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Mouse hepatitis virus A59 increases steady-state levels of MHC mRNAs in primary glial cell cultures and in the murine central nervous system

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Infection of mixed glial cell cultures with mouse hepatitis virus (MHV)-A59 results in an approximately six-fold increase in the level of major histocompatibility complex (MHC) class I mRNA. *In situ* hybridization of glial cell cultures infected with MHV-A59 again showed enhanced MHC mRNA expression, both in infected and uninfected cells. These results extend our earlier finding that MHC surface antigens are enhanced on astrocytes and oligodendrocytes after MHV-A59 infection and suggest that this enhancement is a result of an increase in the steady-state level of MHC mRNA. We further demonstrate that increases in MHC mRNA occur in the murine central nervous system (CNS) following infection *in vivo*. Northern blot analysis of RNA from the brains of infected animals showed transient expression of both MHC class I and class II mRNA over the first 14 days of infection. Expression coincided with viral replication and clearance. *In situ* hybridization of brain sections from infected animals showed that class I and class II expression was widespread throughout all portions of the brain and in uninfected as well as infected cells. Viral RNA, in contrast, was observed in small foci of cells and mostly within the limbic system. Thus enhancement of MHC mRNA was not restricted either to areas of infection or inflammation. The spatial relationship between viral and MHC expression supports our hypothesis that a soluble mediator is involved in the mechanism of the increase in MHC levels. The fact that MHC induction occurs *in vivo* as well as *in vitro* suggests MHC may be important in the mechanism of MHV-induced disease.

Key words: Coronavirus; mouse hepatitis virus (MHV); major histocompatibility complex (MHC); encephalitis; demyelination.

Introduction

Mouse hepatitis virus (MHV), a member of the Coronaviridae, has been widely studied as a model of human neurologic infections. MHV strains A59 and JHM cause an acute necrotizing encephalitis in mice after intracerebral inoculation. Animals surviving the acute infection often develop a chronic disease characterized by demyelination of neurons in the central nervous system (CNS).[1–5]

Recovery from the acute encephalitis requires an active cell-mediated immune response. It has been shown that $CD4^+$, MHC class II-restricted T cells prevent death

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in JHM-infected mice and that $CD8^+$, major histocompatibility complex (MHC) class I-restricted T cells are important in viral clearance.[6–9] In addition, MHC class I-restricted T cells, possibly suppressor/cytotoxic T cells (CTL), have been isolated from mice infected with JHM.[10,11]

The mechanism of MHV-induced demyelination is not well understood. Early evidence suggested that demyelination resulted from direct viral lysis of oligodendrocytes.[12–14] In contrast, Watanabe *et al.*[15] demonstrated adoptive transfer of subacute demyelination with T cells from JHM-infected rats. More recently, Wang *et al.*[16] showed that demyelination could be prevented by irradiation of JHM-infected mice and that such mice would undergo demyelination after transfer of T cells from infected mice. Although these data strongly suggest that immune-mediated mechanisms may be active in demyelination, they do not rule out completely the possibility of viral cytotoxicity to oligodendrocytes.

We have shown previously that MHV-A59 infection of glial cells *in vitro* causes an enhanced expression of surface MHC, class I molecules on astrocytes and oligodendrocytes[17,18] and that this enhancement is probably mediated by a soluble factor, synthesized by astrocytes.[17] In persistently infected glial cells, MHC-enhancement is dependent on continual virus production.[19] We show here that MHC-enhancement may be observed at the mRNA level in glial cultures and that CNS infection of mice by A59 also increases the steady-state levels of MHC mRNA. Elevated expression occurs in both infected and uninfected cells, both *in vivo* and *in vitro*, supporting the hypothesis that a soluble factor is involved in the induction.[17] The increases in MHC expression during infection of the murine CNS as well as in glial cell cultures suggests a potential role for MHC class I molecules in the pathogenesis of MHV-A59-induced disease.

Results

We demonstrated previously that MHC class I antigens are expressed at high levels on the surface of MHV-A59 infected astrocytes and oligodendrocytes when compared to uninfected cells.[17,18] We wanted to determine if these changes were a result of an increase in MHC-specific mRNAs. We show here that infection of cells in culture as well as *in vivo* causes a rapid increase in both MHC class I and class II mRNAs.

MHC mRNA induction in vitro *in glial cell cultures*

Mixed glial cell cultures were derived by mechanical dissociation of newborn mouse brain. Greater than 90% of the cells in these cultures are positive for glial fibrillary acidic protein (GFAP) as determined by immunostaining, suggesting they are of the astroglial lineage;[19,31] (data not shown). Confluent monolayers of cells were infected with A59 at high multiplicity as described in Materials and methods. At regular intervals, total RNA was isolated from the cells, bound to nylon membranes, and hybridized with single-stranded RNA probes specific for MHC class I mRNA; the amount of probe that hybridized was quantitated by counting sections of the membrane containing individual RNA samples with a scintillation counter. The amount of positive-sense probe that bound to the membrane was used as an estimate of non-specific binding and background. As shown in Table 1, uninfected mixed glial cultures express low levels of class I mRNA. However, these cells respond rapidly to infection with an increase in class I expression that, by day 3, approaches six-fold. Class I transcripts decreased thereafter, returning to levels observed in mock-infected cells or below by day 10.

To verify and extend these results, both virus-infected and mock-infected cultures were examined for class I expression by *in situ* hybridization. These experiments failed

Table 1 MHC class I mRNA in mixed glial cultures infected with A59

Day PI	Total bound CPM			Fold-increase[d]
	Negative sense[a]	Positive sense[b]	Net negative sense[c]	
0	266	180	86	1.0
1	398	179	219	2.5
2	562	176	386	4.5
3	669	171	498	5.8
4	414	164	250	2.9
5	375	171	204	2.4
7	439	166	273	3.2
10	190	165	25	0.3
14	214	175	39	0.5

[a] Negative-sense class I RNA probe.
[b] Positive-sense class I RNA probe.
[c] (Negative sense) − (positive sense).
[d] Increase in cpm compared to day 0.

to show expression of class I mRNA in mock-infected cells hybridized with the negative-sense probe (Fig. 1A). In contrast, a marked increase in MHC class I expression was observed in A59-infected cells (Fig. 1B). Expression was observed in the majority of cells indicating that the elevation was not a result of a minor cell type responding to infection. Control experiments in which infected cells were treated with the positive-sense probe did not reveal significant hybridization (Fig. 1C). We suggest that astrocytes, the major cell type in these cultures, have the ability to elevate class I mRNA expression in response to infection.

Enhancement of MHC mRNA expression in vivo

The increased expression observed *in vitro* following infection, together with results from others showing class I-restricted immune responses involved in viral clearance,[8,9] suggested that MHC class I transcripts would likely be elevated *in vivo* during the acute infection. We inoculated mice intracerebrally with 1 LD_{50} of A59 or, as a control, with an uninfected cell lysate. At regular intervals, mice were sacrificed and total cell RNA was isolated from the brains. Northern blots, hybridized with the MHC class I probe, showed no difference in the level of expression between mock-infected and virus-infected animals sacrificed immediately following inoculation (Fig. 2A). Within 24 h of inoculation, class I transcripts had increased in virus-infected mice but not in mock-infected controls. Expression was maximal, approximately 5 days post-infection (pi), but was still elevated 2 weeks after infection. Slight fluctuations in expression occurred between 3 and 16 weeks pi but the significance of the changes is not clear (Fig. 2B). In contrast, the level of class I expression in mock-infected animals remained constant over the time course of the experiment.

The same blots were used to examine expression of MHC class II mRNA. Again, we found significantly greater levels of expression in infected mice compared to mock-infected controls (Fig. 2A and B) but the kinetics of expression were slower than those for class I. Class II transcripts were first detected at 3 days pi and reached maximal levels 10–14 days pi. However, we observed elevated levels of expression as late as 8 weeks pi.

Viral RNA was first detected 3 days pi, coincident with the expression of MHC class II mRNA (Fig. 2A). However, unlike either class I or class II expression, viral RNA was observed only up to 10 days pi. During subsequent time points when MHC

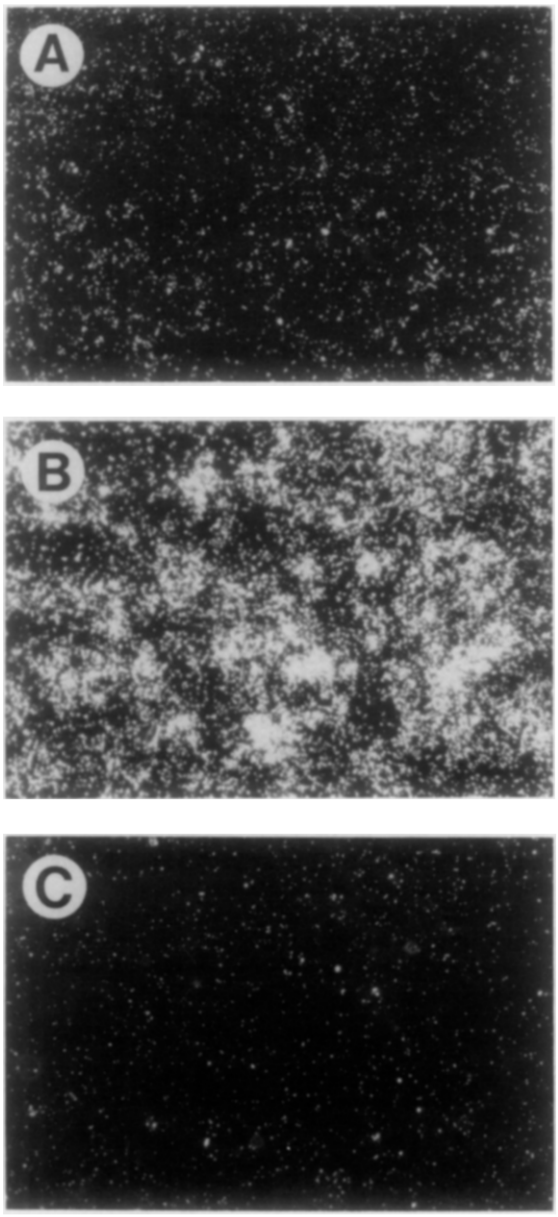

Fig. 1. *In situ* hybridization of primary glial cell cultures with MHC class I probes. Mock-infected (A) or virus-infected cells at 3 days pi (B and C) were fixed and hybridized with single-stranded RNA probes for MHC class I: (A and B) negative-sense probe; (C) positive-sense probe. Photographs were taken under darkfield illumination (50×). Reproduced here at 95%.

expression remained high, viral RNA was not detectable. Genome and all subgenomic mRNAs were detected although the four smallest mRNA species predominated. The kinetics of viral gene expression detected here were consistent with titers of virus in infected brain[4] (data not shown).

Although it was unlikely that the increased expression of MHC genes observed here was a result of variability in the amount of RNA analyzed by Northern blots, we chose to examine the levels of actin mRNA in these samples as a control. Figure 2 shows that while minor differences exist from sample to sample, this variability could not account for extensive increases in MHC gene expression that were observed. In

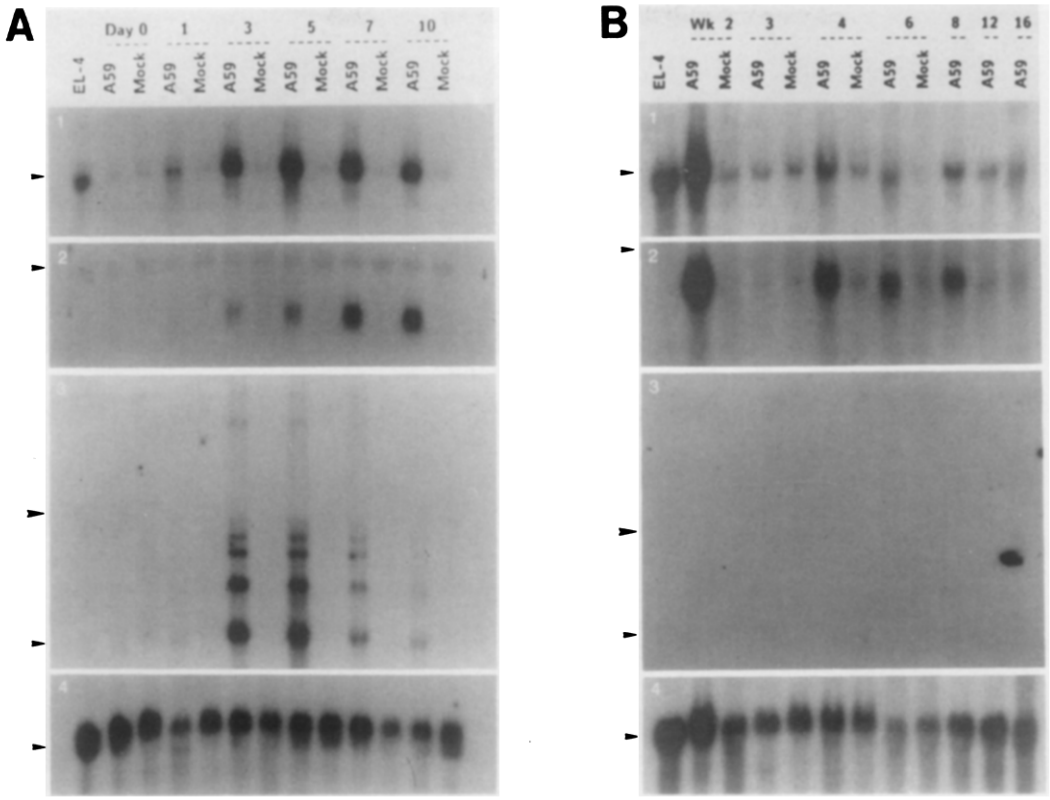

Fig. 2. Northern blot hybridization of total RNA (10 μg) isolated from A59- or mock-infected mouse brain. Mice were sacrificed on the indicated times pi and total RNA was prepared as described in Materials and methods. Each gel was sequentially hybridized with MHC class I (panels 1), MHC class II (panels 2), virus (panels 3), and actin (panels 4) cDNA probes. EL-4 RNA was included as a positive marker for class I expression. (A) Mice examined between 0 and 10 days pi; (B) Mice examined between 2 and 16 weeks pi. Position of 28S (large arrowhead) and 18S (small arrowhead) are indicated. Reproduced here at 95%.

addition, these results demonstrate that actin expression remained relatively constant during the time course of infection, indicating that the elevation of MHC gene expression was not part of a generalized increase in mRNA levels.

In situ hybridization was used to confirm these results and to determine where in the brain MHC or virus mRNA expression occurred and whether the increases in MHC mRNAs were restricted to foci or infection or inflammation. For these experiments, an independent group of mice was inoculated with A59 or an uninfected cell lysate as described above. Mice were selected at various times after infection without regard to signs of disease and sacrificed. Brain sections were prepared and hybridized with single-stranded probes for virus, MHC class I, or class II.

Viral gene expression was focal and largely restricted to the limbic and olfactory systems and is similar to that found when measuring viral antigens in the CNS.[20,21] Viral RNA was initially detected in the olfactory bulbs (Fig. 3A) and in paraventricular areas, most notably the medial and lateral septum (Fig. 3C). Foci of infection were also observed in the caudate putamen (Fig. 3C) and occasionally in cells of the ependyma (Fig. 3C and E). Viral RNA could also be detected in the brain stem by 7 days pi (data not shown). We rarely observed evidence of virus in the cerebral cortex or in the cerebellum. Examination of these sections also showed little inflammation, most of which was observed in the meninges (data not shown). While regions of encephalitis were observed, they were highly focal and few in number.

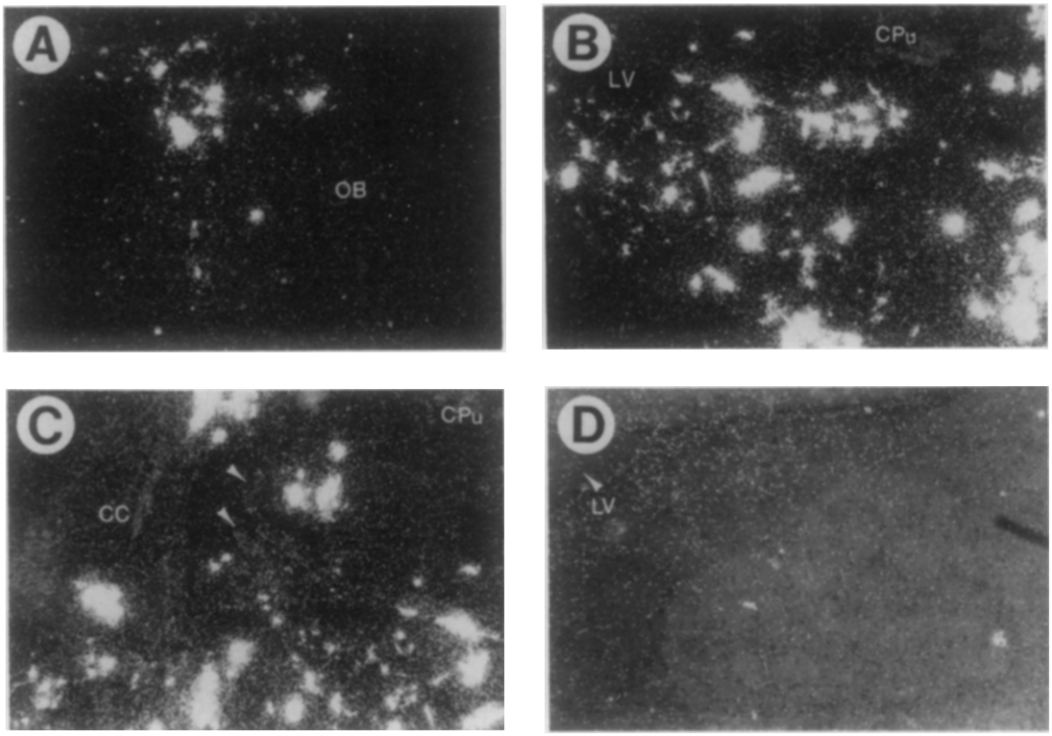

Fig. 3. *In situ* hybridization of cryostat sections of mouse brain with a negative-sense viral RNA probe (clone 320) complementary to the 3′ end of the viral genomic and subgenomic mRNAs. Tissue sections were from virus-infected (A–C) or mock-infected (D) mice sacrificed 3 days after inoculation. Shown are sections through the olfactory bulbs (A) and the lateral ventricle and the medial septum (B–D). CC, corpus callosum; CPu, caudate putamen; LV, lateral ventricle; OB, olfactory bulb. Arrowheads in C and D indicate position of the choroid plexus within the ventricles. Photographs were taken under darkfield illumination (25×). Reproduced here at 92%.

The kinetics of expression of MHC class I mRNA as revealed by *in situ* hybridization was consistent with the results of the Northern blots described above. Expression was largely undetectable in brains of mock-infected controls (Fig. 4) or in virus-infected animals sacrificed immediately following infection (data not shown). However, between 3 and 7 days pi, the number of cells expressing class I transcripts as well as the level of expression consistently increased. While expression did not appear to be restricted to any specific regions or to areas of infection or inflammation, the highest levels of expression were observed within regions previously described as major sites of viral replication[20,21] (Fig. 3). The olfactory bulbs showed extensive expression of MHC class I in the meninges and the olfactory nerve layer (Fig. 4A). Expression was also markedly elevated in the choroid plexus (Fig. 4B) and throughout the medial and lateral septum (Fig. 4C and D). Positive-stranded probes used for controls did not hybridize in sections from virus- or mock-infected animals (data not shown).

Kinetics of class II mRNA expression detected by *in situ* hybridization was also consistent with the results of the Northern blots described above. Mice infected for 1 day or less did not appear to express class II mRNA (data not shown). There was an increase in expression in A59-infected mice by 3 to 5 days pi and was quite elevated near the ventricles, especially near the ependyma (Fig. 5A) and the choroid plexus (Fig. 5C). In contrast, mock-infected mice showed little if any expression of MHC class II mRNA in these areas (Fig. 5B and D). However, we noted that the expression of class II was more focal than that of class I, indicating that inflammatory cells might

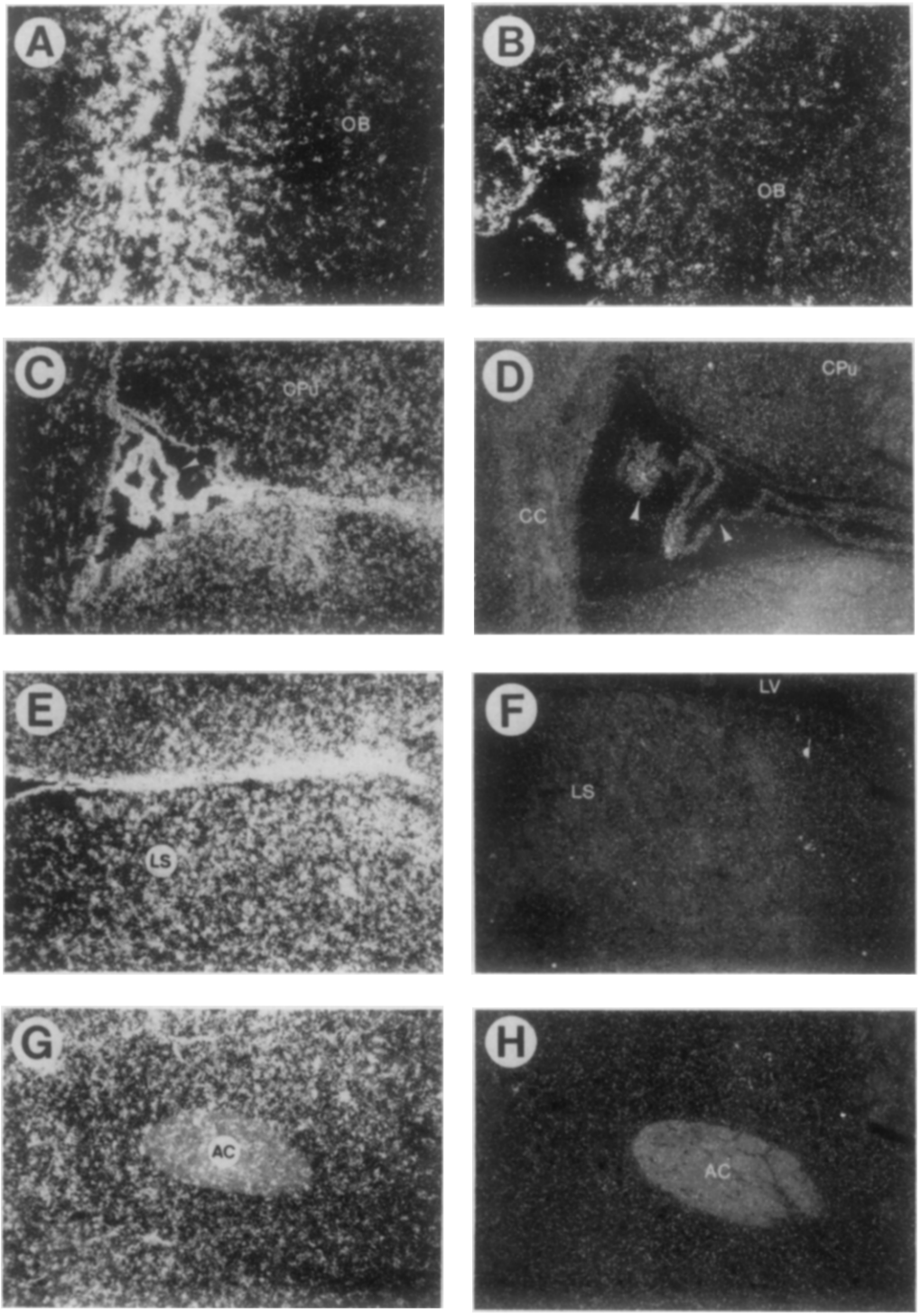

Fig. 4. *In situ* hybridization of cryostat sections of mouse brain with MHC class I RNA probes. Shown are sections from mice sacrificed 3 days after inoculation with virus (A,C,E,G) or with an uninfected cell lysate (B,D,F,H) and hybridized with a negative-sense class I probe: (A,B) olfactory bulb; (C,D) lateral ventricle and choroid plexus; (E,F) medial and lateral septum, and (G,H) the anterior commissure. AC, anterior commissure; CC, corpus callosum; CPu, caudate putamen; LS, lateral septum; LV, lateral ventricle; OB, olfactory bulb. Arrowheads in C and D indicate position of the choroid plexus within the ventricles. Sections are shown under darkfield illumination (25×). Reproduced here at 82%.

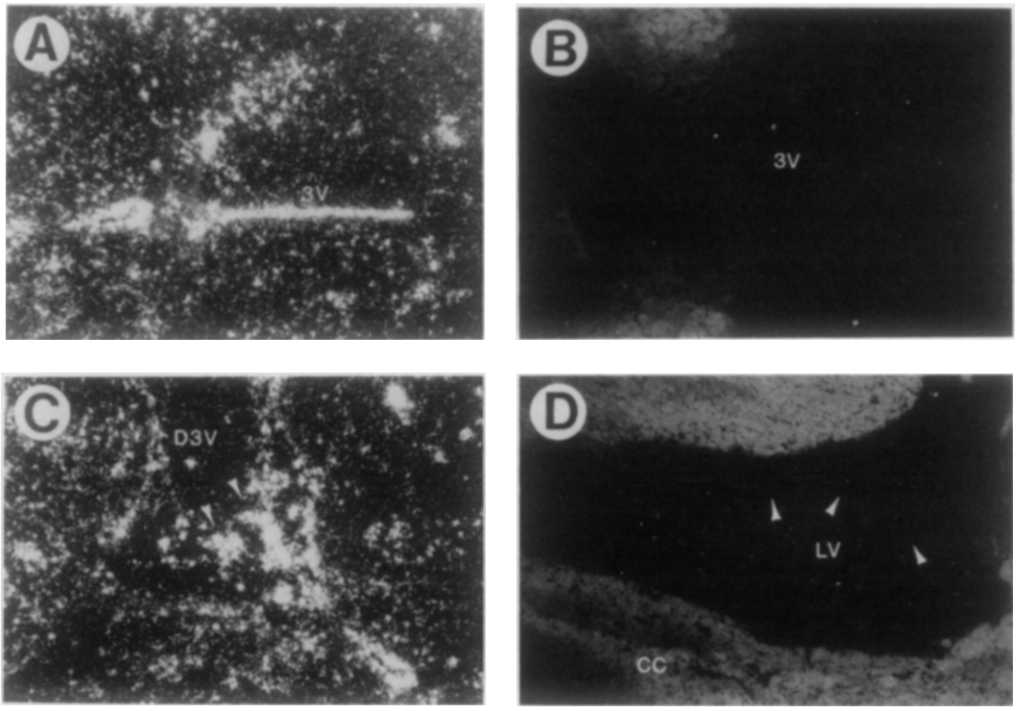

Fig. 5. *In situ* hybridization of cryostat sections of mouse brain with MHC class II RNA probes. Shown are sections from mice sacrificed 7 days after inoculation with virus (A,C) or with an uninfected cell lysate (B,D) and hybridized with a negative-sense class II probe: (A,B) third ventricle, (C) choroid plexus in the dorsal third ventricle, and (D) choroid plexus in the lateral ventricle. 3V, third ventricle; CC, corpus callosum; D3V, dorsal third ventricle; LV, lateral ventricle. Arrowheads in C and D indicate position of the choroid plexus within the ventricles. Sections are shown under darkfield illumination (25×). Reproduced here at 84%.

be contributing to the increased expression. As expected, controls using positive-strand RNA probes failed to hybridize regardless of the inoculum or the time after infection (data not shown).

Areas of the brain that contained viral mRNA always exhibited elevated levels of MHC transcripts. However, high levels of MHC mRNA were found in regions of the brain lacking viral RNA. For example, virus was rarely detected within the choroid plexus (Fig. 3C), yet the expression of MHC mRNA was often high (Figs 4C and 5C). Thus, we conclude that MHC gene expression is not restricted to foci of infection or inflammation and may therefore be modulated indirectly.

Discussion

We have reported previously[18] that infection of either primary mixed glial cultures or of astrocyte-enriched cultures with A59 caused increased expression of MHC class I, but not class II, antigens on the cell surface. Our results here show that infection of mixed glial cell cultures with mouse hepatitis virus causes an accumulation of MHC class I mRNA which presumably leads to the increase of MHC class I antigens on the cell surface. Since more than 95% of cells in these cultures are astrocytes or precursors of astrocytes (as measured by reaction with anti-GFAP serum) and in excess of 80% of the cells are positive for MHC mRNA, MHC-enhancement clearly occurs in astrocytes. This is consistent with our previous double immunofluorescence results showing that antisera that recognize MHC and GFAP or galactocerebroside (GalC) stain the same cells.[18] Microglia, which represent no more than a small percentage of

cells in these primary cultures,[19] may also respond to infection by increasing the level of MHC mRNA and antigen; however, because of their small number in these cultures, microglia cannot account for all of the MHC expression that is observed.

We demonstrate here that CNS infection of C57BL/6 mice by A59 caused a transient increase in the steady-state levels of MHC class I and class II mRNA. Class I mRNA was first observed 1 day after infection, prior to the onset of detectable viral RNA synthesis as well as inflammatory cell infiltration. At the later times post-infection that were examined (3–16 weeks), class I and class II expression showed some variability. For example, neither was expressed at elevated levels 3 weeks pi, but both were present at high levels at 4, 6 and 8 weeks pi. It is tempting to speculate that the increases seen at 4–8 weeks pi might be occurring at times of active demyelination. Elevated levels of MHC mRNA were observed in cells without detectable viral RNA, demonstrating that the enhancement was not occurring solely in infected cells, consistent with the results from primary glial cultures. Furthermore, cells with detectable MHC mRNA were found throughout the brain, while inflammatory cells were found in foci, particularly in perivascular regions. These data along with the observation that MHC class I induction occurs in primary cultures of glial cells (both GFAP-positive and GalC-positive) suggest that it is unlikely that MHC mRNA enhancement in the CNS is only a result of inflammatory cells. However, the rather focal regions of class II expression may result, at least in part, from inflammatory infiltrates in the brain. The increase in MHC expression did not appear to be part of a general effect of infection on cellular mRNA levels since actin mRNA levels remained constant. The results also show that increases in MHC expression during infections *in vivo* are similar to increases observed *in vitro* in infected glial cell cultures.

In these experiments, we found that viral RNA was largely restricted to the olfactory and limbic systems during the early stages of infection. This is consistent with our previous localization of MHV-A59 antigen during acute infection.[20,21] Although these sites are major areas of MHC expression during infection, MHC-specific mRNA was observed also in regions of the brain outside of these systems and devoid of viral RNA. This observation suggests that the induction may be mediated by lymphokines or related soluble factors induced by infection. Our earlier experiments[17,18] showed that a factor capable of inducing MHC expression on glial cells was produced by astrocytes but not oligodendrocytes, microglia, or meningeal fibroblasts *in vitro* following infection with A59. Biochemical and physical characterizations suggested that the factor is not interferon. Although this unidentified factor has only been detected following infections *in vitro*, it is possible that it may have a significant role during infection given the many recent reports describing H2-restricted T cells involved in viral clearance and demyelination (see below). However, we do not discount the possibility that non-CNS cell types (e.g. immune cells) also may produce this factor. Work now in progress is aimed at identifying the potential role of T cells in the induction of MHC expression and its relationship to both the acute and chronic disease.

Other viruses have been identified that either negatively or positively regulate the expression of MHC antigens.[22–29] In the cases of infections of cells with Moloney murine leukemia virus or certain flaviviruses[26,29] control of the MHC mRNA level is thought to be transcriptional. Our results demonstrate higher steady-state levels of MHC mRNA after MHV-A59 infection. Although these results are consistent with increased transcription of MHC genes, we cannot rule out the possibility that the elevation results from changes in the stability of MHC transcripts.

The role of MHC antigens during the acute and chronic stages of the CNS disease caused by MHV is not entirely clear. Clearance of infectious virus usually occurs by 4

weeks pi and, in the case of MHV–JHM, involves both $CD4^+$ and $CD8^+$ T cells.[8,9] This implies a role for both MHC class I and class II in the resolution of the acute disease. The data presented here show that expression of MHC genes was quite pronounced for the first 10 days of infection and that loss of detectable viral RNA coincided with the elevation of MHC mRNAs. Sussman *et al.*[8] identified a $CD4^+$ T cell required for clearance of JHM from the mouse CNS. These cells appeared to be helper-inducer T cells that acted together with class I-restricted $CD8^+$ T cells to effect viral clearance. It was speculated that the $CD8^+$ cells might be cytotoxic T cells (CTLs), not unlikely since the isolation of class I-restricted, $CD8^+$, CTL clones from JHM-infected mice have been reported.[10,11] Class II-restricted T cells mediating a delayed-type hypersensitivity (DTH) have also been identified in JHM-infected mice.[6,7] These T cells protect mice from lethal disease when challenged with JHM but do not prevent growth of the virus in the CNS.

Two mechanisms have been proposed to explain the primary demyelination observed in this system. One suggests that the demyelination is a result of direct cytolytic activity of the virus on oligodendrocytes.[12–14] This hypothesis was supported by the early report that immunosuppression does not alter the course of demyelination in infected animals.[14] A second hypothesis states that the demyelination is immune-mediated and is supported by experiments showing induction of experimental allergic encephalomyelitis (EAE)-like lesions in uninfected animals by transfer of lymphocytes from JHM-infected rats.[15] Lymphocytes from infected rats were sensitized to both myelin basic protein and JHM particles as shown by proliferative responses when cultured in the presence of either antigen. More recent evidence for an immunopathological mechanism of MHV-induced demyelination has been provided by Wang *et al.*[16] who showed that irradiation of mice prevented demyelination by JHM and that demyelination could be restored by adoptive transfer of spleen cells. The activity of the transferred cells appeared to be MHC restricted. Finally, it has been suggested that the acute demyelination observed early in the infection may be a result of cytolysis of oligodendrocytes and the subacute or chronic demyelination to immune-mediated mechanisms.[30]

The increase in MHC antigen expression on astrocytes and oligodendrocytes during MHV-A59 infections is well established.[17–19] We show here that this increase is likely to be the result of an elevation of MHC mRNA. Given the evidence accumulating for an immunopathological mechanism in MHV-induced demyelination,[15,16] it will be important to identify the types and specificities of T cells in the CNS and determine the role of MHC antigens in acutely and chronically infected mice.

Materials and methods

Virus and cells. MHV strain A59 was propagated in a mouse fibroblast cell line (17 Cl-1) and filtered (0.2 μm) prior to use. The LD_{50} of A59 was determined by end-point dilution in 4–6 week old male C57BL/6 mice. Primary mixed glial cell cultures were made from dissociated brains of newborn C57BL/6 mice essentially as described by Lavi *et al.*[31] and were used 10–15 days after plating. These cultures contained 90–95% astrocytes as determined by immunostaining of glial fibrillary acidic protein[31] (data not shown). These cultures also contain a few percent oligodendrocytes (measured by staining with antisera against galactocerebroside) and microglia (measured by phagocytosis of latex beads). Primary glial cultures and 17 Cl-1 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS). EL-4 cells (an MHC-class I positive murine lymphoma cell line obtained from Glen Gaulton, University of Pennsylvania, PA) were maintained in DMEM supplemented with 20% FBS.

Mice. Four to six week-old male, MHV-antibody free, C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). A59 was diluted to 50 LD_{50} per ml (approximately 2.5×10^5 pfu/ml) in phosphate-buffered saline (PBS) containing 0.75% bovine serum albumin

and 20 μl of this was injected into the left cerebral hemisphere of mice. Mock-infected controls received uninfected 17 Cl-1 cell lysates comparably diluted. Virus-infected animals were housed in isolation for the duration of the experiments.

RNA isolation. (a) From glial cell cultures. Cells growing in 60 mm dishes (1.4×10^6 cells) were washed three times with cold Dulbecco's PBS and then lysed in 50 mM Tris-HCl, pH 8/100 mM NaCl/1% Nonidet P-40. The lysate was adjusted to 25 mM EDTA/1%SDS/200 μg Proteinase K per ml and incubated at 37°C for 15 min. After extraction with phenol/chloroform, the RNA was precipitated with ethanol, washed once, and resuspended in water. (b) From brain. Brains were removed and washed once with Dulbecco's PBS. The tissue was homogenized in 4 M guanidinium isothiocyanate (BMB) and layered over a cushion of 5.7 M CsCl. The RNA was pelleted in an SW41 rotor at 32 000 rpm, resuspended in 10 mM Tris-HCl, pH 7.4/1 mM EDTA, extracted with phenol/chloroform, and precipitated as described above. The RNA was washed once, resuspended in water, and quantitated by UV spectroscopy.

Northern blots. RNA (10 μg) was resuspended in 20 mM MOPS/1 mM EDTA/5 mM Na acetate/2.2 M formaldehyde/50% formamide and denatured by heating at 65°C for 10 min. After electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde, the RNA was transferred to nylon membranes (Gene Screen Plus, New England Nuclear) and hybridized with [^{32}P]-cDNA probes in 50% formamide at 42°C according to the manufacturer's suggestions. Membranes were rinsed twice with 2×SSC at room temperature, washed twice with 2×SSC/0.1% SDS at 60°C, twice with 0.1×SSC at room temperature, and exposed to X-ray film at −80°C.

Dot blots. Cytoplasmic RNA was extracted from either mock- or A59-infected glial cells as described above. Samples were adjusted to 6% formaldehyde/50% formamide, denatured, and 10 μg of RNA was spotted onto Gene Screen Plus membranes. RNA probes, transcribed *in vitro* as described below and labeled with [^{32}P]-UTP, were hybridized at 75°C in 5×SSPE (1×SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4)/2×Denhardt's solution [1×Denhardt's is 0.02% (w/v) each ficoll, bovine serum albumin, polyvinylpyrrolidone)/1% SDS/100 μg/ml salmon sperm DNA/50% formamide]. Following hybridization, the membranes were washed three times in 0.1×SSPE/0.1% SDS at 75°C and exposed to X-ray film at −80°C. Bound probe was quantitated by liquid scintillation counting.

In situ *hybridization.* Hybridizations were performed on frozen brain sections or on infected cells as described previously.[32] Briefly, samples were fixed in 3% paraformaldehyde, incubated in 0.1 M Tris/0.1 M glycine, acetylated in 0.1 M triethanol amine-HCl, pH 8/0.25% acetic anhydride, and then dehydrated through graded ethanols. [^{35}S]-labeled RNA probes were allowed to hybridize at 50°C for 4 h in 10% dextran sulfate/1×Denhardt's solution/4×SSC/10 mM DTT/1 mg yeast RNA per ml/1 mg salmon sperm DNA per ml/50% formamide. Following hybridization, samples were washed twice in 2×SSC/50% formamide at 52°C and digested with RNase A in 2×SSC at 37°C. The samples were then rinsed in 2×SSC/50% formamide at 52°C, washed overnight in 2×SSC/0.05% Triton X-100, and dehydrated through graded ethanols. Slides were coated with emulsion (NTB-2, Kodak) and exposed for 3–6 weeks at 4°C.

Nucleic acid probes. DNA probes used for hybridization included (1) a 1.2 kb MHC class I cDNA (pH2II; 33), (2) a 1 kb MHC class II cDNA (obtained from J. Woodward, University of Kentucky, KY), (3) a 3 kb viral cDNA derived from the 3′ end of the A59 genome containing sequences from viral genes 4, 5, 6 and 7 (clone 320; unpublished data), and (4) a previously described actin-specific 3 kb *Eco*RI fragment obtained from R. Weinmann, Wistar Institute.[34] Probes were labeled with [^{32}P]-dCTP by random priming to 5×10^8 cpm/μg or greater.

RNA probes were transcribed *in vitro* in the presence of [^{35}S]-UTP from cDNAs subcloned into pGEM plasmids (Promega) containing promoters for SP6 and T7 RNA polymerases in opposite orientations. Specific activities were generally $1–5\times10^8$ cpm/μg. RNA probes for *in situ* hybridizations were hydrolyzed to approximately 100 base pairs prior to use.

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References

1. Haspel MV, Lampert PW, Oldstone MBA. Temperature-sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination. Proc Natl Acad Sci 1978; 75: 4033–6.
2. Knobler RL, Lampert PW, Oldstone MBA. Virus persistence and recurring demyelination produced by a temperature-sensitive mutant and MHV-4. Nature 1982; 298: 279–80.
3. Koolen MJM, Love S, Wouda W, Calafat J, Horzinek MC, van der Zeijst BAM. Induction of demyelination by a temperature-sensitive mutant of the coronavirus MHV-A59 is associated with restriction of viral replication in the brain. J Gen Virol 1987; 68: 703–14.
4. Lavi E, Gilden DH, Wroblewska Z, Rorke LB, Weiss SR. Experimental demyelination produced by the A59 strain of mouse hepatitis virus. Neurology 1984; 34: 597–603.
5. Lavi E, Gilden DH, Highkin MK, Weiss SR. The organ tropism of mouse hepatitis virus A59 is dependent on dose and route of inoculation. Lab Animal Sci 1986; 36: 130–5.
6. Stohlman SA, Matsushima GK, Casteel N, Weiner LP. *In vivo* effects of coronavirus-specific T cell clones: DTH inducer cells prevent a lethal infection but do not inhibit virus replication. J Immunol 1986; 136: 3052–6.
7. Stohlman SA, Sussman MA, Matsushima GK, Shubin RA, Erlich SS. Delayed-type hypersensitivity response in the central nervous system during JHM virus infection requires viral specificity for protection. J Neuroimmunol 1988; 19: 255–68.
8. Sussman MA, Shubin RA, Kyuwa S, Stohlman SA. T-cell-mediated clearance of mouse hepatitis virus strain JHM from the central nervous system. J Virol 1989; 63: 3051–6.
9. Williamson JSP, Stohlman SA. Effective clearance of mouse hepatitis virus from the central nervous system requires both $CD4^+$ and $CD8^+$ T cells. J Virol 1990; 64: 4589–92.
10. Kyuwa S, Yamaguchi K, Hayami M, Fujiwara K. Characterization of mouse hepatitis virus-reactive T cell clones. In: Lai MC, Stohlman SA, eds. Coronaviruses. New York: Plenum Press, 1987; 391–8.
11. Yamaguchi K, Kyuwa S, Nakanaga K, Hayami M. Establishment of cytotoxic T-cell clones specific for cells infected with mouse hepatitis virus. J Virol 1988; 62: 2505–7.
12. Beushausen S, Dales S. *In vivo* and *in vitro* models of demyelinating disease. XI. Tropism and differentiation regulate the infectious process of coronaviruses in primary explants of the rat CNS. Virology 1985; 141: 89–101.
13. Lampert PW, Sims JK, Kniazeff AJ. Mechanism of demyelination in JHM virus encephalomyelitis. Acta Neuropathol. 1973; 24: 76–85.
14. Weiner LP. Pathogenesis of demyelination induced by mouse hepatitis virus (JHM). Arch Neurol 1973; 28: 298–303.
15. Watanabe R, Wege H, ter Meulen V. Adoptive transfer of EAE-like lesions by BMP stimulated lymphocytes from rats with coronavirus-induced demyelinating encephalomyelitis. Nature 1983; 305: 150–3.
16 Wang F-I, Stohlman SA, Fleming JO. Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated. J Neuroimmunol 1990; 30: 31–41.
17. Suzumura A, Lavi E, Bhat S, Murasko D, Weiss SR, Silberberg D. Induction of glial cell MHC antigen expression in neurotropic coronavirus infections. Characterization of the H-2-inducing soluble factor elaborated by infected brain cells. J Immunol 1988; 140: 2068–72.
18. Suzumura A, Lavi E, Weiss SR, Silberberg DH. Coronavirus infection induces H-2 antigen expression on oligodendrocytes and astrocytes. Science 1986; 232: 991–3.
19. Lavi E, Suzumura A, Murray EM, Silberberg DH, Weiss SR. Induction of MHC class I antigens on glial cells is dependent on persistent mouse hepatitis virus infection. J Neuroimmunol. 1989; 22: 107–11.
20. Fishman PS, Bass JS, Swoveland PT, Lavi E, Highkin MK, Weiss SR. Infection of the basal ganglia by a murine coronavirus. Science 1985; 229: 877–9.
21. Lavi E, Highkin MK, Fishman PS, Weiss SR. Limbic encephalitis after inhalation of a murine coronavirus. Lab Invest 1988; 58: 31–6.
22. Andersson M, Paabo S, Nilsson T, Peterson PA. Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. Cell 1985; 43: 215–22.
23. Burgert H-G, Kvist S. An adenovirus type 2 lycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell 1985; 41: 987–97.
24. Eager KB, Williams J, Breiding D *et al.* Expression of histocompatibility antigens H-2K, -D and -L is reduced in adenovirus-12-transformed mouse cells and is restored by interferon. Proc Natl Acad Sci 1985; 82: 5525–9.
25. Gogusev J, Teutsch B, Morin MT *et al.* Inhibition of HLA class I antigen and mRNA expression induced by Rous sarcoma virus in transformed human fibroblasts. Proc Natl Acad Sci 1988; 85: 203–7.

26. King NJC, Kesson AM. Interferon-independent increases in class I major histocompatibility complex antigen expression follow flavivirus infection. J Gen Virol 1988; 69: 2535–43.
27. Massa PT, Schimpl A, Wecker E, ter Meulen V. Tumor necrosis factor amplifies measles virus-mediated Ia induction on astrocytes. Proc Natl Acad Sci 1987; 84: 7242–5.
28. Vaessen RTMJ, Houweling A, van der Eb AJ. Post-transcriptional control of class I MHC mRNA expression in adenovirus 12-transformed cells. Science 1987; 235: 1486–8.
29. Wilson LD, Flyer DC, Faller DV. Murine retroviruses control class I major histocompatibility antigen gene expression via a trans effect at the transcriptional level. Mol Cell Biol 1987; 7: 2406–15.
30. Erlich SS, Fleming JO, Stohlman SA, Weiner LP. Experimental neuropathology of chronic demyelination induced by a JHM virus variant (DS). Arch Neurol 1987; 44: 839–42.
31. Lavi E, Suzumura A, Hirayama M *et al.* Coronavirus mouse hepatitis virus (MHV)-A59 causes a persistent, productive infection in primary glial cell cultures. Microb Pathogen 1987; 3: 79–86.
32. Chesselet M-F, Weiss LT, Wuenschell C, Tobin A, Affolter HU. Comparative distribution of mRNAs for glutamic acid decarboxylase, tyrosine hydroxylase, and tachykinins in the basal ganglia: an *in situ* hybridization study in the rodent brain. J Comp Neurol 1987; 262: 125–40.
33. Steinmetz M, Moore DW, Frelinger JG *et al.* A pseudogene homologous to mouse transplantation antigens: transplantation antigens are encoded by eight exons that correlate with protein domains. Cell 1981; 25: 683–92.
34. Khalili K, Salas C, Weinmann R. Isolation and characterization of human actin genes cloned in phage lambda vectors. Gene 1983; 21: 9–17.