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Effect of Persistent Mouse Hepatitis Virus Infection on MHC Class I Expression in Murine Astrocytes

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Neurotropic strains of mouse hepatitis virus (MHV) have been used extensively for the study of viral pathogenesis in the central nervous system (CNS), serving as models for human neurological diseases such as multiple sclerosis (MS). MHV strains A59 and JHMV both cause acute and chronic encephalomyelitis and demyelination in susceptible strains of mice and rats. In acute disease, CNS damage is most likely the result of lytic infection in neurons and oligodendrocytes, and death can be prevented by the adoptive transfer of Class I-restricted CD8+ T cells. However, in later stages of the disease induced by some MHV strains, virus tends to be restricted to astrocytes in a nonlytic infection, and the immune response appears to contribute to CNS damage. These data lead us to suggest that the astrocyte may play a central role in the neuropathogenesis of MHV infection. Consistent with this possibility, A59 has been reported to induce the expression of Class I molecules of the major histocompatibility complex (MHC) in glial cells following infection in vivo and in vitro. In this communication, we have examined the influence of persistent infection by both A59 and JHMV on MHC Class I expression in primary murine astrocytes. Persistence was characterized by the presence of intracellular viral antigen and mRNA in the absence of detectable infectious virus particles. Under these conditions, JHMV, but not A59, inhibited constitutive expression of the H-2 K^b molecule, with the magnitude of inhibition increasing with postinfection time. A59 was not able to induce Class I during persistence, presumably due to the lack of infectious virus particles. Class I expression was restored by the addition of gamma-interferon (IFN-γ) to astrocytes persistently infected with either A59 or JHMV. Thus, Class I inhibition is not a permanent consequence of JHMV persistence, and persistence does not interfere with normal signalling pathways for Class I induction.

Key words: astroglia, neurotropic virus, coronavirus, Class I major histocompatibility antigens, persistent infection

INTRODUCTION

The JHM strain of mouse hepatitis virus (MHV-JHM or JHMV) is a highly neurotropic member of the Coronaviridae, which are enveloped RNA viruses with a positive-sensed RNA genome. JHMV and another neurotropic MHV strain, A59, cause acute encephalitis and demyelination in susceptible strains of mice and rats upon intracerebral (i.c.) inoculation (Bailey et al., 1949; Weiner, 1973; Robb et al., 1979; Hirano et al., 1980; Sorensen et al., 1980; Stohlman and Weiner, 1981; Wege et al., 1984). Both viruses have been used as experimental models for the study of virus-induced demyelination, and have also served as models for the study of the autoimmune human demyelinating disease, multiple sclerosis (MS).

During the acute phase of both JHMV and A59 infection, infectious virus can be readily isolated from the central nervous system (CNS) and virus particles or antigen can be detected in a variety of CNS cell types, including neurons, glia, ependymal and endothelial cells (Fleury et al., 1980; Knobler et al., 1981; Fishman et al., 1985; Wang et al., 1992). More selective cellular tropism appears to occur when infection is accomplished at low multiplicity of infection (moi), when mutant viruses with reduced pathogenicity are used for infection, or during the chronic infection that occurs in animals that survive acute encephalitis (Lai and Stohlman, 1992). Neurons are less likely than oligodendrocytes to be targets under these conditions, and several reports have indicated that viral antigen most likely persists in astrocytes

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following in vivo infection (Weiner, 1973; Haspel et al., 1978; Dubois-Dalcq et al., 1982; Perlman and Ries, 1987). In addition, temperature-sensitive (ts) mutants of A59, which show reduced pathogenicity, also show restricted replication in astrocytes compared with that of wild type A59 (van Berlo et al., 1986). In vitro studies support these observations, since mixed glial cell cultures and cultures enriched for astrocytes support a persistent, productive infection following both JHMV and A59 inoculation (Massa et al., 1986; Lavi et al., 1987; van Berlo et al., 1989). By contrast, oligodendrocytes rarely survive in vitro infection. Thus, the astrocyte appears to be a primary target in persistent infection and may participate in the pathogenesis of MHV-induced neurological disease.

It has been well documented that the pathogenesis of MHV infection is influenced considerably by the host immune response (for review, see Lai and Stohlman, 1992). During acute infection, immunosuppression inhibits the clearance of virus from the CNS and changes a nonlethal infection to an acute fatal encephalomyelitis. In addition, the administration of monoclonal antibodies (mAb) specific for any of the structural proteins of JHMV are capable of protecting mice from lethal encephalitis, but the protection from death does not protect mice from demyelinating disease, nor does the treatment reduce virus replication in the CNS (Buchmeier et al., 1984; Nakanaga et al., 1986; Le Conte et al., 1987; Fleming et al., 1989). Reduced virus titers accompany protection from death only following passive transfer of MHC Class I restricted, virus-specific $CD8^+$ T cells, in the presence of $CD4^+$ T cells (Sussman et al., 1989; Williamson and Stohlman, 1990; Yamaguchi et al., 1991). Thus, T cells play important roles in virus clearance and pathogenesis, and involve a role for both Class I and Class II MHC antigens in the outcome of virus infection.

In mice, the H-2 complex encodes the highly polymorphic K, D and L molecules, which are noncovalently associated with β2 microglobulin, or β2m (Hood et al., 1983; Klein, 1986). Class I molecules bind foreign peptides to form a specific target structure that can be recognized by $CD8^+$ cytotoxic T-lymphocytes (CTL; for review see Yewdell and Bennink, 1982; Rammensee et al., 1993). In the CNS, constitutive expression of Class I is very low or absent in vivo, contributing to the notion that the CNS is immunoprivileged (Berah et al., 1970; Barker and Billingham, 1977; Wong et al., 1985). However, various reports indicate that Class I may be expressed on astrocytes, oligodendrocytes, microglia and endothelial cells, though the most consistent observations have been made for microglia or endothelial cells (Wong et al., 1985; Suzumura et al., 1986a; Male et al., 1987; Hickey and Kimura, 1988; Massa et al., 1993). In vitro, Class I has also been clearly shown to be expressed in astrocytes, either constitutively, increasing with time after initial plating (Schnitzer and Schachner, 1981; Wong et al., 1985; Massa et al., 1989, 1993) or following the addition of interferons (IFNs) to the culture medium (Wong et al., 1984, 1985; Fontana et al., 1986). Although all IFNs stimulate Class I expression in a variety of cell types, IFN-γ is usually the most potent (Revel and Chebath, 1986). Finally, in some experimental circumstances the ability of astrocytes to express MHC molecules, in addition to other properties, allows them to function as antigen-presenting cells for $CD8^+$ CTL and $CD4^+$ helper T cell activities (Fontana et al., 1986; Sedgwick et al., 1991).

MHC expression in glial cells can also be induced by A59 infection. In C57BL/6 mice, i.c. inoculation of A59 results in Class I and Class II expression in astrocytes and oligodendrocytes as early as 2 days postinfection (p.i.; Suzumura et al., 1986b). Class I mRNA transcripts increase within 24 hr p.i., before viral RNA and infectious virus are detected (Gumbold and Weiss, 1992). In addition, Class I expression does not appear to be restricted to infected cells, suggesting the involvement of the soluble factor previously reported to exist in glial cultures infected with A59 in vitro (Suzumura et al., 1988). Primary glial cell cultures prepared from A59-infected mice continue to express significant levels of MHC Class I and produce infectious virus up to 90 days p.i. (Lavi et al., 1989). By contrast, A59 infection of mixed glial cells in vitro resulted in Class I mRNA expression in astrocytes that was maximal at 3 days p.i., decreasing progressively to control levels by day 10 p.i. (Gumbold and Weiss, 1992). Thus, there may be significant differences in Class I expression in acute and persistent infection.

Since astrocytes are capable of acting as antigen-presenting cells for in vitro immune responses and are primary targets of persistent infection by MHV in vivo, we initiated studies to examine the influence of persistent MHV infection on MHC Class I expression in astrocytes. Although it is well documented that A59 induces Class I expression in murine astrocytes, we have recently found that JHMV lacks this activity during acute infection (Gilmore et al., 1994). In this communication, we report that during persistent infection, JHMV, but not A59, significantly inhibits constitutive Class I expression in astrocytes. In addition, A59 is no longer capable of stimulating Class I expression during persistence. Thus, Class I expression in persistent infection does in fact differ from that in acute infection by both A59 and JHMV. Interestingly, the effect of virus infection on Class I expression also differs between A59 and JHMV. However, in both cases, there is no change in the astrocyte response to IFN-γ during persistent infection. We suggest that one

interpretation of these data is that astrocytes are capable of acting as antigen-presenting cells in an immune response in spite of the persistent presence of virus, as long as they receive the appropriate signal from IFN-γ. IFN-γ is secreted by activated T cells, which are known to infiltrate the CNS during MHV infection (Williamson et al., 1991) and have been detected in demyelinating lesions in the brains of MS patients (Traugott et al., 1982; Hofman et al., 1986; Hauser et al., 1986). The data have potential to contribute to our overall understanding of the mechanisms of immune responsiveness in the brain.

MATERIALS AND METHODS

Primary Astrocyte Cultures

As a source of astrocytes, mixed glial cells were isolated from the brains of newborn C57BL/6 mice (Bantin and Kingman, Fremont, CA) according to McCarthy and deVellis (1980). Briefly, on postnatal days 0–3, single cell suspensions were prepared from dissected cerebral hemispheres and allowed to grow to confluence. Cells were cultured in DMEM/Ham's F12 (1:1; JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (FCS; Gemini Bioproducts, Inc., Calabasas, CA), 15 mM Hepes, 2.5 mM L-glutamine and penicillin-streptomycin (100 units/μg/ml). Microglia and oligodendroglia were removed from confluent culture by mechanical shaking at days 12–15 in vitro (DIV). The resulting preparations were enriched 95% or greater for cells staining for glial fibrillary acidic protein (GFAP) by immunoperoxidase or immunofluorescent staining procedures (described below).

Virus Strains and Infection

The derivation, propagation and sources of MHV-A59, and the large plaque morphology variant of MHV-4 known as JHM-DL (JHMV) have been described (Stohlman et al., 1982). The murine astrocytoma DBT was used for growth and plaque assay of the virus preparations and cell supernatants (Stohlman and Weiner, 1981). Persistent infection was established in astrocytes infected with JHMV or A59 (moi = 1–2) by the addition of MHV-specific polyclonal antiserum to the culture medium beginning on day 7 p.i. Polyclonal antiserum, prepared as described (Gilmore et al., 1987), was used at a final concentration of 1:200. Unless otherwise indicated, infected cells were maintained in the presence of polyclonal antiserum for the duration of the studies. Persistent infection was defined as the presence of intracellular viral antigen or mRNA in the absence of infectious virus in culture supernatants or cells.

Infectious Center Assays

The number of astrocytes yielding infectious virus was determined on days 3, 7, 14 and 28 p.i. to provide a measure of the relative efficiency of infection by A59 and JHMV. Briefly, astrocytes were trypsinized into single cell suspensions and, following 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 hr at 37°C prior to the addition of agarose at 0.6% in RPMI 1640 supplemented with 2% heat-inactivated FCS, 20 mM Hepes and 100 units/100 μg/ml penicillin/streptomycin. Plaques were counted after 48 hr incubation at 37°C. For days 14 and 28 p.i., medium was changed to omit polyclonal MHV antiserum 48 hr prior to collection of supernatants.

Antibodies

Class I molecules were identified using hybridoma supernatants containing 10–20 μg/ml of monoclonal antibodies (mAb) specific for K^b (AF6.88.5.3; ATCC HB158), D^bL^d (28-14-8S; ATCC HB 27), K^d (SF1-1.1.1; ATCC HB 159) and D^d (34-5-8S; ATCC HB 102). Supernatants were used at a concentration previously determined to be optimal for the detection of INF-γ-induced Class I in astrocytes, usually at 10–20 μg/ml. The percentage of GFAP-positive astrocytes was measured using polyclonal anti-GFAP antibody (rabbit anti-bovine GFAP, Dakopatts, Denmark). Possible contamination of enriched cultures by cells of macrophage/monocyte lineage was determined using a mixture of mAb specific for Mac-1 (hybridoma M1/70.15, ATCC TIB 128), F4/80 (ATCC HB 198) and T-200, the mouse isoform of human CD45, which recognizes cells of bone marrow origin (hybridoma M1/9.3.4HL.2, ATCC TIB 122). Polyclonal rabbit anti-galactocerebroside (Gal C, kindly provided by Dr. M. Smith, Stanford University, Stanford, CA) was used to identify contamination by olidodendrocytes. JHMV antigen was identified in infected cells using J.3.3., a mAb with specificity for both the A59 and JHMV nucleocapsid (N) protein (Fleming et al., 1983).

Radioimmunoassay

The expression of Class I molecules was measured by radioimmunoassay (RIA) in A59- and JHMV-infected astrocytes plated in 96-well plates at a density of 10^4 cells/well, with mock-infected cells serving as controls. On days 3, 5, 7, 14 or 28 p.i., cells were replated at 10^4 viable cells/well and assayed 24 hr later. Medium was removed and mAbs added in triplicate. Assay buffer consisted of 0.1 M phosphate-buffered saline (PBS)/0.3% bovine serum albumin (BSA). After 60 min incubation at room temperature, mAbs were removed, cells were

washed in assay buffer and ^{125}I-labelled protein A (30 μCi/μg; ICN Biomedicals, Costa Mesa, CA) added at 20,000 cpm/well. Unbound radiolabelled protein A was removed after 60 min by extensive washing. Cells were then detached with 0.5% trypsin/0.2% EDTA and transferred to the wells of Immulon Removawell strips (Dynatech Laboratories, Alexandria, VA) for gamma counting. Data are presented either as the percent increase in Class I expression in infected cells compared with uninfected controls, or as mean cpm of triplicate determinations. Again, polyclonal antiserum was removed from the astrocytes 48 hr prior to assay.

Immunoperoxidase Staining

The number of infected cells and cell phenotypes in astrocyte cultures were identified using avidin-biotin immunoperoxidase staining kits (Vectastain, Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions, as described by Wang et al. (1992). Cells were plated in tissue culture chamber slides (Nunc, Inc., Naperville, IL), infected with virus the following day, and fixed on days 3, 7, 14, or 28 p.i. in acetone: methanol (1:1). Background staining was determined in cells stained in the absence of primary antibody. Results are expressed as the percent of stained cells relative to the total number of nuclei in 3 nonoverlapping fields viewed at 400× magnification.

Flow Cytometry

Indirect immunofluorescent staining was used to evaluate Class I expression in astrocytes by fluorescence-activated cell sorting (FACS). Cells were trypsinized into single cell suspensions, adjusted to 10^5 to 10^6 cells/tube, and incubated at 4°C for 60 min in primary antibody diluted in wash buffer. After washing, the appropriate FITC-labelled secondary antibody (goat anti-mouse or rat IgG $F(ab)_2$; Jackson Immunoresearch Laboratories, West Grove, PA) was added for 30 min, again at 4°C. 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, Mountain View, CA). Again, background fluorescence was determined in cells stained in the absence of primary antibody.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to identify the presence of viral mRNA in persistently infected astrocytes no longer showing evidence of infectious virus release into the medium. Total cellular RNA was isolated by acid-guanidinium thiocyanate phenol-chloroform extraction as described by Chomczynski and Sacchi (1987). First strand cDNA synthesis was accomplished in a reaction mixture containing 2 μg total RNA, 2 μg oligo dT, 40 U AMV reverse transcriptase, 200 nM deoxynucleotide triphosphates (dNTP), 25 units RNAse inhibitor and 5X incubation buffer in a total volume of 20 μl for 1 hr at 42°C. Unless otherwise indicated, all reagents for cDNA synthesis were from Boehringer-Mannheim (Indianapolis, IN). For PCR, oligonucleotide primers were selected to amplify a 137 bp fragment corresponding to the nucleocapsid (N) gene of JHMV (Skinner, 1983). The 5′ sense primer (5′-CCGAATTCATGTCTTTTGTTCCTTGGGCAAGAA-3′) corresponds to nucleotides 84–107. The 3′ anti-sense primer (5′-CCGAATTCATGTCTTTTGTTCCTTGGGCAAGAA-3′) corresponds to nucleotides 1425–1446. The PCR reaction mixture contained 250 ng of each oligonucleotide primer, 100 ng cDNA, 2 mM dNTPs, 0.5 nM $MgCl_2$, 10X PCR buffer and 1.5 units Taq polymerase in a total reaction volume of 20 μl. All reagents for PCR were from Perkin-Elmer Cetus (Norwalk, CT). Amplification was performed in 25 cycles of denaturation at 92°C (1 min), annealing at 55°C (2 min) and elongation at 72°C (2 min) in a thermal cycler (Perkin-Elmer Cetus). The correct size of the PCR products was determined in 1% agarose gels stained with ethidium bromide and specificity confirmed by Southern blot analysis using ^{32}P end-labelled primers, or an N-specific cDNA probe obtained from Dr. M. Lai (University of Southern California, Los Angeles, CA).

In Situ Hybridization

For in situ hybridization studies, cells were plated in Nunc tissue culture chamber slides and infected for 1 hr at 37°C with JHMV at an moi = 1. At days 3, 7, 14 and 28 p.i., cells were washed twice in PBS, air-dried for 15 min and stored at −20°C until use. Slides were fixed for 5 min in 3% paraformaldehyde in 0.1 M PBS, pH 7.4, containing 0.02% diethylpyrocarbonate. Following three 5 min washes in PBS, cells were acetylated by a 10 min exposure to 0.1 M triethanolamine containing 0.25% acetic anhydride. Prehybridization occurred for 1 hr at RT in 50% formamaide, 1X Denhardt's solution, 0.5 mg/ml herring sperm DNA, 5% dextran sulfate, 4X SSC and 0.25 mg/ml yeast tRNA. MHV transcripts were detected using the N-specific 3′ antisense oligonucleotide primer labelled with digoxigenin 11-dUTP, using the Genius System (Boehringer-Mannheim) according to the manufacturer's directions. Hybridization was carried out overnight at 42°C following the addition of the labelled probe at a concentration of 300 ng/ml. Washes consisted of 2X SSC, 1X SSC (1 hr each, at RT) and 2 rinses in 0.5X SSC (30 min each, the first at 37°C). Final detection was accomplished by the addition of antidigoxigenin-alkaline phosphatase conjugate, again according to the manufacturer.

RESULTS

Establishment and Characteristics of Persistent Infection in Primary Murine Astrocytes

Persistent infection was established in primary astrocytes following inoculation with A59 or JHMV and subsequent culture in the presence of polyclonal MHV antiserum from 7–28 days p.i. Tables I–III illustrate the characteristics of the infection at various p.i. intervals. In Table I, it is demonstrated that JHMV readily establishes a chronic infection in astrocytes in the absence of polyclonal antiserum, with low levels of infectious virus being released into the medium throughout the period tested. Infected cultures could be maintained for as long as 6–12 months p.i. with little change in the titer of virus recovered from the supernatants. Cell fusion was minimal at 20% or less. In the presence of polyclonal antiserum, however, infectious virus was reduced to almost undetectable levels on day 14, and was not detectable on day 28 p.i. Antiserum could be excluded from the medium at this time with no recovery of infectious virus for at least the following 4 weeks (data not shown). The number of cells producing infectious JHMV or A59 shows the same trend, decreasing from 20% at day 3 p.i., to 0.4% at day 14 p.i., and finally becoming undetectable by 28 days p.i. (Table II). The number of cells staining for N protein in both A59- and JHMV-infected astrocytes decreased more slowly, from approximately 38% at day 3 to 20–22% at day 28 p.i. (Table III). Interestingly, the number of cells showing expression of viral RNA by in situ hybridization increased with time in culture from 70% at day 3 to 95% at day 28 p.i. (Table III). Viral RNA was detectable at least as late as day 53 p.i. by RT-PCR (Fig. 1). These data not only indicate that A59 and JHMV establish long-standing persistence, but also lead us to suggest that viral RNA synthesis continues in the absence of viral protein synthesis and of assembly into infectious particles. They further lead us to suggest that persistent infection established by this method is characterized by restricted viral protein synthesis in the majority of infected cells, rather than simply a reduced number of infected cells.

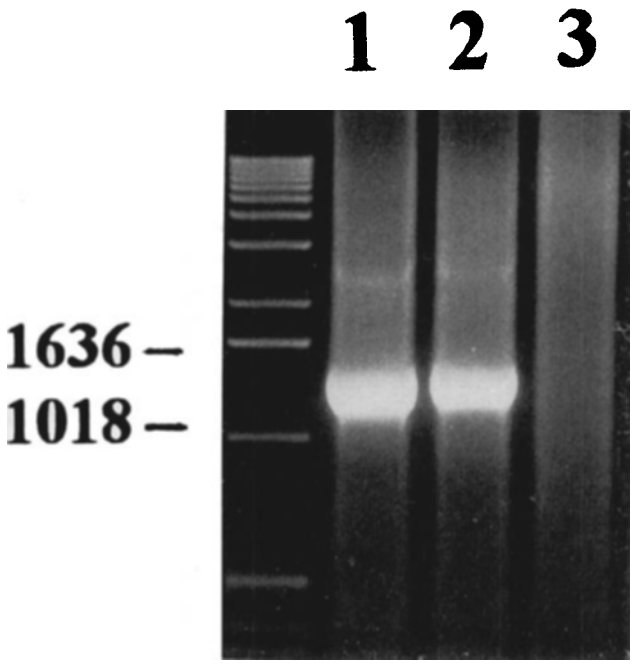

Fig. 1. MHV mRNA persists as late as 53 days p.i. in primary astrocytes from neonatal C57BL/6 mice. RT-PCR amplification yields a 1,372 bp product of N-specific oligonucleotides (**lane 1**). **Lane 2:** JHMV-infected oligodendrocyte cell line as a positive control. **Lane 3:** Uninfected primary astrocytes as a negative control.

TABLE I. Recovery of Infectious Virus in Supernatants From Astrocytes Infected With JHMV*

	Days postinfection			
Antiserum	3	7	14	28
–	5.2×10^{2}[a]	6.2×10^{2}	2×10^{3}	5×10^{2}
+	n.d.[b]	n.d.	32	0

*Astrocytes were infected on day 0 (moi = 1) and polyclonal MHV antiserum added on day 7 p.i. at a final concentration of 1:200.
[a]Titers are expressed as plaque-forming units/ml (pfu/ml). MHV antiserum was removed from tissue culture 48 hr prior to assay.
[b]n.d., not done.

JHMV, But Not A59, Reduces Constitutive Class I Expression in Persistently Infected Astrocytes

The effect of persistent JHMV and A59 infection on the expression of MHC Class I molecules in primary astrocytes was determined throughout the course of infection by RIA and immunofluorescence staining for FACS analyses. Figures 2A and B present RIA data demonstrating that uninfected astrocytes express Class I K^b molecules constitutively, with the level of expression increasing with time in culture. The D^b Class I molecule was also constitutively expressed, though at lower levels than K^b (data not shown). The control anti-K^d and D^d antibodies showed no staining. Figure 2A shows that the constitutive expression of K^b is reduced significantly in astrocytes persistently infected with JHMV. Thus, Class I expression was at least 70% below that observed in uninfected control cultures (Table IV). By contrast, A59-infected astrocytes exhibited very little change in Class I expression during persistent infection on days 14 and 28 p.i. However, during acute infection (days 3 and 5 p.i.), Class I was

TABLE II. Percentage of Cells Producing Infectious Virus at Various p.i. Intervals

	Days postinfection			
Virus	3	7[a]	14	28
JHMV	20	25	0.45	0.02
MHV-A59	19	15	0.42	0

[a]Polyclonal MHV antiserum was added on day 7 p.i. and removed 48 hr prior to assay.

TABLE III. Percentage of Cells Expressing MHV N Protein or mRNA

		Days postinfection			
Protein/RNA	Virus	3	7	14	28
Protein[a]	JHMV	37[b]	42	28	20
	MHV-A59	38	44	35	22
mRNA[c]	JHMV	70	85	85	95

[a]MHV nucleocapsid protein was detected by immunoperoxidase staining using J.3.3, and N-specific mAb.
[b]Results are expressed as percent stained cells relative to the total number of cells evaluated. For each condition, 3 nonoverlapping fields were evaluated at 400X magnification.
[c]Nucleocapsid mRNA was detected in in situ hybridization as described in Materials and Methods.

TABLE IV. Constitutive MHC Class I (K^b) Expression Is Reduced During Persistent Infection in JHMV-, But Not A59-Infected Astrocytes

	Days postinfection			
Virus	3	5	14	28
JHMV	+6[a]	+18	−79	−74
MHV-A59	+2,000	+2,132	+50	+9

[a]K^b expression was measured by RIA using mAb AF6.88.5.3. Results are expressed as percent increase in Class I expression in infected cells compared with that in uninfected cells.

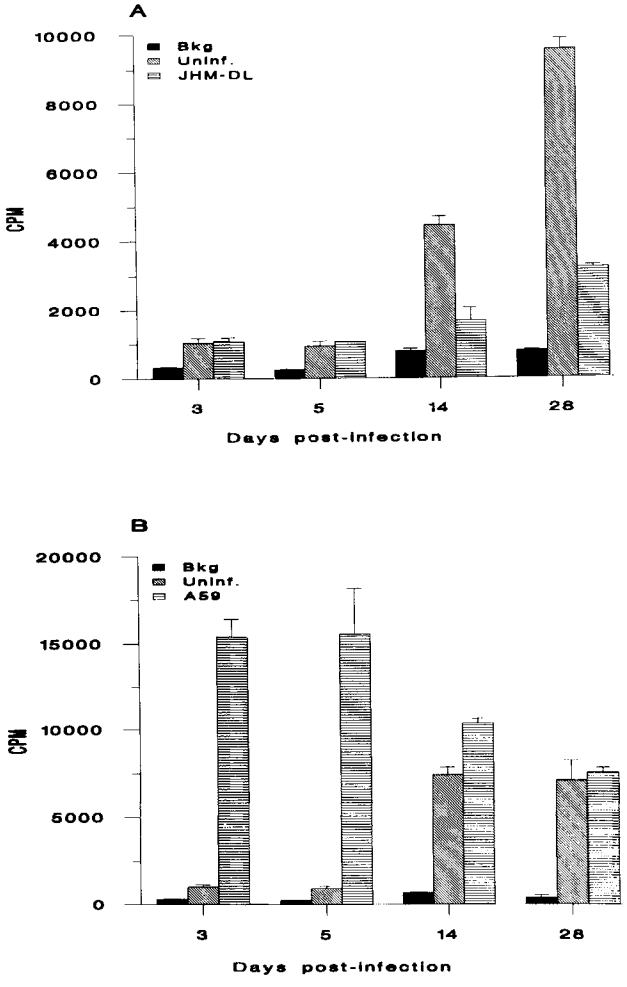

Fig. 2. H-2K^b expression in JHMV- (**A**) and A59- (**B**) infected astrocytes was measured by RIA at 3, 5, 14 and 28 days p.i. Data are presented as counts per minute ± standard deviation of ^{125}I from triplicate wells. Background (Bkg) represents infected cells assayed in the absence of K^b specific mAb, and uninfected controls were assayed in the presence of K^b mAb.

increased dramatically (Fig. 2B), reaching levels up to 2,000% over that in uninfected cultures (Table IV). The lack of Class I induction correlated with the decrease in infectious virus titers that occurred at day 14 p.i. (Table II), indicating that Class I induction by A59 depends on the presence of infectious virus. Additional studies in our laboratory have confirmed this requirement for infectious virus (Gilmore et al. 1994).

FACS analyses confirmed the constitutive expression of K^b in uninfected astrocytes (Fig. 3A) and also documented that JHMV infection did not significantly alter the percentage of cells expressing Class I antigen or the intensity of staining on day 5 p.i. (Fig. 3B). In addition, Figure 3C illustrates the induction of K^b by A59 on day 5 p.i. To rule out the possibility that the presence of polyclonal antiserum was responsible for the observed changes in Class I expression, culture medium was changed to omit the antiserum 48 hr prior to assay. However, uninfected astrocytes were also cultured in MHV antiserum in parallel experiments, and FACS analyses at 14 and 28 days p.i. showed no effect on Class I expression in these cells (data not shown). This leads us to suggest that MHV antiserum supports the establishment of persistent infection without an effect on Class I. Thus, A59 and JHMV differ significantly in their ability to influence Class I expression in acute and persistent infection in vitro.

IFN-γ Restores Class I Expression in Astrocytes Persistently Infected With JHMV and A59

It has been well documented that IFN-γ induces Class I expression in a variety of cell types, including

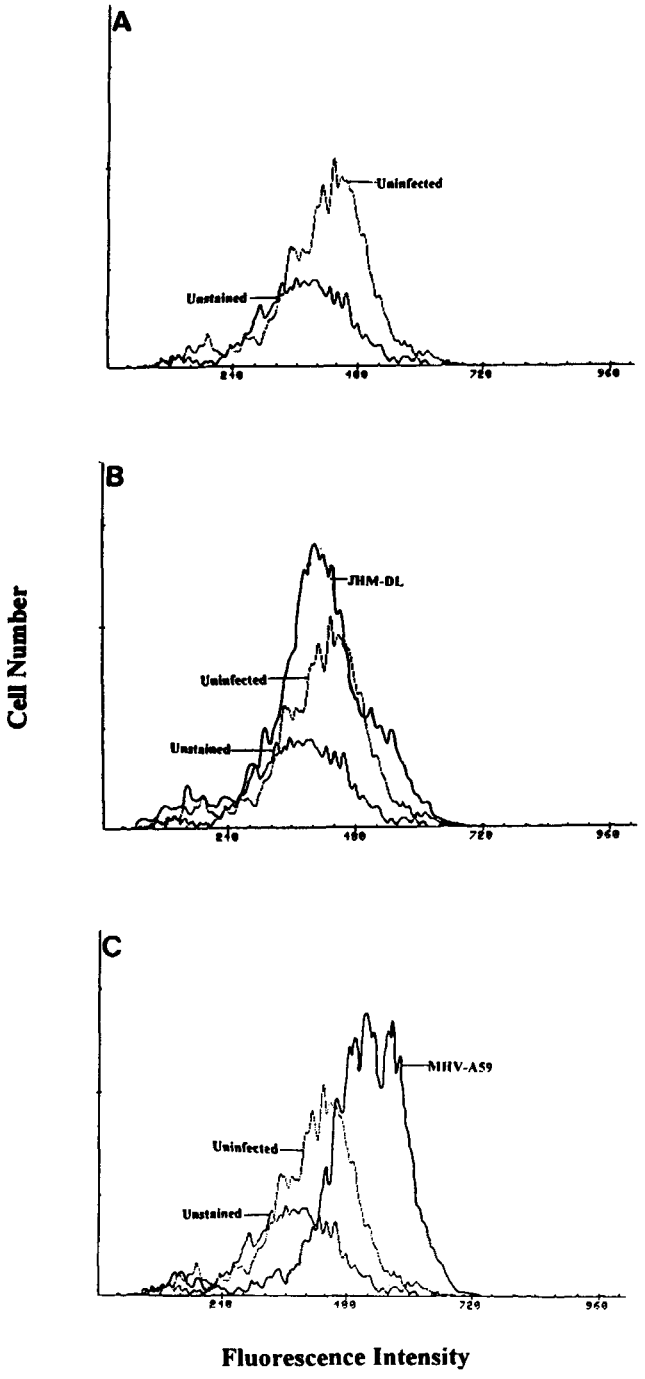

Fig. 3. FACS analysis of the expression of MHC Class I molecules on astrocytes 5 days p.i. **A:** Uninfected astrocytes. **B:** JHMV-infected cells. **C:** A59-infected astrocytes. Unstained cells received FITC-conjugated secondary antibody in the absence of primary antibody.

astrocytes. To determine whether infected astrocytes retain their responsiveness to IFN-γ, recombinant murine IFN-γ was added to A59- and JHMV-infected cells at 14 and 28 days p.i. Forty-eight hours later, Class I expression was measured by flow cytometry and RIA. Table V illustrates that JHMV-infected astrocytes express significant levels of Class I in response to IFN-γ, comparable to that observed in uninfected, IFN-γ-treated astrocytes. FACS analyses revealed that the number of Class I-positive cells increased 9-fold at 14 days p.i., 6-fold at 28 days p.i. and 14-fold at 53 days p.i. An increase in the number of Class I molecules/cell was indicated by a significant increase in mean fluorescence intensity (MFI; Table V and Fig. 4). A similar, if not more dramatic increase in Class I expression following IFN-γ treatment was observed in A59-infected astrocytes as well (Table VI). These data indicate that Class I down-regulation in infected astrocytes is not a permanent consequence of infection, and can be reversed by the addition of exogenous IFN-γ.

TABLE V. IFN-γ Treatment Reverses Down-Modulation of Constitutive Class I (K^b) Expression in Astrocytes Persistently Infected With JHMV

		Days postinfection	
Method	Treatment	14	28
FACS	Uninfected − IFN-γ	11 (354)[a]	25 (473)
	Uninfected + IFN-γ[b]	67 (519)	65 (581)
	Infected − IFN-γ	6 (365)	12 (441)
	Infected + IFN-γ	56 (507)	67 (587)
RIA	Uninfected + IFN-γ	+223[c]	+235
	Infected − IFN-γ	−79	−75
	Infected + IFN-γ	+174	+199

[a]Values represent percent positive cells with mean fluorescence intensity in parentheses.
[b]Astrocytes were treated with 100 U/ml of recombinant murine IFN-γ for 48 hr prior to staining.
[c]Values represent percent increase in Class I relative to uninfected control cultures as indicated for Table IV.

DISCUSSION

In this communication, we report that A59 and JHMV differ considerably in their ability to influence the expression of MHC Class I molecules in both acute and persistent infection in primary C57BL/6 astrocytes. When cultured in the absence of polyclonal antiserum specific for MHV, both viruses cause a chronic infection in which a continuous low level of infectious virus is produced and released into the medium. Cell fusion is minimal, suggesting a relatively nonlytic infection, and the astrocyte cultures could be maintained for as long as 6–12 months p.i. Under these conditions, A59, but not JHMV, induced the expression of the K^b Class I molecule. In additional studies in our laboratory using A59/JHMV recombinant virus strains, we have found that the Class I-inducing activity most likely resides in the 3′ end

TABLE VI. IFN-γ Restores MHC Class I Expression in A59-Infected Astrocytes*

Days p.i.	IFN-γ	K^b	D^d
3	−	+2000	+7
	+	n.d.[a]	n.d.
5	−	+2132	0
	+	n.d.	n.d.
14	−	+62	0
	+	+288	0
	Uninfected + IFN-γ	+235	0
28	−	+9	0
	+	+148	0
	Uninfected + IFN-γ	+83	0

*Class I expression was measured by RIA using K^b-specific mAb AF6.88.5.3 and D^d-specific mAb 34-5-8S. Data are expressed as percent increase relative to that of uninfected control cultures.
[a]n.d., not done.

of the A59 genome, which encodes the structural proteins S, M and N (Gilmore et al., 1994). Class I expression required the presence of infectious virus, and appeared to be restricted to infected cells, indicating that Class I expression is a direct consequence of A59 infection. These data are in agreement with those published by Lavi et al. (1989), who reported that Class I induction in mixed glial cells cultured from A59-infected mice depended on the presence of infectious virus.

When persistent infection was established by the addition of MHV-specific antiserum, A59 no longer induced Class I expression in astrocytes. This is presumably due to the lack of infectious virus, which is almost undetectable within one week of the addition of antiserum to the medium. This also occurs in spite of the presence of viral antigen in 20–35% of cells and viral RNA in 85–95% of cells at days 14–28 p.i. These data lead us to believe that infectious A59 particles, and not a residual viral protein or RNA, are responsible for inducing Class I expression. The mechanism by which this occurs is currently unknown.

Unlike A59, JHMV was not only unable to stimulate Class I, but inhibited the constitutive Class I expression that occurs in astrocyte cultures. This effect appears to be independent of infectious virus, since the inhibition occurred at days 14, 28 and 53 p.i., when infectious virus was not present. In addition, we observed no inhibition of constitutive K^b expression on days 3 and 5 pi, prior to the addition of MHV antiserum and during productive infection.

The lack of Class I expression has long been considered to facilitate the establishment of persistent virus infection, and perhaps represents a mechanism by which persistence is maintained (Oldstone, 1989; Joly et al., 1991). In the CNS, the relative lack of Class I expression has also been considered beneficial to the host in that it represents a means by which an immune response can be regulated in a tissue with limited regenerative capacity. Thus, it is possible that Class I down-regulation by JHMV, or the lack of Class I-inducing activity, may contribute to the establishment of its persistence. We can also speculate that the lack of Class I-inducing activity in persistent A59 infection may help to maintain its persistence once it has been established, and once infectious virus release is controlled.

There have been numerous reports that several other viruses, including adenovirus, human cytomegalovirus (HCMV) and mouse CMV (MCMV), inhibit the expression of Class I molecules in host cells (Maudsley and Pound, 1991). The inhibition may occur at the transcriptional level as for adenovirus (Schrier et al., 1983), post-transcriptionally as in adenovirus and HCMV infection (Andersson et al., 1985; Burgert et al., 1987), or by reducing the stability of Class I molecules as for HCMV (Beersma et al., 1993). In adenovirus infection, the E19 protein inhibits Class I molecules in the endoplasmic reticulum, preventing transport to the cell surface (Andersson et al., 1985; Burgert et al., 1987), while HCMV virions bind to β2m, causing infected cells to fail to synthesize mature Class I molecules (Browne et al., 1990). Although the mechanism by which JHMV inhibits constitutive Class I expression is not known, there is evidence that it blocks the synthesis of host cell proteins in non-neural cells during acute infection (SA Stohlman, University of Southern California, personal communication). This block may be general, affecting all host cell mRNAs, eventually leading to death, or it may be selective, blocking the synthesis of some proteins while up-regulating others. This is consistent with data reported by Oldstone et al. (1982) for LCMV, which inhibits differentiated functions of pituitary cells without influencing the functions that regulate cell survival. In addition, we have found that persistent JHMV infection in the mouse oligodendroglioma, G2620, is associated with reduced MHC Class II expression in the absence of an effect on Class I, and furthermore, is associated with the up-regulation of mRNA specific for the myelin-associated protein CNPase (Gilmore et al., 1990). Whether or not persistent infection exerts similar selective influences on specific astrocyte functions, and whether a specific viral RNA or protein is involved, are topics for future studies in our laboratory.

The difference in the ability of JHMV and A59 to induce Class I expression during acute infection seems to indicate that Class I may play different roles in their pathogenesis. In acute infection, the primary difference between A59 and JHMV is in the severity of encephalitis and the time at which disease begins and death ensues. The strain of A59 used in these studies is highly neurotropic at very low pfu, inducing severe encephalitis be-

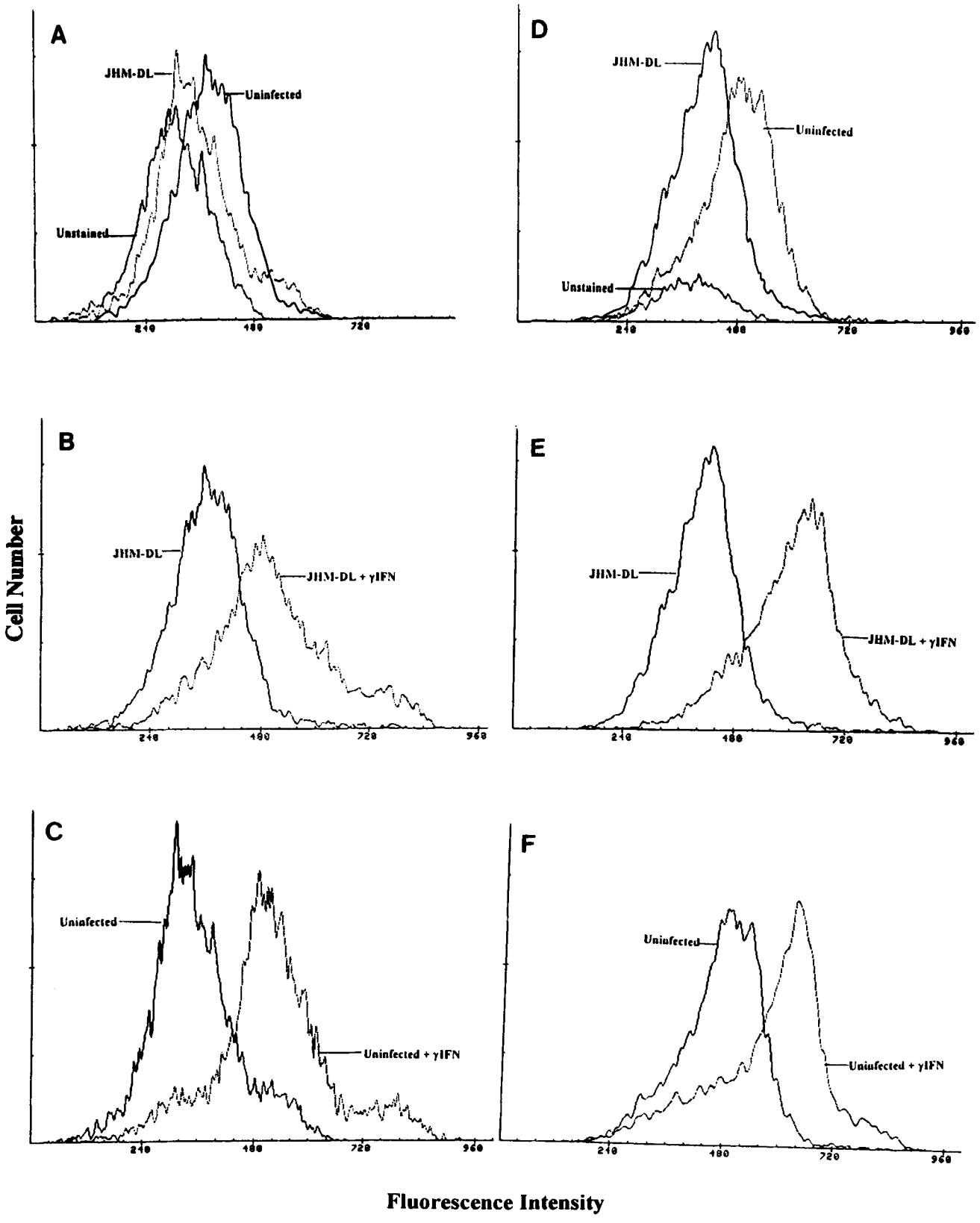

Fig. 4. Class I expression is restored by IFN-γ in astrocytes persistently infected with JHMV. At day 14 p.i. (**A–C**) and day 28 p.i. (**D–F**), astrocytes were stained for K^b and analyzed by FACS as described in Figure 3. The percentage of cells and the mean fluorescence intensity of H-$2K^b$ expression are also summarized in Table V.

ginning on day 4 p.i. and resulting in death in 95–100% of C57BL/6 mice by day 6 p.i. By contrast, the onset of encephalitis following i.c. inoculation with the same amount of JHMV occurs on day 6–7 p.i., with death occurring on days 9–11 p.i. at similar incidence. It is possible that A59-induced Class I expression may accelerate damage in the CNS such that the virus-mediated cell lysis is accompanied by a vigorous Class I-restricted T cell mediated lysis of infected cells. Just as a lack of MHC Class I expression facilitates virus persistence and limits tissue damage, an increase in Class I expression may accelerate immune-mediated, virus-stimulated tissue destruction (Oldstone, 1989). However, mice infected with either virus eventually succumb to an otherwise seemingly identical disease. Thus, it could also be argued that A59 simply replicates more efficiently than JHMV, growing to higher titers in the CNS in a shorter period of time. In support of this possibility, recombinant viruses containing the A59 leader RNA predominate over those with JHMV leader sequence following i.c. infection with both (Lai and Stohlman, 1992). Thus, the difference in Class I expression during acute infection by A59 and JHMV may not play an important role in the ultimate type of disease they cause, but may be more likely to contribute to their speed of progression. Similar observations have been made for JHMV isolates that differ in quantity of the hemagglutinin-esterase (HE) protein (Yokomori et al., manuscript submitted).

Perhaps one of the more interesting findings in these studies is that IFN-γ can rescue Class I expression in both JHMV- and A59-infected astrocytes. Thus, persistent infection does not permanently alter the Class I gene or the signalling mechanisms by which it is normally induced. Furthermore, the data lead us to suggest that astrocytes may be induced to express Class I as a bystander effect of a systemic virus-specific immune response upon entry into the CNS, resulting in local inflammatory "hot spots." Such an activity may explain the ability of CNS disease to relapse in JHMV infection, as reported by Shubin et al. (1990). IFN-γ has also been shown to restore Class I expression in mouse cell lines transformed by adenovirus 12 (Eager et al., 1985). Several additional cytokines generated during the course of an immune response, including TNFα and IFNα/β, are known to regulate MHC expression in glial cells (Wong et al., 1985; Lavi et al., 1988), and may act as participants in MHV-induced neuropathogenesis.

In conclusion, we have provided evidence that MHC Class I expression in murine astrocytes is influenced significantly by A59 and JHMV in either or both acute and persistent infection. The precise mechanisms of Class I induction by A59 in acute infection, Class I inhibition by JHMV in persistent infection and rescue of Class I expression by IFN-γ in both A59 and JHMV persistent infection remain important areas for future investigation.

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