Published September 1, 1994

Coronavirus Induction of Class I Major Histocompatibility Complex Expression in Murine Astrocytes Is Virus Strain Specific

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Summary

Neurotropic strains of mouse hepatitis viruses (MHV) such as MHV-A59 (A59) and MHV-4 (JHMV) cause acute and chronic encephalomyelitis and demyelination in susceptible strains of mice and rats. They are widely used as models of human demyelinating diseases such as multiple sclerosis (MS), in which immune mechanisms are thought to participate in the development of lesions in the central nervous system (CNS). The effects of MHV infection on target cell functions in the CNS are not well understood, but A59 has been shown to induce the expression of MHC class I molecules in glial cells after in vivo and in vitro infection. Changes in class I expression in infected cells may contribute to the immunopathogenesis of MHV infection in the CNS. In this communication, a large panel of MHV strains was tested for their ability to stimulate class I expression in primary astrocytes in vitro. The data show that the more hepatotropic strains, such as MHV-A59, MHV-1, MHV-2, MHV-3, MHV-D, MHV-K, and MHV-NuU, were potent inducers of class I expression in astrocytes during acute infection, measured by radioimmunoassay. The K^b molecule was preferentially expressed over D^b. By contrast, JHMV and several viral strains derived from it did not stimulate the expression of class I molecules. Assays of virus infectivity indicated that the class I–inducing activity did not correlate with the ability of the individual viral strain to replicate in astrocytes. However, exposure of the viruses or the supernatants from infected astrocytes to ultraviolet light abolished the class I–inducing activity, indicating that infectious virus is required for class I expression. These data also suggest that class I expression was induced directly by virus infection, and not by the secretion of a soluble substance into the medium by infected astrocytes. Finally, analyses of A59/JHMV recombinant viral strains suggest that class I–inducing activity resides in one of the A59 structural genes.

Mouse hepatitis viruses (MHV)[1] are members of the Coronaviridae, which cause gastrointestinal, neurological, and respiratory diseases in a wide variety of mammalian species (1–7). Although some MHV strains, such as A59 and MHV-3, can cause both gastrointestinal and neurological disease in mice and rats, many tend to induce pathology that is restricted to either system, prompting their classification as hepatotropic or neurotropic. There has been considerable interest in the study of the more neurotropic strains of MHV, such as JHMV and A59, because of their ability to produce demyelinating lesions that resemble the demyelinating plaques observed in the human neurological disease, multiple sclerosis (MS). In addition, MHV and human coronavirus isolates are capable of inducing demyelination in primates (8), and coronavirus RNA and antigen have been detected in demyelinating lesions in the brains of MS patients (9, 10).

As Coronaviridae, all MHV strains are enveloped viruses that contain a single-stranded, positive-sense RNA genome of 31-kb that is expressed as 7 or 8 mRNAs encoding both structural and nonstructural proteins (11). The structural proteins have been well characterized, and include the nucleocapsid (N) protein, which interacts with the viral RNA, and two envelope glycoproteins, the spike (S) and membrane (M) proteins. The S protein forms the virion surface spikes and is responsible for binding the cellular MHV receptor, inducing cell-to-cell fusion and providing a target for neutralizing antibodies. It is the least conserved of the structural proteins among the MHV strains (12–16). M interacts with the N protein–RNA complex, and may be involved in virus assembly. It is the most conserved structural protein among the MHV. A third envelope glycoprotein, hemagglutinin–esterase (HE), has been identified in MHV-S and several JHMV isolates,

[1] *Abbreviations used in this paper:* CNS, central nervous system; GFAP, glial fibrillary acidic protein; i.c., intracerebral; M, membrane; MHV, mouse hepatitis virus; m.o.i., multiplicity of infection; MS, multiple sclerosis; N, nucleocapsid; p.i., post infection; S, spike; VSV, vesicular stomatitis virus.

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and may participate in the pathogenesis of JHMV infection in the central nervous system (CNS) (11, 17). Nonstructural proteins include an RNA-dependent RNA polymerase, and three additional proteins that do not appear to be required for viral replication, but whose functions and biological activities have not been identified.

After intracerebral (i.c.) inoculation in mice and rats, the parent strains of both JHMV and A59 cause an acute encephalomyelitis and demyelination from which a varying number of animals survive to exhibit chronic demyelination (1–7, 11). The severity of the disease varies with the individual virus strain and with the age, genetic background, and immune status of the infected host. The neuropathological features of acute infection can be somewhat distinguished from those of chronic disease by a prevalence of gray matter involvement in which virus replicates in neurons, oligodendrocytes, and astrocytes to cause extensive CNS damage (18–21). In chronic disease, lesions are more prevalent in the white matter and consist of primary demyelination with axonal sparing (2, 11). Infectious virus is rarely isolated during chronic JHMV disease, though viral RNA can be detected by reverse transcriptase (RT)-PCR in the brains of infected mice as late as 2 yr post infection (p.i., Fleming, J., personal communication). Viral antigen and RNA tend to be restricted to astrocytes in chronic infection (2, 22–25), though there is evidence that neurons are also affected (25). This pattern of white matter involvement is also characteristic of infection with attenuated or mutant strains of A59 (26, 27) or JHMV (25, 27–29), which cause demyelination in the relative absence of encephalitis. In vitro, JHMV and A59 cause a lytic infection in primary oligodendrocyte cultures that is rapidly self-limiting, but both virus strains can establish a productive and relatively nonlytic, acute, and long-term chronic infection in mixed glial cell cultures and in cultures enriched for astrocytes (30–32). These data support early reports that acute demyelination is the result of virus-mediated oligodendrocyte death (2, 11), but more importantly, they also suggest that the astrocyte plays an important role in acute encephalitis and chronic demyelination.

Currently, there is very little known about the ability of MHV strains to influence specific activities of the host cells that they infect. Early reports indicate that A59 infection enhances the expression of MHC class I molecules in primary cultures of murine glial cells (33) and that the enhancement also occurs in the brain after i.c. infection in C57Bl/6 mice (34). Since class I molecules play a key role in the interaction between infected target cells and $CD8^+$ cytotoxic T cells (CTL; 35, 36), any change in their expression after virus infection has potential implications for the outcome of the infection. Cells in the CNS generally express little, if any, constitutive class I antigen in vivo, but astrocytes have been consistently reported to express both class I and class II antigens in vitro after the addition of IFN-γ and/or TNF-α to the culture medium (37–42). Several reports indicate that astrocytes are capable of acting as antigen-presenting cells for in vitro antigen- or allospecific $CD4^+$ and $CD8^+$ T cell responses (43, 44), which suggests that they also have potential to participate in immune responses occurring within the confines of the CNS. Since clearance of infectious JHMV from the CNS requires class I–restricted $CD8^+$ T cells (11, 45–47), it is of particular interest to characterize the effect of MHV infection on class I expression in one of its principal cellular targets.

In this communication, we have examined the ability of a panel of MHV strains to induce class I expression in cultures enriched at least 95% for astrocytes. The data indicate that class I expression occurred in response to all the MHV strains tested except JHMV and virus strains derived from JHMV. Additional studies show that class I expression requires the presence of infectious virus. Finally, the testing of A59/JHMV recombinant viral strains suggests that the class I–inducing activity resides in the 3′ end of the A59 genome, possibly in one of the genes encoding the structural proteins.

Materials and Methods

Primary Astrocyte Cultures. Astrocytes were isolated from mixed glial cell cultures prepared from the brains of newborn C57Bl/6 mice (Bantin and Kingman, Fremont, CA) at postnatal day 0–3 according to McCarthy and deVellis (48). Briefly, single cell suspensions were prepared from cerebri dissected free of brain stems and cerebelli, plated at 3–5 brains per T-75 flask and allowed to grow to confluence at 12–15 d in vitro. Culture medium consisted of DMEM/Ham's F12 (1:1; JRH Biosciences, Lenexa, KS) supplemented with 10% FCS (Gemini Bioproducts, Inc., Calabasas, CA), 15 mM Hepes, 2.5 mM L-glutamine, and penicillin/streptomycin (100 U/ml-100 μg/ml). At confluence the cultures were mechanically shaken to dislodge microglia and oligodendroglia, resulting in preparations enriched 95% or greater for cells staining for glial fibrillary acidic protein (GFAP). Immunoperoxidase or immunofluorescent staining (described below) revealed that the cell preparations contained ~2–4% of cells of microglia/macrophage lineage, expressing F4/80, T-200, the mouse equivalent of human common leukocyte antigen (CD45), and/or Mac-1 surface markers.

Coronavirus Strains and Infection. Table 1 presents a summary of the MHV strains used and the principal types of diseases they cause in mice. The derivation, propagation, and sources of MHV-A-59, JHM-DL, JHM-DS, MHV-1, MHV-2, MHV-3, MHV-D, MHV-K, and MHV-Nuu have been described (12). The neutralization-resistant MHV-4 strains 2.2-V-1 and 2.2/7.2-V-2 (28, 29) were the kind gift of Dr. John Fleming (University of Wisconsin, Madison, WI). The development and properties of JHM-X and the JHM/A59 recombinant MHV strains have been reviewed (49). All virus strains were propagated using the murine astrocytoma DBT as previously described (6). Virus titers were determined for each virus preparation using DBT as indicator cells.

Infectious Center Assays. The number of infected astrocytes was determined on day 3 p.i. to provide a measure of the relative efficiency of infection by several MHV strains. Briefly, astrocytes were trypsinized into single cell suspensions and, after washing, were plated on DBT cell monolayers at 0.1, 1, 10, 100, and 1,000 cells/60 mm petri dish. Cells were allowed to attach for 1 h at 37°C before the addition of agarose at 0.6% in RPMI 1640 supplemented with 2% heat-inactivated FCS, 20 mM Hepes, and penicillin/streptomycin. Plaques were counted after 48 h incubation at 37°C.

Virus Inactivation by Exposure to Ultraviolet Light. Inactivation of virus was accomplished by exposure to UV light under a UVP transilluminator (UVP Inc.,; San Gabriel, CA) at 7 mW/cm^2 for

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Table 1. *MHV Strains and Their Characteristics*

Virus strain	Characteristics
JHM-DL (MHV-4)	Lethal encephalitis, demyelination
JHM-DS (MHV-4)	Demyelination, encephalitis
JHM-X (MHV-4)	Lethal encephalitis, demyelination
2.2-V-1 (MHV-4)	Nonlethal demyelination
2.2/7.2-V-2 (MHV-4)	Nonpathogenic CNS infection
MHV-A59	Lethal encephalitis, demyelination, hepatitis
MHV-NuU	Nonpathogenic enteric infection
MHV-K	Nonlethal myeloproliferative disease, hepatitis
MHV-D	Nonlethal hepatitis, enteritis
MHV-1	Nonlethal mild hepatitis
MHV-2	Lethal hepatitis
MHV-3	Lethal hepatitis, choroidoependymitis

30 min. Virus inactivation was confirmed as lack of virus infectivity in plaque assays using DBT cells.

Antibodies. To identify class I molecules in primary astrocyte cultures, mAbs specific for K^b (AF6.88.5.3), D^bL^d (28-14-8S), K^d (SF1-1.1.1), and D^d (34-5-8S) were obtained from the American Type Culture Collection (ATCC HB 158, HB 27, HB 159, and HB 102, respectively; Rockville, MD) and used as tissue culture supernatants. Optimal antibody concentrations were determined by RIA or FACS® (Becton Dickinson & Co., Mountain View, CA) analyses in preliminary experiments using either astrocytes or lymphocytes from the appropriate haplotype. The percentage of GFAP-positive astrocytes was determined in the enriched cultures using polyclonal anti-GFAP antibody (rabbit antibovine GFAP; Dakopatts, Glostrup, Denmark). Cells of macrophage/monocyte lineage were identified using a mixture of mAb specific for Mac-1 (hybridoma M1/70.15, ATCC TIB 128), F4/80 (ATCC HB 198), and T-200, which recognizes all cells of bone marrow origin (hybridoma M1/9.3.4HL.2, ATCC TIB 122). The possible presence of oligodendrocytes was identified using polyclonal rabbit antigalactocerebroside (Gal C, a gift from M. Smith, Stanford University, Stanford, CA). Finally, viral antigen was identified using J.3.3, a mAb specific for the N protein of JHMV that crossreacts with all of the MHV strains used in this study (12).

RIA. The expression of class I molecules was measured in astrocytes cultured in flat-bottomed 96-well plates at a density of 10^4 cells/well and infected with various virus strains at a multiplicity of infection (m.o.i.) of 1–2. Mock infected cells served as controls. On day 3 or 5 p.i., cells were washed twice using a wash buffer of 0.3% BSA in 0.1 M PBS before the addition of 50 μl of the appropriate mAbs in triplicate. After a 60 min incubation at room temperature, cells were washed three times, followed by the addition of 20,000 cpm/well of ^{125}I-labeled protein A (30 μCi/μg; ICN Biomedicals, Costa Mesa, CA). At the end of a second 60-min incubation, cells were extensively washed in PBS to remove unbound radiolabeled protein A and detached with 0.5% trypsin-0.2% EDTA (JRH Biosciences). Data are presented as cpm bound radioactivity ± SD, or percent increase in class I expression in infected cells compared with that in uninfected controls, corrected for background binding in the absence of antibody. A result was considered positive when the percent increase in expression was 100% or greater. Nonspecific staining was identified by the inclusion of K^d-specific mAb SF1-1.1.1. In some experiments, supernatants from infected cells were substituted for virus and RIA performed to detect class I 3 d later.

Immunoperoxidase and Immunofluorescent Staining. Cell phenotypes and the number of infected cells were determined by immunoperoxidase staining in astrocytes cultured in tissue culture chamber slides. Avidin-biotin immunoperoxidase staining kits (Vectastain; Vector Laboratories Inc., Burlingame, CA) were used according to the manufacturer's instructions as previously described (21). Cells were infected with virus 1 d after plating and fixed on day 3 or 5 p.i. in acetone/methanol (1:1). The number of positively stained cells in each well was determined in three fields/well at a magnification of 20× and reported as the percentage of the number of total cells in the same fields. Background staining was determined in cells stained in the absence of primary antibody.

Class I expression was also evaluated by FACS® using an indirect immunofluorescent staining procedure. Briefly, single cell suspensions of astrocytes were adjusted to 10^5 to 10^6 cells/tube before the addition of class I–specific primary antibodies. FITC-labeled goat anti–mouse IgG $F(ab)_2$ was used as secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescence data were collected on 3×10^3 to 10^4 viable cells, indicated by forward light scatter intensity, using a FACStar cell sorter (Becton Dickinson & Co.). Again, background fluorescence was determined in cells stained in the absence of primary antibody.

Detection of Interferons. The possible presence of IFN in the supernatants of infected astrocytes was determined by bioassay in a vesicular stomatitis virus (VSV) neutralization assay using vital dye uptake as a spectrophotometrically measured endpoint in L929 cells (50, 51). Supernatants were collected from infected and control uninfected astrocytes on day 3 and 7 p.i., UV irradiated, and added in triplicate to L929 cells plated at 10^5 cells/well in 96-well plates. After a 24-h incubation at 37°C and 7% CO_2, supernatants were aspirated and cells infected with VSV (Indiana strain) at approximately 10 tissue culture infectious doses ($TCID_{50}$)/well. Control wells included uninfected L929 cells (cell control) and VSV-infected L929 cells cultured in the absence of interferon (virus control). At 24–36 h p.i., when CPE was maximum in the control wells, cells were stained for 2 h at 37°C with 0.004% neutral red. They were then lysed to release retained dye using 0.01 M HCl in 30% ethanol, and ODs measured at 540 nm in an automated microELISA reader (Dynatech Laboratories, Inc., Chantilly, VA). IFN concentrations were deduced by comparison of sample ODs with those of standard concentrations of recombinant mouse IFN-γ (Genzyme Corp., Cambridge, MA) and murine IFN-α/β (Lee Biomolecular, San Diego, CA).

Immunoneutralization was also used to identify the possible presence of IFN in UV-irradiated supernatants of A59-infected astrocytes. For this purpose, rat monoclonal anti–mouse IFN-γ antibody (hybridoma R4-6A2; ATCC HB 170) or rabbit polyclonal anti–mouse IFN-α/β (Lee Biomolecular) was added to the astrocytes before the addition of A59, and class I expression measured by RIA on day 3 p.i. Anti-IFN-γ (20–60 μg/ml) completely inhibited the protective activity of 10 U/ml of recombinant mouse IFN-γ, whereas 10–30 U/ml anti-IFN-α/β was effective in neutralizing 20 U/ml purified mouse IFN-α/β.

Detection of TNF. TNF activity was measured in a cytotoxicity assay using actinomycin D–treated L929 fibroblasts as targets, again with vital dye uptake as a spectrophotometric endpoint. Serial 2-fold dilutions of UV-irradiated supernatants, or 10-fold dilutions

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of murine recombinant TNF-α (Genzyme Corp.), as a standard were added to L929 monolayers in 96-well plates in the presence of actinomycin D (8 μg/ml; Sigma Chemical Co., St. Louis, MO). After an 18-h incubation at 37°C, 7% CO_2, neutral red was added as described for the IFN assay, and OD read at 540 nm.

Results

MHV-A59, but Not JHM-DL, induces MHC Class I Expression in Primary Cultures of Murine Astrocytes. To investigate the effects of coronavirus infection on MHC class I expression in primary astrocytes, cultures were routinely used at 20–30 d in vitro, or 6–12 d after mechanical shaking to remove oligodendrocytes and microglia. Fig. 1 shows that A59 induced abundant class I expression in astrocytes, measured by RIA using the K^b-specific mAb AF6.88.5.3. Class I expression was routinely measured on days 3 and 5 p.i. after infection at an m.o.i. of 1, though it was detectable within 24–48 h p.i. K^b was preferentially expressed over D^b after A59 infection. This was not due to a lack of inducibility of the D^b molecule, since both K^b and D^b were expressed spontaneously with time in culture, and also in response to 100 U/ml of IFN-γ (data not shown). Under these conditions, K^b was expressed earlier than D^b, and usually at significantly higher levels. In infected astrocytes, the class I–inducing activity of A59 was consistently 30–50% more potent than the single dose of 100 U/ml of IFN-γ (Fig. 2). Finally, FACS® analyses revealed that K^b was expressed on ~50% of the astrocytes in the infected cultures.

By contrast, the large plaque morphology variant of JHMV designated JHM-DL did not stimulate class I at all, or at best, stimulated minimal expression at 10–20% over that observed in uninfected cells (Fig. 1 and 2). These data indicate that the ability to induce class I expression is not a general property of MHV strains.

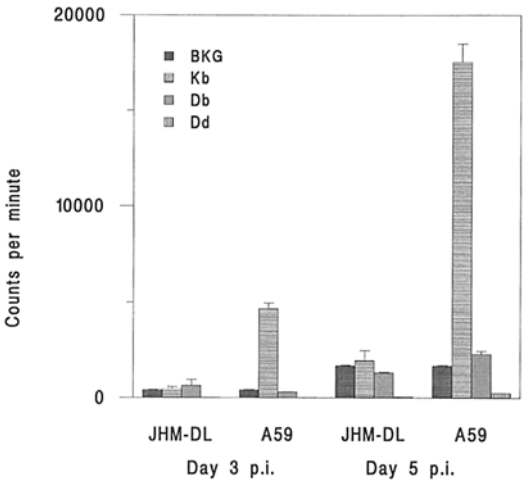

Figure 1. MHC class I is induced in primary murine astrocytes by A59, but not JHM-DL. Class I expression was measured on days 3 and 5 p.i. using mAb specific for K^b, D^b, and D^d as described in Materials and Methods. Data are represented as mean cpm ± SD of triplicate determinations.

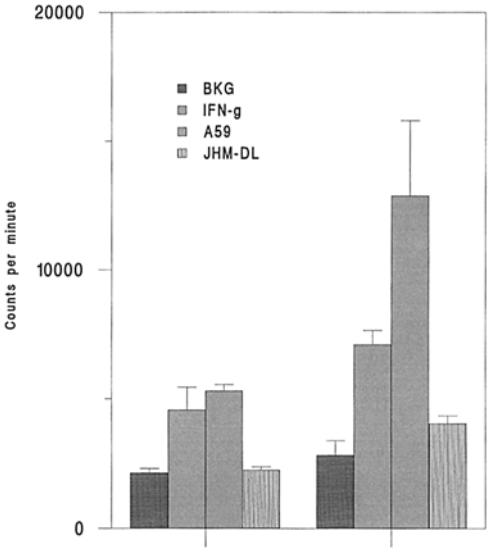

Figure 2. MHC class I (K^b) induction in primary astrocytes by A59 relative to that of IFN-γ. Recombinant IFN-γ was added to astrocytes for 48 h at 100 U/ml before RIA at days 3 and 5 p.i. Data represent mean cpm ± SD of triplicate determinations.

Virus Strains Related to or Derived from JHM-DL Do Not Stimulate Class I Expression. To determine whether the lack of ability to stimulate class I expression in astrocytes is specific for JHMV, a panel of virus strains derived from wild-type JHMV or from JHM-DL were tested. The results indicate that none of the JHMV strains isolated in our laboratories stimulated class I (Table 2). In addition, JHM-X, which was derived from JHMV passage in Japan and has been shown to have extensive deletions in the S gene (11), did not affect class I expression. By contrast, all of the viral strains in a panel of more hepatotropic isolates of MHV, including MHV-NuU, MHV-K, MHV-D, MHV-1, MHV-2, and MHV-3, were effective stimulators of class I expression. The magnitude of stimulation, expressed as percent change relative to uninfected cells, varied with the individual viral strain 2–10-fold over that in uninfected cells. A59 and MHV-2 were the most potent inducers of the class I K^b molecule, and both inconsistently induced a low-level expression of D^b. It should be mentioned that the K^d-specific mAb SF1-1.1.1 occasionally yielded a low level of nonspecific binding; however, it was significantly lower than the specific binding observed with the K^b-specific antibody.

Studies with Recombinant A59/JHMV Strains Map the Genes Responsible for Class I Expression to the 3′ End of the Genome. To determine whether a specific A59 gene or genes are responsible for the class I–inducing activity, a panel of recombinant viruses between A59 and JHMV that retain various portions of A59 sequences were tested. The results, represented in Fig. 3 and summarized in Fig. 4, indicate that all strains that retain A59 sequences at the 3′ end of the recombinant genome were potent class I inducers. Thus, CA13 and CA43, which retain ~30–40% JHMV sequences at the 3′ end, were completely devoid of class I–inducing activity. This end of the MHV genome contains the genes encoding the structural

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Table 2. *MHV Strains Differ in Their Ability to Induce MHC Class I Expression in Murine Astrocytes**

Experiment	Virus strain	K^b	D^b	D^d/K^d
1	JHM-DL	10	9	0/–‡
	JHM-DS	7	10	0/–
	JHM-X	0	25	0/–
	2.2-V-1	0	0	0/–
	2.2/7.2-V-2	0	0	0/–
	A59	322	0	0/–
2	JHM-DL	7	29	0/–
	A59	958	110	0/–
	A59H	1,042	0	0/–
	MHV-2	822	0	0/–
3	A59	359	–	–/0
	MHV-NuU	331	–	–/65
	MHV-K	281	–	–/13
	MHV-D	257	–	–/93
	MHV-1	258	–	–/0
	MHV-3	369	–	–/40
4	JHM-DL	17	11	0/–
	A59H	786	8	0/–
	MHV-2	955	94	0/–

* MHC Class I expression was measured by RIA on day 3 p.i. (m.o.i. = 1) using mAbs AF6.88.5.3 (K^b), 28-14-8S (D^b), 34-5-8S (D^d), and SF1-1.1.1 (K^d). Values are expressed as percent change relative to uninfected cells stained with the indicated mAb. Positive results were defined as an increase in class I expression greater than or equal to 100% over that observed in uninfected cells. Descriptions of assay procedures are included in Materials and Methods.

‡ (–) Not tested.

proteins S, M, and N in addition to two nonstructural proteins, which suggests that the class I–inducing activity resides in one of these proteins. Often, though not exclusively, the most potent inducers of class I were strains that retain the highest percentage of A59 sequences at the 3′ end, while those retaining the least A59 character, or exhibiting more than one crossover site for recombination, were significantly less potent. In addition, it was observed in three out of four experiments that RL1 was ~50% less potent than EL3, as illustrated in Fig. 3 *B*. EL3 retains slightly more A59 sequences in the S gene than RL1. This observation suggests that class I–inducing activity may involve S. However, it was not possible to attribute the class I–stimulating activity to an individual gene or gene product, since none of the recombinants had crossover sites that sufficiently isolated individual A59 from JHMV genes. However, it was clear that if JHMV sequences were retained at the 3′ end, class I–inducing activity was abolished, and that 5′ retention of A59 sequences did not salvage it.

The Ability of MHV to Induce Class I Is Not Dependent on Replication Efficiency. In our laboratory, A59 grows to higher titer in vitro than the JHMV strains in DBT cells, often exceeding JHMV growth 10–20-fold. In addition, recombinant viruses containing A59 leader predominate over those containing JHMV leader, suggesting that A59 leader provides a growth advantage (52). Thus, it was important to determine whether the lack of class I inducing activity by JHMV was due to poor replication efficiency in primary astrocytes. For this purpose, the number of astrocytes staining for viral antigen was determined by immunoperoxidase staining, and the yield of infectious viral particles/cell was measured in infectious center assays. The data, presented in Table 3, indicate that on day 3 p.i., 53% of the JHM-DL–infected astrocytes were positive for viral antigen, compared with 45% of cells infected with A59. In addition, cells infected with the recombinant virus CA13, which retains 3′ JHMV and 5′ A59 sequences and does not stimulate class I expression, showed 48% antigen positive staining. Cells infected with CA13 produced the highest level of infectious virus at 0.51 plaques/cell, in spite of its inability to stimulate class I expression (Table 3). By contrast, EL3, which was an effective class I inducer, replicated at relatively low efficiency at 0.08 plaques/cell. Thus, class I–inducing activity was not a function of the ability of the virus to infect or replicate in pri-

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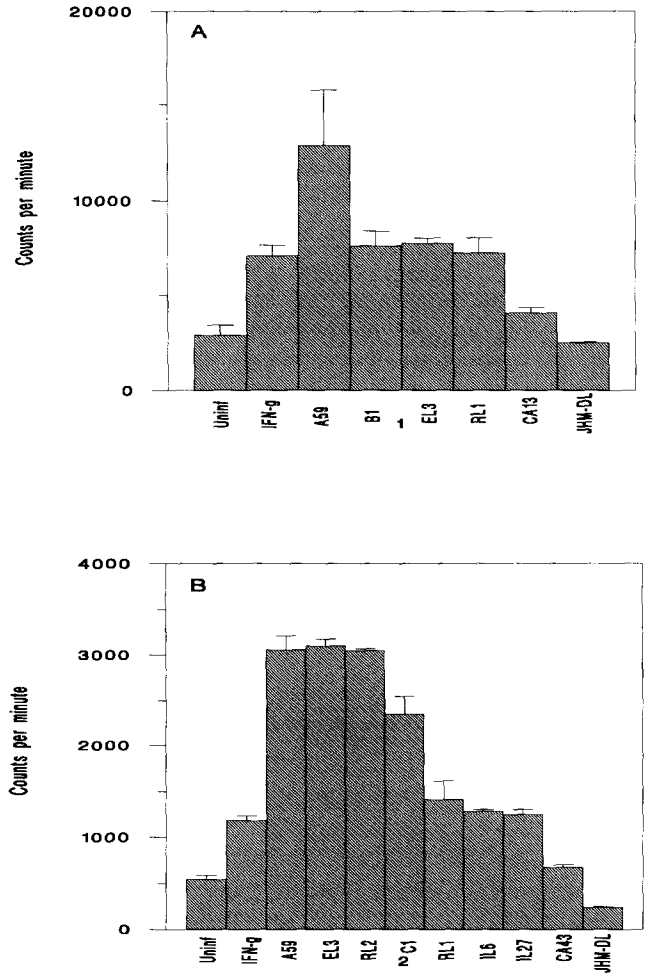

Figure 3. MHC class I (K^b) induction in primary astrocytes infected by A59 and recombinant A59/JHMV coronavirus strains. Data represent mean ± SD of triplicate determinations. Data are also included for uninfected astrocytes exposed to IFN-γ (100 U/ml) for 48 h before assay on day 3 p.i. *A* and *B* are two experiments that are representative of four experiments yielding similar results.

Table 3. *Efficiency of Infection of Coronavirus Strains at 3 d p.i.*

Virus strain	Percent cells infected*	No. plaques/cell‡
JHM-DL	53	0.20
A59	45	0.19
B1	40	0.18
EL3	41	0.08
RL1	42	0.14
CA13	48	0.51

* Determined by immunoperoxidase staining using an antibody specific for the nucleocapsid protein of JHMV.
‡ Measured in infectious center assays as described in Materials and Methods.

mary astrocytes, nor did it appear to depend on the presence of A59 leader.

Class I-inducing Activity Is a Direct Consequence of A59 Infection. It has been reported that class I induction in A59-infected glial cell cultures is not a direct consequence of infection, but is instead due to the release of a soluble factor into the medium that requires continual virus production (33, 53, 54). The presence of a non-IFN-like soluble factor was demonstrated in supernatants from A59-infected glial cells that had been exposed to UV light to inactivate the virus. Similarly, our data show that UV-inactivated A59 and A59-like recombinant coronavirus strains are not able to induce class I activity in purified astrocytes (Table 4). Virus inactivation in the UV-treated virus preparations was confirmed by plaque assay using DBT cells (data not shown). However, class I–inducing activity was not detected in the supernatants collected on days 3 and 7 p.i. from A59-infected astrocytes and exposed to UV light to inactivate the virus (Fig. 5), while supernatants that were not exposed to UV light and thus, contained infectious virus, were able to induce class I. Induction was not inhibited by the addition of antibodies specific for IFN-γ or IFN-α/β to the astrocytes before A59 infection (Table 5). Finally, there was no evidence of the presence of TNF-α in UV-treated supernatants of A59-infected astrocytes (data not shown). These data suggest that class I induction is a direct consequence of A59 infection itself, and does not occur indirectly as a result of the release of a soluble class I inducer into the astrocyte medium.

Discussion

In this communication, we report that the ability of A59 to stimulate MHC class I expression during acute infection in primary murine astrocytes, previously observed by Suzumura et al. (33, 53, 54), is not an inherent property of MHV strains. It is interesting to note that the strains that did not stimulate class I are derivatives of the highly neurotropic MHV-4, or JHMV strain, whereas most of the MHV strains that did are the more hepatotropic strains. However, class I–inducing

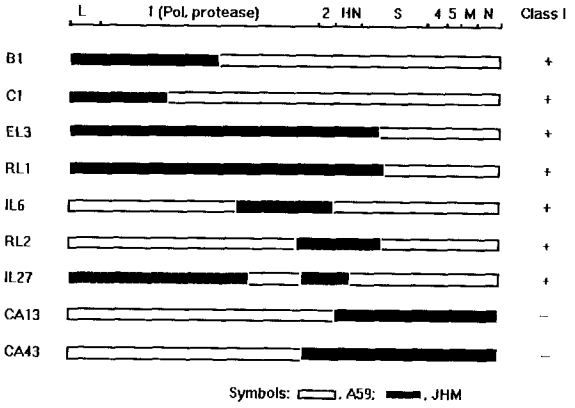

Figure 4. Genetic structure of the recombinant A59/JHMV strains used. Class I–inducing ability is indicated for each strain.

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Table 4. *Induction of MHC Class I by A59 and A59-like Recombinant Coronavirus Stains Requires Infectious Virus**

Experiment	Virus strain	K^b	D^b	D^d
1	A59	301	-	0
	A59 UV‡	0	-	7
2	A59	363	0	0
	A59 UV	8	2	0
3	A59	4,091	0	0
	B1	2,544	0	0
	EL3	2,007	0	0
	RL1	1,610	0	0
	B1 UV	0	0	0
	EL3 UV	0	0	0
	RL1 UV	0	0	0

* MHC class I was measured by RIA on day 3 p.i. (m.o.i. = 1-2) using mAbs as defined in the legend for Fig. 1. Values are expressed as percent change relative to uninfected cells stained with the appropriate mAb.
‡ UV refers to virus inactivated by exposure to ultraviolet light as described in Materials and Methods.

ability could not be exclusively attributed to selective tissue tropism, since A59 and MHV-3, which readily establish both CNS and hepatic infections, were potent inducers of class I expression in astrocytes. Lack of class I–inducing activity in JHMV was also not due to poor replication efficiency in astrocytes, since JHMV infected the same percentage of cells and produced similar levels of infectious virus as the class I–inducing MHV strains (Table 3). Thus, some other MHV characteristic must be responsible for class I–inducing activity, or the lack thereof.

Since the expression of class I genes is regulated by interferons (55), it seemed possible that class I induction might

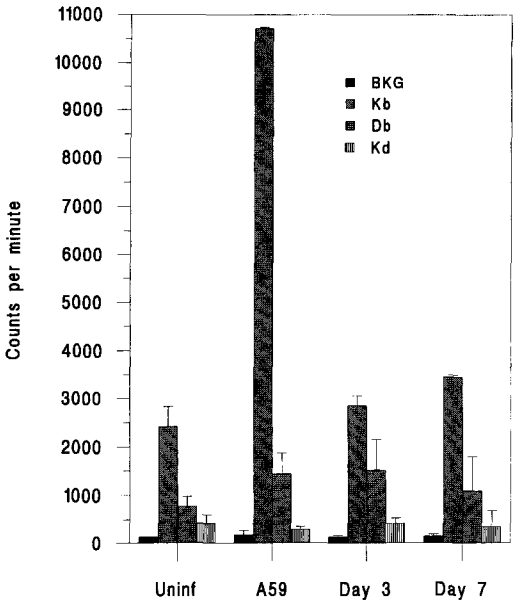

Figure 5. Relative to uninfected control cultures, MHC class I is not induced by UV-treated supernatants collected on days 3 and 7 p.i. from MHV-A59 infected astrocytes. Supernatants were added to fresh astrocyte cultures and incubated for 3 d before RIA. Controls included supernatants from MHV-A59–infected cells that had not been UV treated. Nonspecific class I expression was measured using a K^d-specific mAb. Data are expressed as mean cpm of triplicate determinations, with error bars indicating SDs.

Table 5. *Class I Induction by MHV-A59 Is Not Inhibited by Antibodies to IFNs**

Experiment	Virus strain	Antibody	K^b	D^d
1	JHM-DL	-	-22	0
	MHV-A59	-	280	0
	MHV-A59	IFN-γ‡	230	0
	MHV-A59	IFN-α/β§	335	0
2	JHM-DL	-	-35	30
	MHV-A59	-	338	31
	MHV-A59	IFN-γ‖	282	0
	MHV-A59	IFN-α/β	338	4

* Antibodies to IFNs were added before the addition of virus, and class I expression measured by RIA on day 3 p.i. Values are expressed as percent change relative to uninfected cells stained with the appropriate mAb.
‡ Anti–mouse IFN-γ added at 60 μg/ml.
§ Anti–mouse IFN-α/β added at 10 U/ml.
‖ Anti–mouse IFN-γ added at 20 μg/ml.

Published September 1, 1994

correlate with the ability of MHV strains to stimulate IFN production. This possibility gains credibility from reports that JHMV is a notoriously poor inducer of interferon after in vitro (56) and in vivo (57) infection. However, Suzumura et al. (53) did not detect IFNs in UV-treated supernatants from A59-infected mixed glial cells in spite of the presence of class I–inducing activity, and our findings indicate that A59-induced class I expression was not inhibited by antibodies to IFN-α/β or -γ (Table 4). Unlike Suzumura et al. (53), we did not find that class I–inducing activity was retained after UV-inactivation of A59 or the A59-like recombinant MHV strains (Table 4), and it was not detected in supernatants from A59-infected astrocytes that were UV-treated to inactivate infectious virus (Table 5 and Fig. 5). In addition, we have recently found that class I expression is no longer observed when A59 replication is controlled by the culture of persistently infected astrocytes in the presence of MHV-specific polyclonal antiserum, in spite of the persistence of viral protein and RNA (58). Overall, our data suggest that class I induction by MHV in astrocytes requires infectious virus and does not involve the secretion of a soluble factor. In this respect, it is interesting to note that the class I–inducing activity originally reported by Suzumura et al. (33, 53) was also dependent on continuous virus production in glial cell cultures prepared from the brains of infected mice (54). However, it is possible that our lack of detection of class I–inducing activity in the supernatants could reflect culture, or other technical conditions.

Although it was not possible to attribute class I–inducing activity to a single gene or gene product using the A59/JHMV recombinant virus strains, the data clearly indicate that class I–inducing activity maps to the 3′ end of the A59 genome, which contains the genes encoding the primary structural proteins S, M, and N, and two poorly understood nonstructural proteins. However, there are some indications that S is the most likely candidate. For example, recombinant strains which retain more A59 sequences in the S gene tended to be more potent than those retaining less A59, or more JHMV character. In addition, the S gene shows the greatest antigenic divergence of the structural proteins between A59 and JHMV (12–16) and so might be more likely to exhibit differences in activity. This possibility is supported by data indicating that A59 and JHMV differ considerably in their ability to cause receptor-independent fusion and syncytium formation (59). In addition, preliminary data suggest that A59 and JHMV may show selective binding to the cellular receptors for MHV (60), which are members of the carcinoembryonic antigen (CEA) family of proteins (61).

The mechanism by which A59, MHV-3, and the A59-like recombinant MHV strains induce class I expression in astrocytes is currently unknown. Massa et al. (62, 63) have shown that MHC class I promoter activity is upregulated in astrocytes treated with IFN-γ and that the upregulation is associated with an increase in the binding activities of the MHC class I regulatory element (MHC-CRE) and the IFN consensus sequence (ICS). Whether or not these regulatory elements are engaged by A59 infection remains to be determined.

Perhaps one of the more interesting findings in these studies is the prevalence of K^b expression over that of D^b after MHV infection, and to a lesser extent, IFN-γ treatment. Differential modulation of H-2K and H-2D molecules has been reported to follow in vitro JHMV infection in mouse brain endothelial cells, and the data indicate that the nature of the regulation was dependent on the mouse strain and did not necessarily correlate with susceptibility to JHMV-induced CNS disease (64). Thus, K was decreased and D increased in endothelial cells from susceptible BALB/c mice, but both were upregulated in susceptible B10.S and (BALB/c × SJL)F1 and resistant SJL endothelial cells. Differential expression of K and D molecules has not always been observed after virus infection of neural cells; both K and D molecules appear to be equally enhanced by West Nile virus infection in astrocytes from CBA/H mice (65), and the murine neuroblastoma C1300 does not show differential K and D expression when persistently infected with measles virus (66). However, the possibility that differential modulation of class I molecules may be linked to CNS disease, or may be a marker of susceptibility to virus-induced CNS disease, is suggested by studies using Theiler's murine encephalomyelitis virus (TMEV; 67, 68). After i.c. TMEV inoculation, resistant B10 mice showed minimal class I expression in the CNS that did not differ between K and D, whereas susceptible B10.Q and B10.RBQ mice showed a greater increase in D expression compared with that of K (67).

The inability of JHMV to stimulate class I expression in C57BL/6 astrocytes in the current studies was not reported for astrocytes from BALB/c, CXJ-8, SJL, and B10.S mice by Joseph et al. (69). In these experiments, JHMV infection at an m.o.i. of 0.1 was followed by a two- to threefold increase in the expression of H-2K molecules, measured by FACS® analyses, but astrocytes from C57BL/6 mice were not tested. The disparity in our findings may again reflect differences in the regulation of class I expression that are mouse strain specific. However, it is also possible that the disparity may be due to the cellular composition of the astrocyte cultures used for infection. In their report, Joseph et al. (69), did not indicate whether or not they purified the astrocytes from mixed glial cell cultures. In our hands, mixed glial cells that have not been subjected to mechanical shaking for purification of astrocytes show up to 30% contamination by microglia, or cells staining for macrophage/monocyte surface antigens (data not shown). Microglial cells readily express class I molecules both in vivo and in vitro (70–72), and so might be expected to express class I after JHMV infection. The cultures used in our experiments routinely show 95% or greater purity, containing 2–4% microglial cells, and ~50% express class I after A59 infection. Thus, class I expression in these cultures occurs primarily in astrocytes, with little, if any contribution by microglia. Finally, it should also be mentioned that class I expression in astrocytes, like that in endothelial cells, does not appear to be linked to susceptibility to JHMV-induced

CNS disease, since both BALB/c and C57BL/6 develop encephalitis and demyelination in spite of their differences in class I inducibility after JHMV infection. In addition, astrocytes from SJL mice express class I after JHMV infection in spite of being resistant to CNS disease.

The different effect of A59 and JHMV on class I expression in astrocytes suggests that class I may play different roles in their pathogenesis. As discussed by Maudsley et al. (73), the classical picture of MHC expression after virus infection is that of an increase that is most likely mediated by the release of IFNs by infiltrating immune cells, and is thought to facilitate the ability of T cells to recognize the infected cells and control or eliminate the infection. Unfortunately, clearance of virus from infected tissue by CTLs is often accompanied by significant cellular destruction, which is not well tolerated in tissues with limited regenerative capacity, especially the CNS. Thus, the ability of a virus to stimulate class I expression in host cells may facilitate a rapid CTL response that in turn accelerates tissue destruction originally begun by virus-mediated cell lysis (74). This may be the case for A59 infection, since the A59 strains used in these experiments cause a severe encephalitis after i.c. inoculation that begins on day 4 p.i., and results in death of the majority of infected mice by day 6–7 p.i. (our unpublished data). However, it is not known if the infiltration of T cells into the CNS after i.c. A59 infection is associated with the onset of encephalitis. By contrast, JHMV causes a similar clinical disease, but the onset of encephalitis is delayed in comparison with that of A59, beginning on day 6–7 p.i. (21). Death occurs on day 9–11 at a similar incidence. The onset of encephalitis coincides with the appearance of immune cells in the CNS, which, after JHM-DS infection, peaks on days 7–9 p.i. (75). It is possible that class I expression is upregulated at this time by the secretion of interferon by the infiltrating immune cells, facilitating JHMV-specific, CTL-mediated tissue destruction. Thus, the difference in the ability of JHMV and A59 to stimulate class I expression in astrocytes may contribute to the speed of disease progression in the CNS. It is interesting to note that Fazakerley et al. (25) reported that the JHMV variant, V5A13.1, is neuroattenuated relative to parental JHMV by its slower rate of spread in the CNS of BALB/c mice. Variant V5A13.1 differs from parental JHMV by the deletion of 142 amino acids in one of the subunits of the S protein (16, 76), suggesting that S plays an important role in the rate of virus spread in the CNS. In this respect, it would be of interest to test the ability of neuroattenuated A59 strains to stimulate class I expression in astrocytes.

In conclusion, we present data indicating that significant differences exist among MHV strains in their ability to stimulate class I expression in murine astrocytes, and that these differences may contribute to their ability to cause CNS disease. Since class I expression has been reported on astrocytes in the brains of MS patients (77), the data have implications for understanding the role of class I in human CNS disease.

We wish to thank Michael Lai for the recombinant virus strains and Steve Stohlman, Michael Lai, and Minnie McMillan for helpful critiques of the data and manuscript. We are also grateful for help with the manuscript provided by Sonia Garcia.

This work was supported by National Institutes of Health grant NS-18146-12.

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Received for publication 30 March 1994 and in revised form 7 June 1994.

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