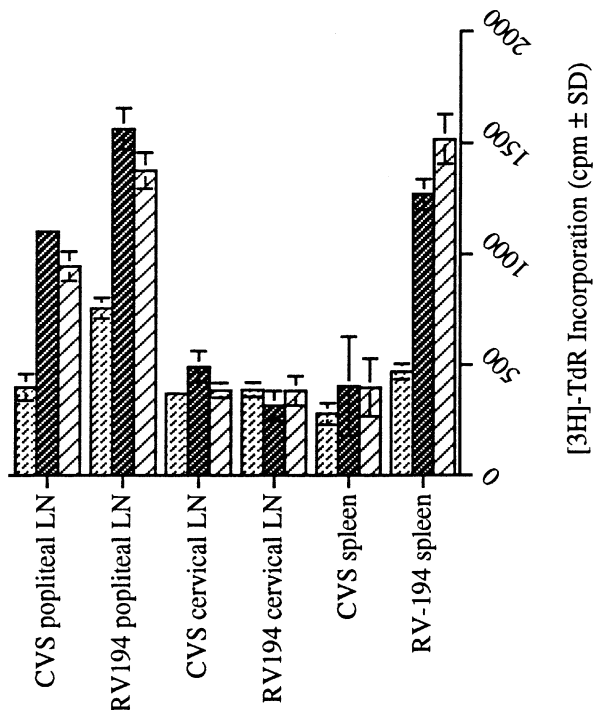


Figure 3 T helper cell response upon infection of mice with virulent CVS-24 or avirulent RV194-2 virus. Groups of mice were infected as described in the legend to Figure 1 and euthanized 7 days postinfection. Lymphocytes were harvested from lymph nodes or spleen and co-cultured for 24 h with medium (▨), 5 µg/mL (▤) or 1 µg/mL (▥) of ERA-BPL virus. Supernatants were tested for induction of proliferation of CTLL-2 indicator cells. Data show the mean [3H]thymidine incorporation in counts per minute for triplicate samples. The horizontal bars represent ± 1 standard deviation (s.d.).

and choroid plexus (Figure 5B and D). MHC class II expression gradually decreased after 8 days p.i., although signals were still detectable at 20 days p.i. MHC class II expression was minimal in CVS-11 infection with expression noted in only rare mononuclear inflammatory cells (Figure 5A and C). However, the amount of the inflammatory changes in the brain was similar in CVS-11 and RV194-2 infections (data not shown). This observation, however, contrasts with the difference in the lymphocytic infiltration in the CSF caused by CVS-24 compared with RV194-2 infection seen after footpad inoculation.

Discussion

Avirulent rabies virus variants have been observed to spread less efficiently in the central nervous system and infect a smaller number of neurons than their virulent parent viruses (Dietzschold *et al*, 1985; Jackson, 1991, 1994). Marcovistz *et al* (1994) found that both IFN and antibody production was higher in mice after intracerebral inoculation of the avirulent RV194-2 virus in comparison with infection with the virulent CVS-11 parent virus. In the current studies, the antibody response and the splenic T helper cell response to RV194-2 were also higher than these responses to CVS-24 1 week after infection by identical doses via the footpad. Both viruses induced local (popliteal lymph nodes) and systemic (spleen) cytolytic T cell responses that were similar in magnitude, whereas CVS-24 induced a stronger cytolytic T cell reaction in the CSF after footpad inoculation than the avirulent (RV194-2) virus, and, in addition, induced a better cytolytic T cell response in cervical lymph nodes that drain the CNS. A similar stronger response to CVS-11 infection was not observed in the leptomeninges after intracerebral inoculation of the CVS parent and avirulent (RV194-2), viruses, possibly reflecting the different routes of inoculation, the difference in neuroadapted CVS-24 *versus* cell culture-adapted CVS-11, or the observation that inflammatory infiltrates in CSF may differ from those in the parenchyma or leptomeninges (Moench and Griffin, 1984). Upon infection with either CVS-24 or RV194-2 virus, cytokine secreting T cells could be isolated from popliteal lymph nodes. These lymph nodes do not drain the CNS where viral replication occurs. Both CVS-24 and avirulent variant viruses induced a comparable T helper cell response in popliteal lymph nodes, which likely reflects activation of T cells by presentation of defective (non-infectious) viral particles present in the inoculated virus preparation that had not invaded the nervous system. Neither of the viruses induced T helper cells detectable in cervical lymph nodes, suggesting that either activation of this T cell subset was not initiated in the CNS or, alternatively, that activated

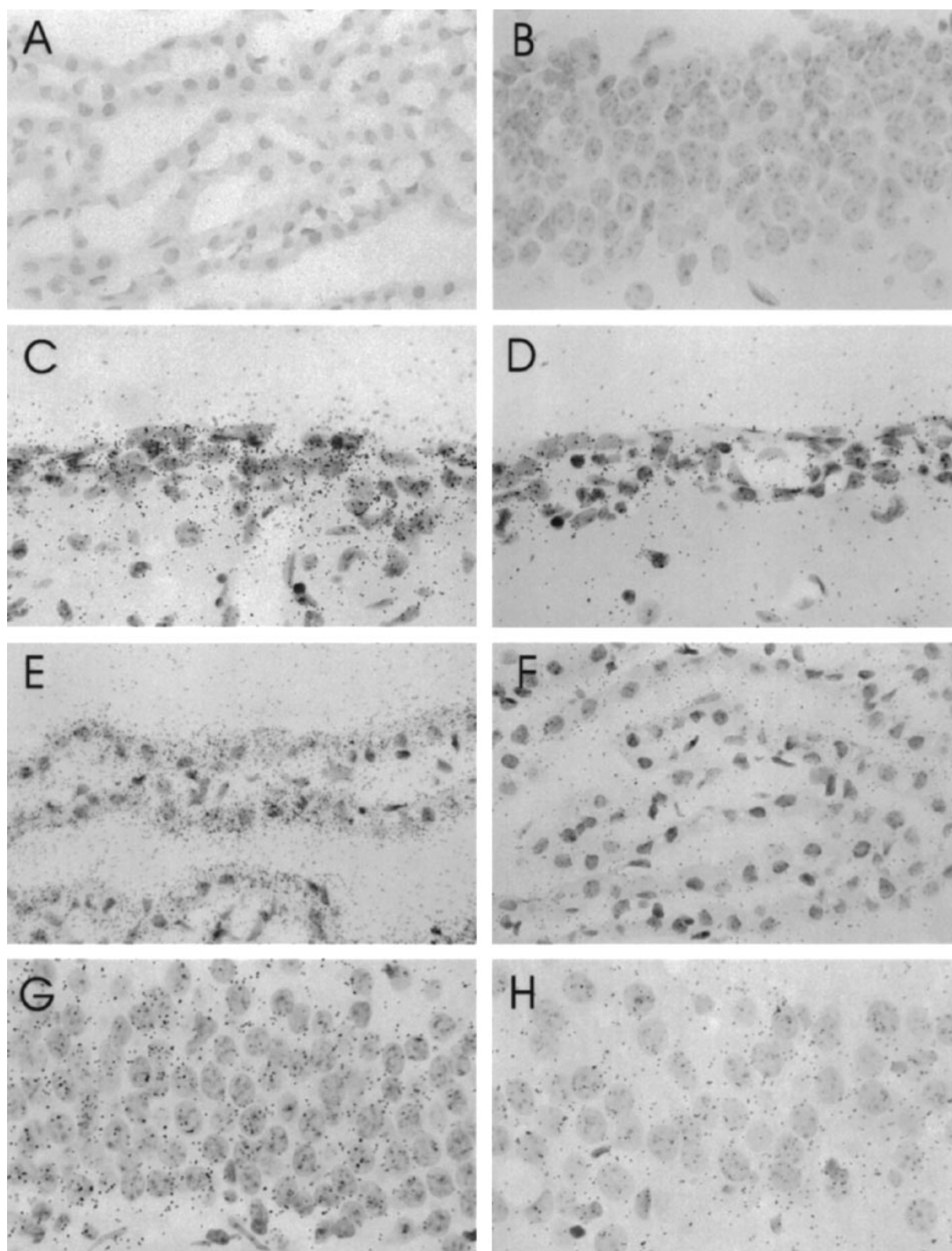


Figure 4 *In situ* hybridization for MHC class I mRNA in mock-infected controls (A and B) and CVS-11 (C, E and G) and RV194-2 (D, F and H) infections in the choroid plexus (A, E and F), dentate gyrus of the hippocampus (B, G and H), and leptomeninges (C and D) 8 days after intracerebral inoculation. Few background grains are present over choroid plexus epithelial cells (A) and neurons in the dentate gyrus of the hippocampus (B) of mock-infected mice. In CVS-11 infection many more grains are present over inflammatory cells infiltrated into the leptomeninges, choroid plexus epithelial cells, and neurons in the dentate gyrus of the hippocampus than in RV194-2 infection (5C–H). (A) $\times 460$; (B) $\times 580$; (C and D) $\times 525$; (E and F) $\times 420$; (G and H) $\times 600$.

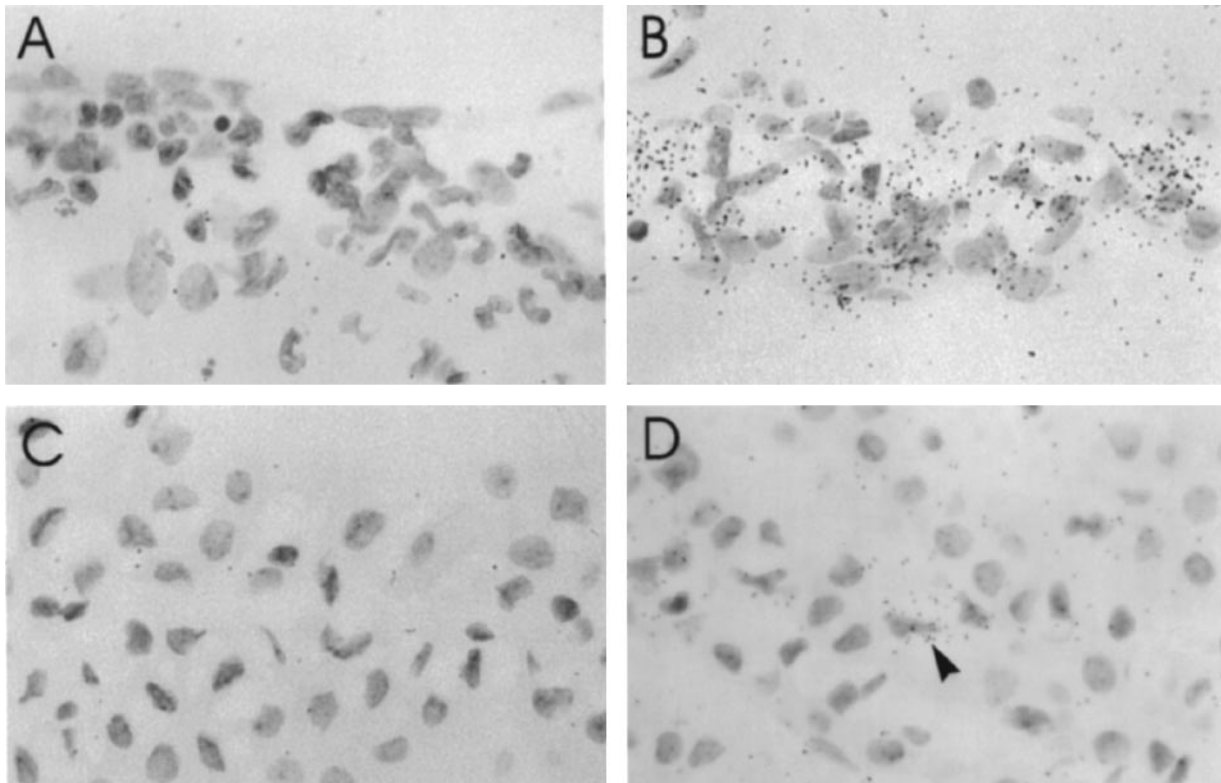


Figure 5 *In situ* hybridization for MHC class II mRNA in CVS (A and C) and RV194-2 (B and D) infections in the leptomeninges (A and B) and choroid plexus (C and D) 8 days after intracerebral inoculation. Signals in CVS-11 infection (A and C) were similar to mock-infected mice. In RV194-2 infection many grains are seen over inflammatory cells infiltrated into the leptomeninges (B) and over occasional inflammatory cells in the choroid plexus (D). (A and B) $\times 750$; (C and D) $\times 650$.

CD4⁺ T helper cells failed to infiltrate the CNS. RV194-2 virus induced a strong splenic cytokine secreting T cell response following inoculation by the peripheral route, whereas spleens of CVS-24-infected mice failed to respond to restimulation with inactivated virus. In addition to these observed immunobiological responses to the respective virulent and avirulent viruses, significant differences were observed in the present study in the expression of MHC class I and class II mRNAs in the brains of mice infected with CVS-11 and RV194-2 viruses. These differences may be important aspects of the respective dominant neurovirulence phenotypes of these viruses in this mouse model.

In both CVS-11 and RV194-2 infections following intracerebral inoculation, MHC class I expression was observed by *in situ* hybridization in uninfected neural cell types such as choroid plexus epithelial cells, leptomeningeal cells, and neurons in the dentate gyrus of the hippocampus. This suggests that while neural tissue is generally considered to be 'immunologically privileged', neural cells are at least capable of MHC class I expression even if they may resist lysis by virus-specific CTLs (Joly *et al*, 1991). No correlation was noted between the distribution of infected neurons, detected by immunoperoxidase staining for rabies virus antigen,

and the distribution of MHC class I mRNA expression in these neurons. The early and widespread distribution of MHC class I mRNA signals in infected neurons suggests stimulation by a soluble factor such as a cytokine, and IFN- γ has been recognized as an important inducer of MHC class I expression (Lampson and Fisher, 1984; Skoskiewicz *et al*, 1985; Wong *et al*, 1985). Gombold and Weiss (1992) also observed early and widespread expression of MHC class I mRNA in mouse hepatitis virus A59 infection without restriction to areas of infection or inflammation. Similarly, Gogate *et al* (1996) found no correlation between the degree of infection and the expression of MHC class I mRNA in neurons in fatal human cases of subacute sclerosing panencephalitis. Expression of MHC class I molecules on the surface of infected cells in association with viral peptides may result in immune killing by cytolytic T cells, which occurs by apoptosis (Atkinson and Bleackley, 1995). Widespread apoptotic cell death was observed in CVS-infected mice (Jackson and Rossiter, 1997), although the relative role of immune mechanisms and the more direct apoptosis-inducing effects of rabies virus infection has not yet been determined. In particular, it would be of interest to know whether the moderately greater expression of MHC class I mRNA in CVS-11 infection contributes

to the difference in neurovirulence of these viruses and whether there is a correlation with the enhanced lymphocytic infiltration in the CSF observed in CVS-24-infected mice, even though the enhanced lymphocytic infiltration only occurred when virus was inoculated via the footpad and not intracerebrally. β_2 -Microglobulin is needed for cell surface expression of MHC class I molecules, yet Xiang *et al* (1995) found that β_2 -microglobulin-deficient mice demonstrated mildly increased mortality after intracerebral inoculation of the avirulent RV194-2 virus, but no increased mortality in GKO mice that lack expression of IFN- γ . This is a paradoxical observation that does not explain the effect of the moderately greater MHC class I expression in CVS-11 infection. Notwithstanding, T cell-mediated cytolysis may be crucial only for the resolution of infections with noncytopathic viruses, while soluble mediators, including antibodies and cytokines, are important for the control of cytopathic viruses such as the rhabdovirus vesicular stomatitis virus (Kägi and Hengartner, 1996). Although MHC class I expression may play a role in recovery from RV194-2 infection, it is doubtful that the mechanism involves enhanced virus-infected killing by cytolytic T cells. However, it has been demonstrated that cytolytic T cells may release cytokines following antigen recognition, including IFN- γ and tumor necrosis factor- α , which promote intracellular viral inactivation (Guidotti and Chisari, 1996). Thus, while the role of MHC class I expression in neuronal cells in CVS-11 infection is not yet clear, a direct consequence of the expression may be to retain virus-specific T cells, which cross the blood brain barrier, in the brain (Wekerle *et al*, 1986). Hanlon *et al* (1989) observed that raccoons vaccinated with a vaccinia-rabies virus glycoprotein (VRG) recombinant and subsequently subjected to a viral challenge developed a mild CSF pleocytosis. This suggests that the brain may retain virus-specific T helper cells after immunization.

Marked MHC class II mRNA expression was observed in inflammatory cells in RV194-2 infection, but not in CVS-11 infection. Gombold and Weiss (1992) also observed class II mRNA expression in focal areas of the brain in mouse hepatitis virus A59 infection and they speculated that there was likely involvement of inflammatory cells. The marked difference in MHC class II expression between RV194-2 and CVS-11 infections suggests that T-helper cells may play a critical role in stimulating immune responses that are important for recovery from the avirulent RV194-2 infection, and might be correlated with protection against RV194-2 infection for reasons that we do not know. Immune mechanisms appear to be essential in recovery from infection by avirulent rabies virus variants, since infection is lethal in athymic nude, RAG1 (complete lack of antigen-specific T or B cells), and cyclophosphamide-treated mice (Fla-

mand *et al*, 1984; Xiang *et al*, 1995). Stereotaxic brain inoculation of avirulent rabies virus variant Av01 likely results in a fatal infection because this route of viral entry circumvents the immune stimulation that occurs with other routes of inoculation, including intracerebral inoculation which is associated with intravenous administration of the inoculum (Mims, 1960; Yang and Jackson, 1992). The widespread stimulation of inflammatory cells expressing MHC class II mRNA in RV194-2 infection suggests that this response is also mediated by soluble factors such as cytokines. In summary, the differential expression of MHC class I and class II mRNAs in the virulent CVS-11 and avirulent RV194-2 virus infections may be important in establishing the respective neurovirulence phenotypes of these viruses in this mouse model.

Materials and methods

Viruses

All viruses were from The Wistar Institute, which is a World Health Organization Collaborating Center for Rabies. The cell culture adapted strain of fixed rabies virus CVS-11 and avirulent rabies virus variant RV194-2 (Dietzschold *et al*, 1983) were grown in BHK-21 cells and plaque purified. The Evelyn Rokitniki Abelseth (ERA) strain of rabies virus was grown on BHK-21 cells, purified by gradient centrifugation, and inactivated with β -propiolactone (ERA-BPL virus) as described (Wiktor, 1973). The CVS-24 strain of rabies virus, which has been adapted for higher mouse neurovirulence in comparison with CVS-11, was propagated in suckling mouse brain. The Copenhagen strain of vaccinia virus and vaccinia virus recombinants, VRG (expressing the rabies virus glycoprotein) (Wiktor *et al*, 1984) and VRNS (expressing the rabies virus phosphoprotein [P/NS]) (Larson *et al*, 1992), were propagated in HeLa cells, purified, and titrated as described (Wiktor *et al*, 1984).

Animals and virus inoculations

Six-week-old female ICR mice (Charles River Canada, Inc., St Constant, Quebec, Canada) were used to study antigen distribution and MHC class I and class II mRNA expression in brain. C3H/He mice (Jackson Laboratory, Bar Harbor, Maine, USA) were used for immunologic studies. A viral dose of 9.3×10^5 plaque forming units (p.f.u.), determined on BHK-21 cells, of CVS-11 or RV194-2 was administered intracerebrally in 0.03 mL of phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). Uninfected control mice were inoculated intracerebrally with PBS with 2% FBS. Seven to 14-week-old female C3H/He mice were inoculated subcutaneously in the footpad with identical doses of 10 LD₅₀ of the CVS-24 strain of rabies virus

and the RV194-2 avirulent virus (based on titrations of both viruses in suckling ICR mice).

Preparation of tissue sections for immunoperoxidase staining and in situ hybridization

Mice were anesthetized with methoxyflurane and perfused with buffered 4% paraformaldehyde. Brains of 3–6 infected mice were removed at daily intervals and immersion-fixed in the same fixative for 18 h at 4°C. Tissue sections (6 µm) were prepared in the coronal plane after dehydration and embedding in paraffin. Studies were performed on 2–3 animals inoculated with each of the viruses and uninfected controls at indicated time points. Immunoperoxidase staining was performed on 60 infected mice and *in situ* hybridization was performed on 25 infected mice.

Immunoperoxidase staining

Sections from daily time points were stained for rabies virus antigen by the avidin-biotin-peroxidase complex (ABC) method using polyclonal rabbit anti-rabies virus serum as primary antibody as previously described (Jackson and Wunner, 1991). Tissues from uninfected mice were used as controls and normal rabbit serum was substituted for the primary antibody on tissues as another control, and background staining was minimal.

In situ hybridization

³⁵S-UTP labeled RNA probes were used for localizing the MHC mRNAs in tissues. Single-stranded RNA probes were synthesized in the presence of [³⁵S]UTP (Dupont NEN) with SP6/T7 RNA polymerases from linearized plasmids containing 1.2 kb MHC class I cDNA (possibly encoding the L^d molecule) and 1.0 kb MHC class II cDNA (encoding the A^k molecule) (Gombold and Weiss, 1992). The MHC cDNAs were obtained from J Gombold and S Weiss (University of Pennsylvania, Philadelphia, PA, USA). Probes complementary to the class I and class II mRNAs were synthesized for the detection of mRNAs and also probes of the same sense as mRNA were synthesized as controls. The ³⁵S-labeled RNA probes were reduced in size by alkaline hydrolysis. The specific activities of the probes ranged from 1.4 × 10⁸–3.7 × 10⁸ d.p.m./µg. Tissue sections were deparaffinized and sequentially immersed in 0.2 N HCl for 20 min, 2 × SSC for 30 min, 10 µg/mL proteinase K in 10 mM Tris-HCl, pH 7.5, 2 mM CaCl₂ for 15 min at 37°C, and 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl buffer, pH 8.0, for 10 min. The sections were dehydrated in a graded series of alcohols and subsequently air dried. Hybridization was carried out for 4 h at 45°C. The hybridization solution contained 0.2 ng/µL of ³⁵S-labeled RNA probes, 50 mM dithiothreitol (DTT), 0.3 M NaCl, 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate,

0.2 mg sheared salmon sperm DNA/mL, 0.125 mg/mL tRNA, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% Triton X-100. After hybridization, the slides were rinsed three times in 4 × SSC with 10 mM DTT at room temperature for 20 min, once in 2 × SSC with 10 mM DTT for 5 min at 4°C, once in 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 10 mM DTT at 65°C for 15 min, once in 0–8 µg/mL RNase (Boehringer-Mannheim, Mannheim, Germany) in RNase buffer at 37°C for 30 min, twice in RNase buffer with 10 mM β-mercaptoethanol at 37°C for 15 min, once in 2 × SSC at room temperature for 30 min, and once in 0.1 × SSC at 63°C for 15 min, dehydrated in a series of graded alcohols containing 0.3 M ammonium acetate and air dried. Controls for the specificity of the reactions included tissue sections that were treated with 50 µg/mL RNase A (Boehringer-Mannheim) prior to hybridization and hybridization with probes of the same sense as mRNA.

The slides were dipped in Kodak NTB 2 nuclear track emulsion (Eastman Kodak Company, Rochester, NY, USA) diluted 1:1 with 0.6 M ammonium acetate and exposed at 4°C for 10 days. The slides were then developed with D19 developer (Eastman Kodak Company) for 5 min, fixed with 30% sodium thiosulfate for 5 min, and counterstained with hematoxylin.

Analysis of cerebrospinal fluid

Groups of 5 C3H/He mice, which were inoculated in the footpad with CVS-24 or RV194-2, were given a parenteral overdose of phenobarbital. Once mice were unconscious, they were exsanguinated and the foramen magnum was surgically exposed. A small hole was pierced into the dura mater and cerebrospinal fluid (CSF) was harvested with mild suction using a glass capillary tube. Approximately 5–10 µL of CSF was obtained per mouse. The fluid was analyzed by light microscopy counting mononuclear cells using a hemacytometer.

Enzyme linked immunoadsorbent assay (ELISA)

Antibody titers to rabies virus were determined by an ELISA on plates coated with inactivated ERA virus as described (Xiang and Ertl, 1992).

Lymphokine release assay

Splenocytes and T lymphocytes from popliteal lymph nodes on groups of 3–5 mice per assay, which were infected with CVS-24 or RV194-2 7 days previously via the footpad, were cultured at 6 × 10⁶ cells per well in 1.5 mL of DMEM supplemented with 2% FBS and 10⁻⁶ M 2-mercaptoethanol in 24-well plates with or without 250 µL of ERA-BPL virus at 5 µg and 1 µg of virus per mL and the cell-free supernatants from these cultures were cocultured with CTLL-2 cells (IL-2) as previously described (Xiang and Ertl, 1992). Proliferation of

the indicator cells was assessed 48–72 h later by a 6-h pulse with 0.5 μ Ci of [3 H]thymidine (Ertl *et al*, 1991).

Cytolytic T lymphocyte (CTL) assay

Lymphocytes from three mice infected with either CVS-24 or RV194-2 7 days earlier were cultured *in vitro* for 5–6 days with ERA-BPL virus. Lymphocytes were then tested at different effector to target cell ratios on L929 cells infected with vaccinia virus (Copenhagen strain) or vaccinia virus recombinants, VRG and VRNS, in a standard 51 Cr-release assay as previously described (Larson *et al*, 1992).

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Acknowledgments

We thank James Gombold and Susan Weiss (University of Pennsylvania, Philadelphia, PA, USA) for plasmids containing MHC class I and class II cDNA. This work was supported by Research Grant AI 33683 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health and by a Consortium Agreement with The Wistar Institute and Queen's University.

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