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Expression of Interferon-γ by a Coronavirus Defective-Interfering RNA Vector and Its Effect on Viral Replication, Spread, and Pathogenicity

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A defective-interfering (DI) RNA of the murine coronavirus mouse hepatitis virus (MHV) was developed as a vector for expressing interferon-γ (IFN-γ). The murine IFN-γ gene was cloned into the DI vector under the control of an MHV transcriptional promoter and transfected into MHV-infected cells. IFN-γ was secreted into culture medium as early as 6 hr posttransfection and reached a peak level (up to 180 U/ml) at 12 hr posttransfection. The DI-expressed IFN-γ (DE-IFN-γ) exhibited an antiviral activity comparable to that of recombinant IFN-γ and was blocked by a neutralizing monoclonal antibody against IFN-γ. Treatment of macrophages with DE-IFN-γ selectively induced the expression of the cellular inducible nitric oxide synthase and the IFN-γ-inducing factor (IGIF) but did not affect the amounts of the MHV receptor mRNA. Antiviral activity was detected only when cells were pretreated with IFN-γ for 24 hr prior to infection; no inhibition of virus replication was detected when cells were treated with IFN-γ during or after infection. Furthermore, addition of IFN-γ together with MHV did not prevent infection, but appeared to prevent subsequent viral spread. MHV variants with different degrees of neurovirulence in mice had correspondingly different levels of sensitivities to IFN-γ treatment *in vitro,* with the most virulent strain being most resistant to IFN-γ treatment. Infection of susceptible mice with DE-IFN-γ-containing virus caused significantly milder disease, accompanied by more pronounced mononuclear cell infiltrates into the CNS and less virus replication, than that caused by virus containing a control DI vector. This study thus demonstrates the feasibility and usefulness of this MHV DI vector for expressing cytokines and may provide a model for studying the role of cytokines in MHV pathogenesis. © 1997 Academic Press

INTRODUCTION

Interferon-γ (IFN-γ) is a pleiotropic cytokine produced by activated $CD4^+$ and $CD8^+$ T cells and natural killer cells (Trinchieri and Perussia, 1985; Pestka and Langer, 1987; Ijzermans and Marquet, 1989), which exerts both antiviral and immunomodulatory effects. These include the activation of mononuclear phagocytes, enhancement of the generation of oxygen-free radicals, modulation of class I and II major histocompatability complex (MHC) antigen expression, and promotion of differentiation of both T and B cells (for reviews, see references by Pestka and Langer, 1987; Benveniste, 1992). It plays an important role in the early phase of many viral infections (Wheelock, 1965; Wong and Goeddel, 1986; Leist *et al.,* 1989; Klavinskis *et al.,* 1989; Feducchi and Carrasco, 1991; Ramsey *et al.,* 1993; Heise and Virgin IV, 1995; Rodriguez *et al.,* 1995), inhibiting the replication of a variety of viruses prior to activation of antiviral effector cytotoxic T lymphocyte (CTL) or antibodies. Because of its antiviral activity, IFN-γ has been implicated in virus clearance and resolution of viral infection (Ramshaw *et al.,* 1992). Resistance to IFN-γ may lead to incomplete viral clearance and contribute to the establishment of persistent infection (Moskophidis *et al.,* 1994). By contrast, IFN-γ is also involved in inflammatory processes. IFN-γ induces the expression of many other inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), and acts synergistically with these cytokines (Wong and Goeddel, 1986). The multitude of immunomodulatory effects of IFN-γ makes it a particularly interesting cytokine for studying viral pathogenesis. In the central nervous system (CNS), no cells constitutively express IFN-γ. During encephalomyelitis, for example as a result of mouse hepatitis virus (MHV) infection, activated NK cells and T cells which pass through the blood–brain barrier into the CNS express IFN-γ (Bukowski *et al.,* 1983; Pearce *et al.,* 1994). In addition to its effects on mononuclear cells, IFN-γ acts upon cells of the CNS, such as astrocytes, microglia, and macrophages (Benveniste, 1992).

MHV, a murine coronavirus, causes a variety of diseases in rodents, such as hepatitis, enteritis, and neurological diseases, depending on the viral strain (Cheever *et al.,* 1949; Gledhill and Niven, 1955; Ishida *et al.,* 1978). Even within the well-studied neuropathogenic JHM strain, different variants cause different disease patterns, ranging from acute fatal encephalitis to chronic demye-

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lination (Stohlman *et al.,* 1982; Lai and Stohlman, 1992). The DL variant derived from the parental JHMV causes an acute, fulminant, necrotizing encephalomyelitis with minimal or no demyelination. By contrast, the neuroattenuated variant 2.2-V-1 derived from DL produces a nonfatal encephalomyelitis with extensive demyelination (Fleming *et al.,* 1986, 1987; Wang *et al.,* 1992). Disease outcome also depends on the genetic background, the developmental stage, and the immunological status of the host. Previous studies have shown that immunocompetent mice infected with MHV exhibited increased expression of a number of cytokines, including IL-1, IL-6, TNF-α, and IFN-γ, in the CNS at the time of viral clearance (Pearce *et al.,* 1994). However, the role of these cytokines in MHV pathogenesis is not fully understood. For example, it has been suggested that IFN-γ may not be necessary for induction of the MHC class I molecules on neural cells *in vivo* (Pearce *et al.,* 1994), a prerequisite to CTL-mediated clearance (Stohlman *et al.,* 1995). However, IFN-γ treatment ameliorates MHV-induced disease (Smith *et al.,* 1991), suggesting that either the antiviral role or the immunomodulatory role of IFN-γ is a critical component of MHV infection.

MHV contains a single-strand, positive-sense RNA genome of 31 kb (Lee *et al.,* 1991). It undergoes rapid recombination, probably due to its large RNA genome and the special properties of its RNA-dependent RNA polymerase (Lai, 1992). Similarly, defective interfering (DI) RNAs are frequently generated in MHV-infected cells. Recently, recombinant DI RNAs have been developed which can replicate in the presence of a helper MHV (Makino *et al.,* 1988a, 1991; Van der Most *et al.,* 1991). We have modified an MHV DI RNA and developed an expression vector. This DI RNA contains both the 5′- and the 3′-ends, an internal region of the parental MHV genome (Makino *et al.,* 1988b), and an intergenic (IG) sequence, which is a recognition signal for subgenomic mRNA transcription, followed by an exogenous gene. Upon transfection of this DI RNA into MHV-infected cells, a subgenomic mRNA is synthesized and the inserted gene expressed. This system has been used to express the chloramphenicol acetyltransferase (CAT) protein and the coronavirus structural protein hemagglutinin/esterase (HE) in MHV-infected cells (Liao and Lai, 1994; Liao *et al.,* 1995). These proteins are expressed only in infected cells during virus replication, thus providing some degree of targeted gene expression. Furthermore, the expressed HE protein can be incorporated into virus particles, and the expression can be detected in serial virus passages (Liao *et al.,* 1995). Thus, this DI RNA expression system provides an alternative to an infectious full-length cDNA clone, which is still not available, for studying the molecular biology and pathogenesis of coronaviruses.

In the present study, we have used this DI RNA system to express the murine IFN-γ gene. The expressed IFN-γ exhibited antiviral activity, prevented virus spread *in vitro,* and altered viral pathogenesis in mice. This system may allow studies of the interaction between MHV and the host's immune system by expressing immunoregulatory proteins at the foci of viral infection.

MATERIALS AND METHODS

Virus and cells

The following virus strains were used in this study: the neuropathogenic MHV strain JHM isolate (DL), which is a large plaque variant derived from the parental JHM strain (Stohlman *et al.,* 1982); the small plaque variant DS (Stohlman *et al.,* 1982); the neutralization-escape mutant 2.2-V-1 (Fleming *et al.,* 1987; Wang *et al.,* 1992), and strain A59, which is both neurotropic and hepatotropic. The murine astrocytoma cell line (DBT) (Hirano *et al.,* 1974) and J774.1 macrophage cell line (obtained from the American Type Culture Collection) were used for *in vitro* experiments. DBT cells were also used for plaque assay.

Plasmid construction

A previously constructed plasmid p25CAT (Liao and Lai, 1994), which contains the plasmid Bluescript (Promega) sequence with a CAT gene inserted behind an IG sequence in the DIssE cDNA (Makino *et al.,* 1988a), was used as the basic DI vector. For cloning the murine IFN-γ gene into the DI vector, a cDNA fragment containing the complete IFN-γ gene (kindly provided by Dr. J. A. Frelinger, University of Rochester) was generated by polymerase chain reaction (PCR) using a pair of primers. The 5′ sense primer (5′-TAACTAGTAATCTAATCTAAACTTTAAGGAATGAACGCTACACACT-3′) contains a restriction enzyme *Spe*I site (underlined), the coronavirus intergenic sequence (in boldface), and the first 16 nucleotides of the IFN-γ open reading frame (ORF). The 3′ antisense primer (5′-TCAGAATTCAATCAGCAGCGACTCCT-3′) contains the last 15 nucleotides of the IFN-γ ORF and a restriction enzyme *Eco*RI site (underlined). After restriction enzyme digestion of the PCR products with *Spe*I and *Eco*RI, a 0.5-kb cDNA fragment was purified by low-melting-point agarose gel electrophoresis and directionally cloned into the *Spe*I and *Eco*RI sites of p25CAT, resulting in pDE-IFN-γ (Fig. 1A). The resulting construct contains the IFN-γ gene placed behind the IG sequence between genes 6 and 7 (IG7) of MHV.

RNA transcription and transfection

Plasmid DNA (pDE-IFN-γ) was linearized with *Xba*I, and RNA was transcribed *in vitro* using T7 RNA polymerase according to the manufacturer's recommended procedure (Promega). RNA transfection was carried out using the DOTAP method (Boehringer-Mannheim) as described previously (Zhang *et al.,* 1994). Briefly, monolayers of DBT cells grown at approximately 70% confluence in 60-mm petri dishes were infected with MHV at

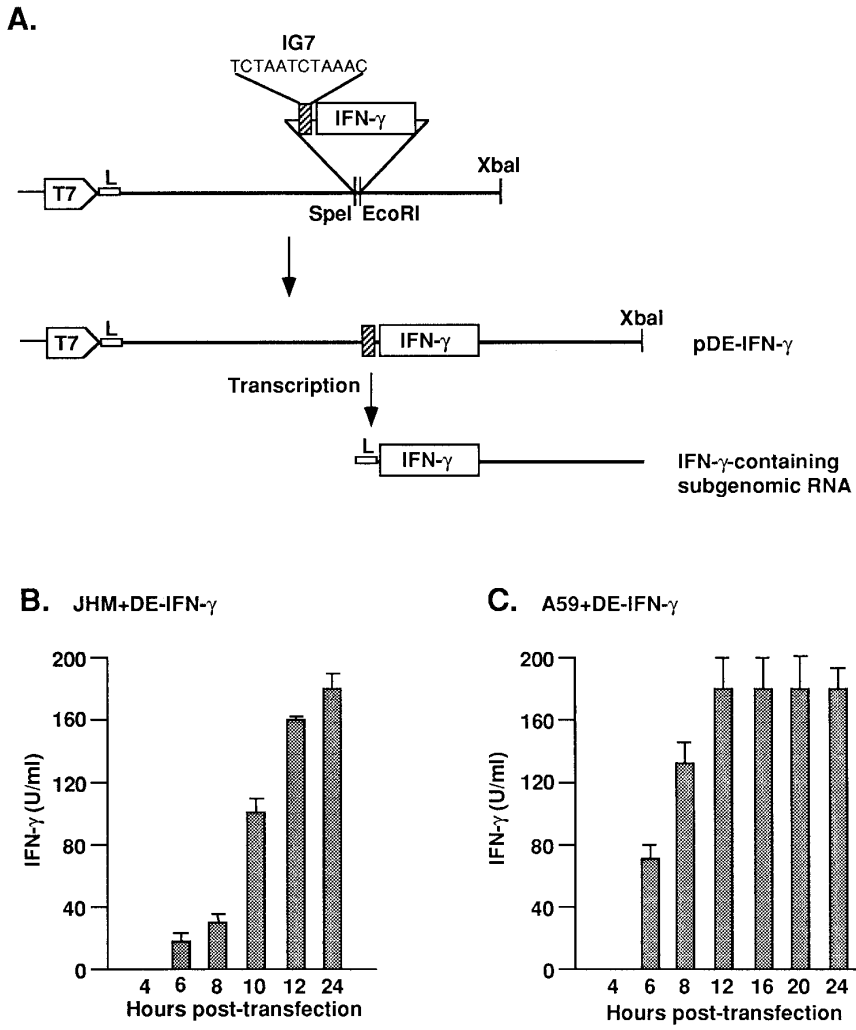

FIG. 1. Structure of the DI vector containing the IFN-γ gene and its expression. (A) The IFN-γ gene was inserted into the *Spe*I and *Eco*RI sites of the DI cDNA in plasmid pBluescript under the control of a coronavirus transcriptional promoter derived from the intergenic sequence (IG7) between genes 6 and 7 of MHV-A59. Restriction enzyme site *Xba*I was used for digestion of the plasmid DNA for *in vitro* run-off transcription. Only the DI cDNA and T7 promoter are shown. The IFN-γ-containing subgenomic RNA transcribed from the IG7 site of the genomic DI RNA is indicated. L, leader RNA. (B and C) Expression of IFN-γ by the DI vector. Culture medium was collected at different time points posttransfection from DBT cell cultures infected with JHM (A) or A59 (B) and transfected with DE-IFN-γ RNAs. IFN-γ was assayed by ELISA. The amounts of IFN-γ are the mean values and standard deviation of three independent experiments.

a multiplicity of infection (m.o.i.) of 5. At 1 hr postinfection, cells were washed with phosphate-buffered saline (PBS) and covered with 2 ml of prewarmed Eagle's minimum essential medium (MEM) containing 1% newborn calf serum (Intragen). Five to ten micrograms of *in vitro* transcribed RNAs were mixed slowly with 10 μl of DOTAP (Boehringer-Mannheim) in HBS buffer (20 m*M* HEPES; 150 m*M* NaCl; pH 7.4), and incubated at room temperature for 10 min. The mixture was then added to the cell culture. The final concentration of DOTAP was 5 μg/ml.

Enzyme-linked immunosorbent assay (ELISA) for IFN-γ

To quantitate expression of IFN-γ, medium was collected at 4, 6, 8, 10, 12, and 24 hr posttransfection from DBT cells infected with JHM or A59 and transfected with DE-IFN-γ RNA. Following centrifugation at 4000 *g* for 30 min, supernatants were tested for IFN-γ using a sandwich ELISA as previously described (Cua *et al.*, 1995). R4-6A2 (anti-IFN-γ) (American Type Culture Collection) serum-free hybridoma supernatant was used to coat 96-well plates. Biotinylated XMG-1.2 (anti-IFN-γ) was obtained from PharMingen. Avidin-peroxidase and *o*-phenylenediamine (OPD) were obtained from Sigma Chemical Co. Recombinant IFN-γ (rIFN-γ) (Zymogen) was used as ELISA standard, and the concentration of IFN-γ is reported in international units per milliliter (U/ml).

MHV replication in the presence of IFN-γ

DBT cells were seeded at a concentration of 5×10^5 cells per well into 24-well plates and incubated for 24 hr

TABLE 1

Primers/Probes for Detection of Cellular mRNAs

Gene	Primer/probe sequences (S, sense; A, antisense, P, probe)	Position (nt)
iNOS	S5′-GCCTTCCGCAGCTGGGCTGT-3′	2168 to 2187
	A5′-ATGTGTAGCACATCCCGAGCC-3′	3449 to 3427
	P5′-AGCTACTGGGTCAAAGACAAGAGGCT-3′	2597 to 2622
IGIF	S5′-ACTGTACAACCGCAGTAATACGG-3′	292 to 314
	A5′-GAGTGAACATTACAGATTTATCCC-3′	726 to 703
	P5′-GTGTTCCAGGACACAACAAGATGGAGTT-3′	586 to 613
MHVR	S5′-ATGGAGCTGGCCTCAGCACATCTC-3′	1 to 24
	A5′-CGCACAGTCGCCTGAGTACGACGA-3′	410 to 387
	P5′-TCGTGCAATTTCTTTGTCTATAGCCGT-3′	243 to 217
HPRT	S5′-GTAATGATCAGTCAACGGGGGAC-3′	[a]
	A5′-CCAGCAAGCTTGCAACCTTAACCA-3′	
	P5′-GCTTTCCCTGGTTAAGCAGTACAGCCCC-3′	

[a] The sequences for HPRT were based on Murphy *et al.* (1993).

at 37° in MEM containing 5% newborn calf serum. J774.1 cells were seeded at a concentration of 5×10^4 cells per well into 24-well plates and incubated for 24 hr at 37° in Dulbecco's modified MEM (DMEM) containing 10% fetal calf serum. Cells were treated with various concentrations of the DI-expressed IFN-γ (DE-IFN-γ) or rIFN-γ and infected with viruses at an m.o.i. of 1, 0.1, 0.01, or 0.001. After virus adsorption for 1 hr, the respective medium with or without IFN-γ was added and the cells were incubated for the indicated periods of time.

Isolation and detection of intracellular mRNAs

To study the effects of IFN-γ treatment on the expression of cellular genes [inducible nitric oxide synthase (iNOS), interferon-γ-inducing factor (IGIF), and MHV receptor (MHVR)], macrophage cells (J774.1) were grown to 90% confluence in 60-mm petri dishes and then treated with medium from cells expressing DE-IFN-γ or DE-CAT, both of which had been irradiated with UV to inactivate helper virus. At 24 and 48 hr after treatment, cells were collected and intracellular RNA was isolated as described previously (Zhang *et al.,* 1994). To determine the effects of MHV infection on the expression of cellular genes, J774.1 cells were infected with MHV-JHM virus at an m.o.i. of 0.01 at 24 hr after IFN-γ treatment. RNA was isolated at 24 hr postinfection. The RNA samples were used for synthesis of cDNAs by reverse transcription (RT) with random priming hexamers (Boehringer-Mannheim). To detect individual genes, cDNA pools were subjected to PCR amplification using gene-specific primers (Table 1). The gene encoding the housekeeping enzyme hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control. The PCR was performed for 20 cycles under the following condition: 95° for 1 min for denaturation, 56° for 1 min for annealing, and 72° for 2 min for extension. PCR products were analyzed by agarose gel electrophoresis.

Dot blot analysis

RT-PCR products were quantitated using the dot blot method previously described (Murphy *et al.,* 1993; Cua *et al.,* 1995). Briefly, PCR-amplified cDNA (10 μl) was denatured in 90 μl of denaturing solution (0.4 *N* NaOH and 25 m*M* EDTA) for 10 min and neutralized by the addition of an equal volume of 1 *M* Tris–HCl, pH 8.0. Samples were transferred to a nylon membrane via a Minifold I Dot Blot apparatus (Schleichel and Schuell), and the wells were washed with 5× SSC (4.38% sodium chloride, 2.2% sodium citrate). Membranes were air-dried and the cDNA was fixed using a Stratalinker UV oven (Stratagene). Following prehybridization [6% 10× SSC, 0.5% sodium dodecyl sulfate (SDS), 0.1 mg/ml salmon sperm DNA] at room temperature for 30 min, ^{32}P-labeled specific probes (Table 1) were added. Following hybridization at 60°, the membranes were washed three times with 2× SSC containing 0.1% SDS for 10 min, air dried, and scanned on an Ambis radioanalytic imaging system (Ambis Systems). Total counts of each duplicate sample for iNOS, IGIF, and MHVR at each time point were normalized to the control HPRT. The blots were further autoradiographed.

Mice

C57BL/6 mice were purchased at 7 weeks of age from The Jackson Laboratory. Mice were infected with 1×10^5 PFU of A59 expressing DE-IFN-γ or DE-CAT. Preliminary experiments showed no difference in virus replication in the CNS comparing parental A59 and A59 virus containing the DE-CAT vector.

Tissues and histology

Virus titers in the CNS were determined by homogenization of half of the brain in PBS followed by plaque assay on monolayers of DBT cells as previously described (Stohlman *et al.,* 1995). The remaining half of the brains were fixed in Clark's solution (75% ethanol, 25% glacial acetic acid), embedded in paraffin, and stained with hematoxylin and eosin to examine the extent of encephalitis or with the immunoperoxidase method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) using the anti-nucleocapsid monoclonal antibody J.3.3. (Fleming *et al.,* 1983) to determine the percentage of virus-infected cells.

RESULTS

Expression of IFN-γ using an MHV DI RNA vector

The murine IFN-γ gene was cloned into the MHV DI RNA vector (Liao *et al.,* 1995) under the control of the MHV IG7 sequence. The resulting RNA, DE-IFN-γ RNA, was transfected into MHV-infected cells, and the production of IFN-γ in the culture medium was detected by ELISA. As shown in Fig. 1B, when MHV-JHM was used as helper virus, IFN-γ was secreted into the medium (20 U/ml) as early as 6 hr posttransfection and increased with time. At 24 hr posttransfection, when cell monolayers were completely lysed, the amount of IFN-γ reached approximately 180 U/ml. When A59 was used as helper virus, the production of IFN-γ was detected at 80 U/ml at 6 hr posttransfection and reached a maximum (approximately 180 U/ml) earlier (at 12 hr posttransfection) (Fig. 1C), consistent with the observation that A59 replicates faster than JHM. These results indicated that MHV DI vector can be used for the production of a secreted cytokine during MHV infection *in vitro.*

Effects of DI RNA-expressed IFN-γ on MHV replication *in vitro*

IFN-γ exerts multiple biological functions both *in vitro* and *in vivo* (Trinchieri and Perussia, 1985; Pestka and Langer, 1987), but its effects on coronavirus infections have not been extensively examined. We first determined whether DI-expressed IFN-γ had antiviral effects on helper viral replication. Virus titers in the medium of DBT cells infected with JHM and transfected with DE-IFN-γ RNA were determined at various time points after infection and compared to DE-CAT RNA-transfected cells. Figure 2 shows that the virus titers in the presence of DE-IFN-γ were lower by approximately half a $\log_{10}$ compared to cultures transfected with the DE-CAT RNA. This difference was small but reproducible, suggesting that IFN-γ exerts at most a weak antiviral effect. The absence of significant anti-viral effect of IFN-γ in this system could be due to the requirement for interferon to modify host cell metabolism prior to infection or it may be that interferon acts at an early stage of viral replication.

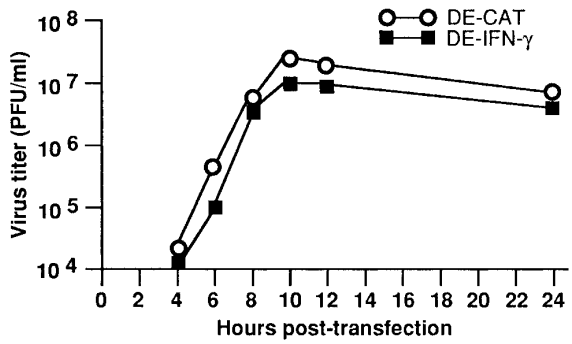

FIG. 2. Effects of expression of DE-IFN-γ on helper virus replication. Cuture medium from DBT cells infected with JHM virus and transfected with either DE-IFN-γ or DE-CAT RNA was harvested at various time points posttransfection, and virus titers were determined by plaque assays.

To distinguish these possibilities, the culture medium harvested from JHM-infected and DE-IFN-γ-transfected cells late in infection was used to infect DBT cells. This medium contained not only JHM virus but also IFN-γ (180 U/ml) (Fig. 1). Therefore, IFN-γ was present throughout the infection, beginning with the initiation of viral infection. No significant differences in virus titer released from the DE-IFN-γ- and DE-CAT-infected cells were detected (both yielded approximately 10^6 PFU/ml) (data not shown). Thus, IFN-γ has little antiviral effect even when present at the initiation of viral infection.

In view of the known mechanisms of action of IFN-α and -β, whose antiviral activities require preadsorption to cells prior to viral infection (Bianzani and Autonelli, 1989), we examined the effects of pretreatment of cells with IFN-γ prior to infection. For this study, the culture medium from JHM-infected and DE-IFN-γ-transfected cells was UV-irradiated to inactivate infectious virus and then used as a source of IFN-γ to pretreat DBT cells. Twenty-four hours later, cells were infected with JHM or A59 virus at m.o.i.'s ranging from 0.1 to 0.001 in the continual presence of DE-IFN-γ. Virus titers were determined at 24 hr postinfection. As shown in Fig. 3A, DE-IFN-γ exhibited a slight inhibitory effect on JHM replication (approximately 1 $\log_{10}$ reducation in virus titer), when an m.o.i. of 0.001 was used; similar results were obtained with A59 virus (Fig. 3A), suggesting that pretreatment of cells with IFN-γ prior to viral infection induces an antiviral state. This inhibitory effect was less pronounced when higher m.o.i.'s were used (data not shown), suggesting that the observed antiviral activity was weak and could be overcome by a higher virus titer.

To further establish that the antiviral effect was due to the specific effects of IFN-γ, the UV-inactivated DE-IFN-γ preparation was preincubated for 2 hr with a hamster neutralizing monoclonal antibody specific for rIFN-γ. Antiviral effects were completely blocked by this treat-

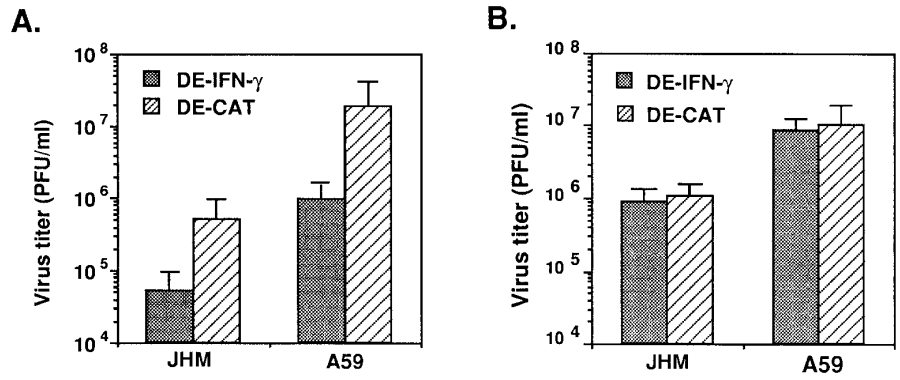

FIG. 3. Effects and specificity of DE-IFN-γ pretreatment on MHV infection. (A) Supernatants from DBT cell cultures infected with JHM virus and transfected with either DE-IFN-γ or DE-CAT RNA were harvested at 24 hr posttransfection and subjected to UV irradiation to inactivate virus. The UV-irradiated supernatants were used either as a source of IFN-γ or as a control (CAT) to pretreat cells for 24 hr, and the cells were then infected with either JHM or A59 at an m.o.i. of 0.001. After virus adsorption, cells were incubated with the same supernatants for 24 hr, and the virus titers in culture medium at 24 hr postinfection were determined by a standard plaque assay. (B) Neutralization assay of IFN-γ. Both UV-irradiated supernatants (IFN-γ and CAT) were incubated with 1 μg/ml of a hamster anti-IFN-γ neutralizing monoclonal antibody for 2 hr at room temperature prior to being used for pretreatment of cells. Subsequent procedures were the same as in (A).

ment (Fig. 3B), demonstrating that IFN-γ, but not the replication of the DI vector itself, was responsible for the antiviral activity. These combined results suggest that IFN-γ has a weak antiviral effect, which was evident only when cells were pretreated with IFN-γ prior to infection.

The relatively weak antiviral effects of IFN-γ also could be due to the possibility that DBT cells do not respond well to IFN-γ. Since it is known that macrophages are particularly sensitive to IFN-γ treatment (Ijzermans and Marquet, 1989), we further determined the inhibitory effects of IFN-γ on MHV replication in an MHV-susceptible macrophage cell line (J774.1). J774.1 cells were pretreated with various concentrations of rIFN-γ for 24 hr before and throughout virus infection. As shown in Fig. 4, both A59 and JHM were inhibited by rIFN-γ by 1 to 2

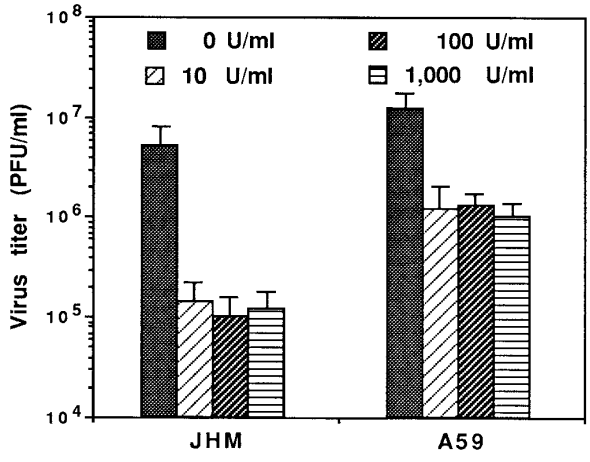

FIG. 4. Effects of rIFN-γ on MHV replication in a macrophage cell line (J744.1). Cells were pretreated with rIFN-γ at various concentrations (0, 10, 100, and 1000 U/ml) for 24 hr. After infection with JHM or A59, cells were incubated for an additional 24 hr in the presence of rIFN-γ at the same concentrations, and the virus titers (PFU/ml) in the culture medium were determined by a standard plaque assay.

$\log_{10}$, similar to the data obtained with DBT cells. Thus, the absence of strong antiviral effects of IFN-γ is not due to nonresponsiveness of cells to IFN-γ.

DI RNA-expressed IFN-γ prevents virus spread

The results described above indicated that antiviral effects of IFN-γ could be demonstrated only when cells were pretreated with IFN-γ before viral infection and when a low m.o.i. was used. They suggested the possibility that IFN-γ could prevent virus spread, if virus initially infects only a small number of cells. To establish an *in vitro* model for studying the potential effects of IFN-γ in preventing virus spread, UV-irradiated culture medium from DE-IFN-γ-transfected cells, which contained IFN-γ at 180 U/ml, was mixed with a very low titer of JHM virus at approximately one infectious particle in each well of a 24-well plate. Cells were observed for cytopathic effects daily for 4 days and the number of fusion plaques was counted. Results of these experiments are presented in Table 2. The number of plaques increased more slowly when the DE-IFN-γ was present (for example, from 1 plaque on Day 1 to 12 plaques on Day 4), as compared to those in the control wells, in which DI-expressed CAT preparation was used (i.e., from 1 plaque on Day 1 to 30 plaques on Day 2 and too numerous to count by Day 3) (Table 2). Initially, the plaque sizes in the presence of IFN-γ were indistinguishable from those of the control wells (data not shown); however, by Day 3 or 4 postinfection, while all plaques in the IFN-γ-treated cultures remained of uniform size, plaques in the absence of IFN-γ became numerous and heterogeneous in size (Fig. 5). These data suggest that the cells were infected at different time points throughout the incubation period and that DE-IFN-γ prevents virus spread to neighboring uninfected cells. However, these differences were not observed when a higher m.o.i. was tested, possibly

TABLE 2

Effects of DI-Expressed IFN-γ on Virus Spread

Treatment[a]	Well[b] No.	No. of plaques[c] on Day 1	Day 2	Day 3	Day 4
DE-IFN-γ	1	1	5	7	12
	2	2	3	9	24
	3	1	6	10	25
	4	1	3	7	18
DE-CAT	1	1	30	UC[d]	UC
	2	1	25	UC	UC
	3	0	3	40	UC
	4	1	18	UC	UC

[a] Medium from cell cultures infected with MHV-A59 and transfected with either DE-IFN-γ RNA or DE-CAT RNA was harvested at 16 hr posttransfection and UV-irradiated to completely inactivate infectious virus. One milliliter of each culture medium was then mixed with JHM virus and added to the cell monolayers, so that an average of 1 PFU per well was present.

[b] Each sample was quadruplicated in 4 wells of a 24-well plate.

[c] Plaques were counted in the liquid medium using a light microscope.

[d] UC, uncountable due to extensive cytopathic effects and detachment of cells.

due to the rapid spread of progeny virus before IFN-γ exhibited its antiviral effect (data not shown). Similar results were obtained when various concentrations of rIFN-γ (50, 100, and 150 U/ml) were used, suggesting that 50 U/ml rIFN-γ is sufficient to prevent virus spread *in vitro* (data not shown).

Comparison of MHV variants for sensitivity to IFN-γ treatment

Sensitivity of different JHM variants to IFN-γ treatment *in vitro* was assessed in an effort to determine whether the IFN-γ sensitivity correlates with the pathogenicity of the virus *in vivo.* Three JHM variants with different degrees of neurovirulence were used: DL (LD_{50} 1–5 PFU), DS (LD_{50} 100–200 PFU), and 2.2-V-1 (LD_{50} 2000–10,000 PFU) (Stohlman *et al.,* 1982, 1995; Fleming *et al.,* 1986, 1987). DL causes little demyelination and infects predominantly neurons whereas variant 2.2-V-1 causes extensive demyelination and infects predominantly glial cells with a particular tropism for oligodendrocytes. Variant DS causes less demyelination than variant 2.2-V-1. DBT cells pretreated with IFN-γ (180 U/ml) for 24 hr were infected, and the same concentrations of IFN-γ were maintained throughout the infection. At 24 hr postinfection, culture medium was collected and virus titer determined by plaque assay. As shown in Fig. 6, a reduction of approximately 2.5 $\log_{10}$ in virus titer was found for 2.2-V-1, 2 $\log_{10}$ for DS, and 1 $\log_{10}$ for DL. Therefore, variant 2.2-V-1 is most sensitive to IFN-γ treatment whereas variant DL is most resistant, suggesting a rough correlation between the virulence of these JHM variants and sensitivity to IFN-γ.

Selective induction of cellular genes by MHV DI RNA-expressed IFN-γ

It has been suggested that IFN-γ induces a number of cellular proteins and enzymes which either act as endoribonucleases to degrade viral RNAs or interfere with viral protein synthesis by blocking the initiation of translation of virus-specific mRNAs (Pestka and Langer, 1987). To investigate whether the MHV DI RNA-expressed IFN-γ can modify the expression of specific cellular proteins, we analyzed the expression of three cellular genes in J774.1 cells before and after IFN-γ treatment. iNOS is a cellular enzyme associated with the antiviral function of TNF and IFN, both of which induce iNOS expression in macrophages (Lyons *et al.,* 1992). IGIF (IL-1γ) is a cytokine secreted from Kupffer cells and activated macrophages, and it induces IFN-γ expression in T cells (Okamura *et al.,* 1995). MHVR is a member of the biliary glycoprotein (BGP)/carcinoembryonic antigen (CEA) family and serves as a receptor for MHV infection (Williams *et al.,* 1991). Treatment of cells with DI-expressed IFN-γ for 24 hr increased the expression of iNOS and IGIF mRNAs. MHV infection did not affect the expres-

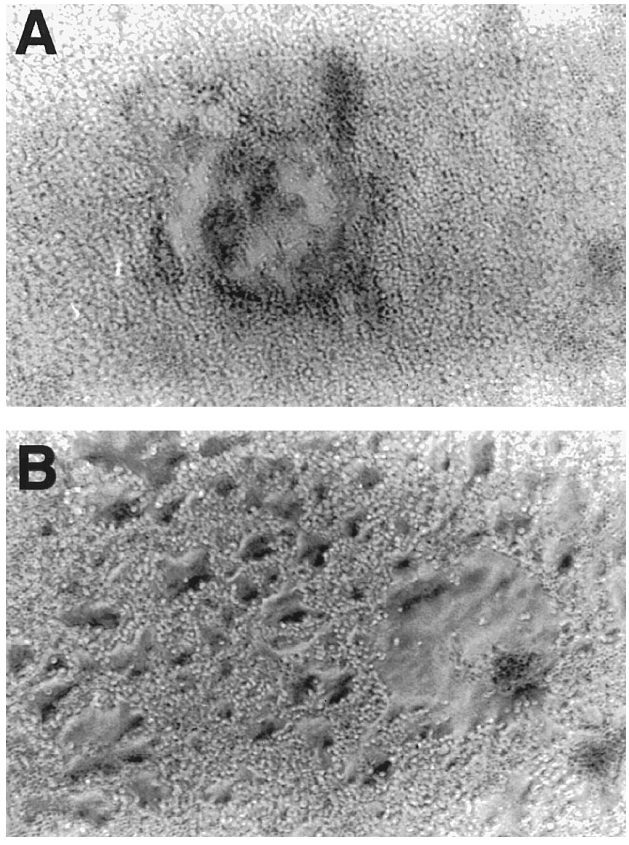

FIG. 5. Morphology of viral plaques in the presence of DI-expressed IFN-γ. Approximately 1 PFU of JHM virus and 180 U/ml of DI-expressed IFN-γ were added to each well of DBT cells in a 24-well plate, and the cytopathic effects were observed on Day 3 postinfection in liquid culture using a light microscope and photographed. Original magnifications, ×100. (A) In the presence of DE-IFN-γ. (B) In the presence of DE-CAT.

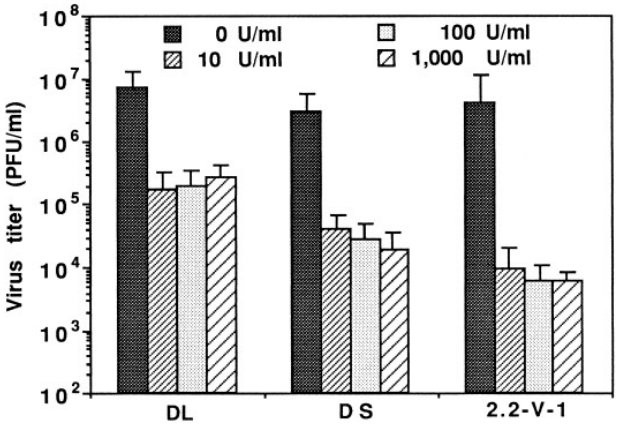

FIG. 6. Effects of IFN-γ on the replication of various MHV-JHM variants in DBT cells. Cells were pretreated with rIFN-γ at various concentrations (0, 10, 100, and 1000 U/ml) for 24 hr and infected with JHM variants (DL, DS, or 2.2-V-1) at an m.o.i. of 0.01, followed by an additional incubation with IFN-γ at the same concentrations. The virus titers (PFU/ml) in the culture medium at 24 hr postinfection were determined by a standard plaque assay.

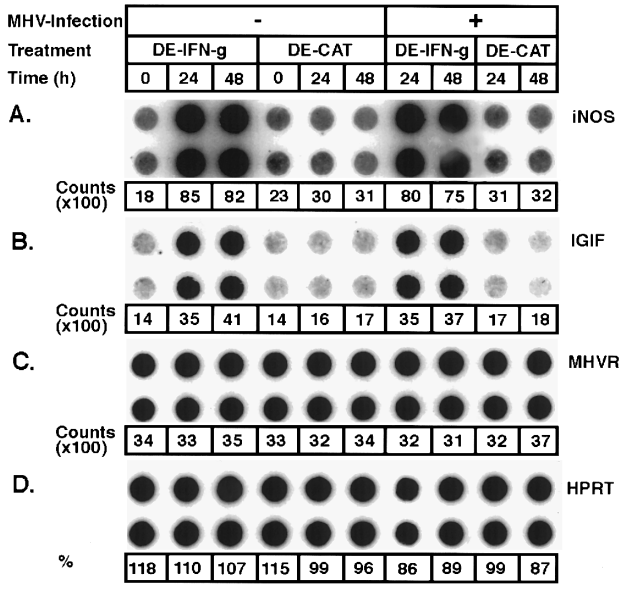

FIG. 7. Effects of DE-IFN-γ treatment on the expression of selected cellular genes. (A) Inducible nitric oxide synthase (iNOS) gene. (B) Interferon-γ-inducing factor (IGIF) gene. (C) Mouse hepatitis virus receptor (MHVR) gene. (D) Hypoxanthine phosphoribosyltransferase (HPRT) gene. Macrophage cell line J774.1 was treated with either DI-IFN-γ or DI-CAT preparation and collected at 0, 24, and 48 hr after treatment. A subset of cells was infected with MHV at 24 hr after treatment and incubated for an additional 24 hr (48 hr after treatment). Cellular RNAs were isolated and amplified by RT-PCR and detected by dot blot with gene-specific ^{32}P-labeled probes (see Materials and Methods for details). Blots (duplicate samples) were scanned on an Ambis radioanalytic imaging system and the total counts of radioactivities in each dot were quantitated. The counts shown in A, B, and C represent the average of the duplicates for each sample ($\times$100) and are adjusted by the factors shown at the bottom of each sample in D for the HPRT as an internal control.

sion of either gene. In contrast, the level of MHVR mRNA was not significantly affected by the IFN-γ treatment nor by MHV infection (Fig. 7C). Therefore, the MHV DI RNA-expressed IFN-γ is biologically active and selectively induces the expression of some cellular genes. Furthermore, the antiviral effect of IFN-γ is not mediated by alteration of MHVR expression.

Expression of IFN-γ alters MHV pathogenicity in mice

To determine if the DE-IFN-γ vector could alter MHV pathogenicity *in vivo,* groups of C57BL/6 mice were infected with 1×10^5 PFU of A59 virus containing either DE-IFN-γ or DE-CAT. Preliminary experiments showed no difference in virus replication in CNS between mice infected with parental A59 virus and those infected with A59-DE-CAT (data not shown). At 6 days postinfection, four mice in each group were sacrificed and the brains were examined for MHV titer and histological changes. The remaining mice in each group were monitored daily for survival. Table 3 shows that there was approximately 2.4 $\log_{10}$ less virus in the CNS of mice infected with A59 expressing DE-IFN-γ vector compared to the mice infected with A59 expressing DE-CAT vector. Correspondingly, all the mice infected with DE-IFN-γ-expressing A59 survived the entire 21-day observation period. By contrast, only one mouse in the group receiving DE-CAT survived to 21 days postinfection. Histological examination showed that there was much less viral antigen in the CNS of mice infected with the DE-IFN-γ-containing virus (Fig. 8). This finding and the lower virus titer in the CNS in this group of mice are consistent with the antiviral effect of IFN-γ. However, both DE-IFN-γ- and DE-CAT-expressing viruses infected the same cell types, i.e., neuron, glial cell, and microglial cell populations. Significantly, while the brains of the DE-CAT-expressing group showed only small numbers of perivascular and subarachnoid mononuclear cells, the brains of the DE-IFN-γ-expressing group showed widespread meningomyeloencephalitis with prominent perivascular cuffs, infiltration of mononuclear cells into the parenchyma, and subarachnoid infiltrates (Fig. 8). This result supports the immunostimulatory effects of IFN-γ. Although this experiment used only a small number of mice, the data suggest that expression of immunomodulatory molecules from the DI vector can alter the pathogenesis of MHV-induced disease.

TABLE 3

Effects of DI-Expressed IFN-γ on Viral Pathogenesis

Inoculum[a]	Virus titer[b]	Live/dead[c]
A59-DE-IFN-γ	2.71 $\pm$ 1.92	4/0
A59-DE-CAT	5.28 $\pm$ 1.25	1/3

[a] Mice were infected intracerebrally with 1×10^5 PFU in a volume of 32 μl.

[b] Virus titer $\times$ $\log_{10}$ PFU/g brain at Day 6 postinfection.

[c] Live/dead determined at 21 days postinfection.

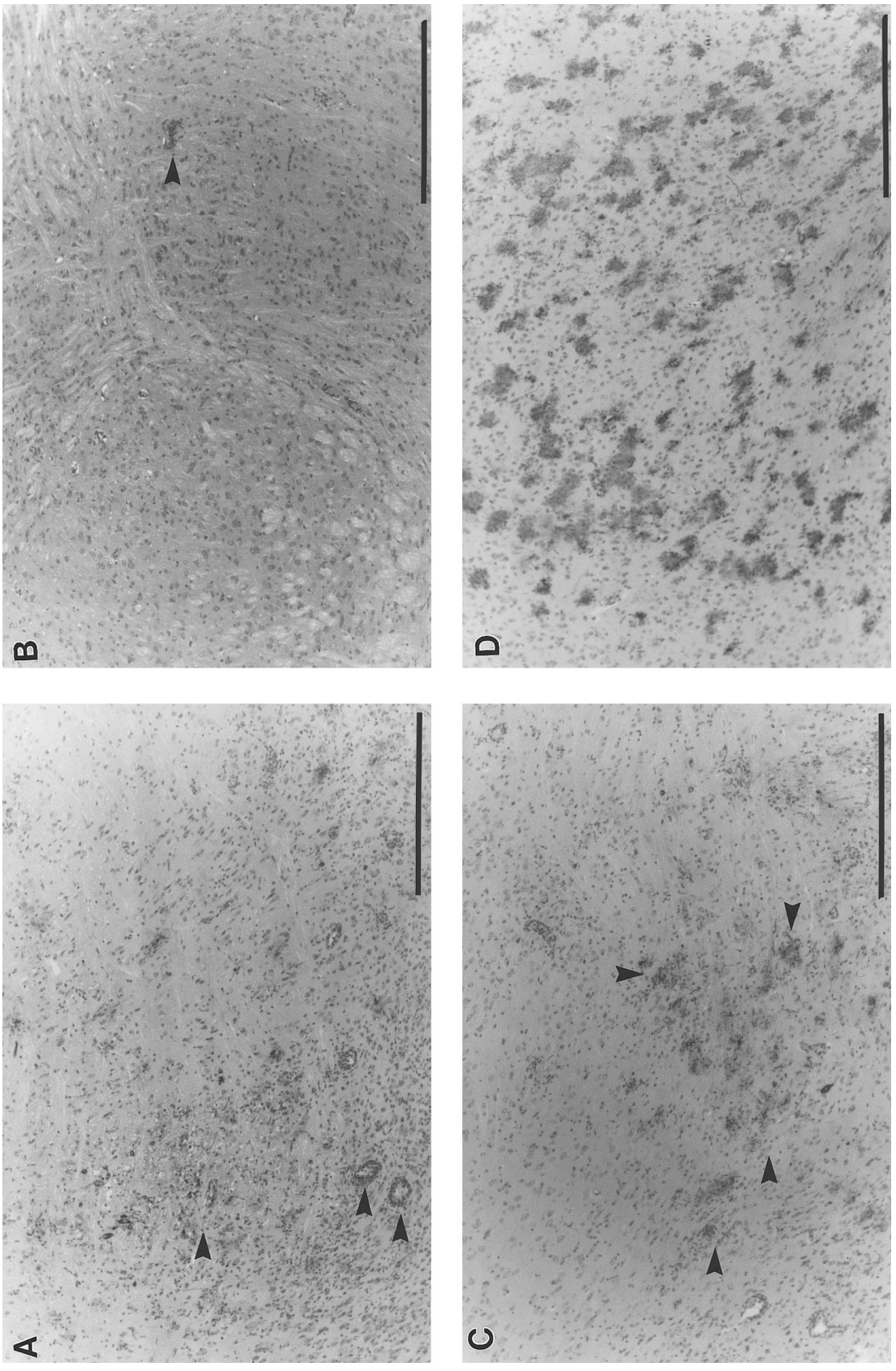

FIG. 8. The effect of DE-IFN-γ vector on the pathogenicity of MHV. C57BL/6 mice were infected with 1×10^5 PFU of A59 virus pools containing DE-IFN-γ or DE-CAT. Four mice from each group were sacrificed at Day 6 postinfection and half of each brain was fixed and embedded in paraffin for histology. Hematoxylin and eosin stained sections (A, B) show that there is prominent encephalitis in the DE-IFN-γ mice (A), which is characterized by the presence of multifocal perivascular and parenchymal mononuclear cell infiltrates (arrowheads), while the DE-CAT mice (B) show only rare mild perivascular infiltrates (arrowheads). Immunoperoxidase-stained (ABC method) sections for viral antigen using anti-N antibody J.3.3 in the DE-IFN-γ mice (C) demonstrate only occasional small foci of antigen-containing cells within the brain (arrowheads), while numerous foci of antigen-containing cells are present throughout the brain in the DE-CAT mice (D). (Bar, 400 μm).

DISCUSSION

This study demonstrates that the MHV DI RNA system can be utilized as a vector to express the IFN-γ gene and that the IFN-γ protein is translated and secreted from infected cells as a biologically active molecule. These data represent the first successful attempt to express a mammalian cellular gene product using a coronavirus DI RNA vector. Thus far, we have demonstrated the feasibility of this DI RNA system for expressing a prokaryotic bacterial gene CAT (Liao and Lai, 1994), a viral structural protein gene HE (Liao *et al.,* 1995), and the mammalian cellular gene IFN-γ (this report). These studies showed a broad range of usage of this DI RNA system for expressing various genes of interest.

Currently, an infectious, full-length cDNA clone of MHV RNA is not available; therefore, it is difficult to unequivocally elucidate the mechanism of pathogenesis of MHV at the molecular level. The development of a DI RNA expression system thus provides an alternative approach, allowing the expression of both viral and cellular genes to be manipulated. Further, this system allows expression of heterologous gene products at the site of viral replication. This system has an advantage over the passive administration of cytokines for studying viral pathogenesis, since cytokines usually have a short half-life, making it difficult to maintain high local concentrations at the site of infection. One drawback of the DI system, however, is its limited expression. The DI RNA cannot be packaged beyond the fourth passage *in vitro* (data not shown). We have attempted to increase retention of the DI RNA via incorporation of a packaging signal. However, the expression level of the gene product was reduced; no significant retention was found (Lin and Lai, 1993). Nevertheless, our data indicated that, during the first several passages, the expression level of IFN-γ was such that a sufficiently high level of IFN-γ can be maintained locally at the beginning of viral infection.

The virulence of several MHV variants correlates with their resistance to IFN-γ treatment, suggesting that IFN-γ may play a role in the pathogenesis of MHV. An earlier study analyzed the effects of IFN-γ during JHM infection using passive transfer of an anti-IFN-γ-antibody (Smith *et al.,* 1991). This treatment significantly enhanced virus replication and resulted in a higher mortality with decreased survival times. IFN-γ treatment of macrophages from A/J mice rendered them partially resistant to MHV3 infection, whereas the macrophages from susceptible BALB/c mice did not respond to IFN-γ, suggesting that the resistance of mice to MHV3 infection involves the sensitivity of macrophages to IFN-γ (Lucchiari *et al.,* 1991; Vassao *et al.,* 1994a,b). IFN-γ was also shown to be more effective than IFN-α/β in inducing an antiviral state in macrophages infected with MHV (Vassao *et al.,* 1994a). These reports support the notion that IFN-γ may play a role in MHV infection.

The molecular basis for the relative IFN resistance of different MHV strains is not yet known. Previous studies have shown that the neutralization-escape mutant 2.2-V-1 of JHM strain has a single nucleotide mutation at position 3340 of the S gene, which results in a leucine to phenylalanine substitution (Wang *et al.,* 1992). Whether this single mutation affects the sensitivity of the virus to IFN-γ remains unclear. In lymphocytic choriomeningitis virus, resistance of various virus strains to IFN-α/β or IFN-γ *in vitro* correlates with their ability to establish persistent infections in adult immunocompetent mice (Moskophidis *et al.,* 1994). One possibility is that IFN resistance allows enhanced viral replication and spread, facilitating exhaustion of antiviral CTL, thereby resulting in virus persistence. Whether MHV utilizes a similar mechanism to modulate its infection in mice is an interesting issue. Correlation between IFN resistance and viral pathogenicity has also been documented for measles virus, adenovirus, and herpes simplex virus type I (Carrigan and Kehl-Knox, 1990; Su *et al.,* 1990; Kalvakolanu *et al.,* 1991).

The *in vitro* experiments showed that the DI-expressed IFN-γ had inhibitory effects on virus spread from initially infected cells to neighboring uninfected cells. The inhibitory effect was more pronouced at a lower m.o.i., which apparently allowed sufficient time for IFN-γ to activate an antiviral state in adjacent uninfected cells. Pretreatment of cells (astrocytoma and macrophages) with IFN-γ is required to induce an antiviral state (Figs. 3 and 4), consistent with previous findings from studies of primary mouse macrophages (Lucchiari *et al.,* 1991) and other target cells (Lewis, 1982). Expression of both iNOS and IGIF mRNA in macrophages was induced by IFN-γ. However, whether these molecules mediate the antiviral effects of IFN-γ is not clear. Recently, it was demonstrated that iNOS expression did not play a significant role in the pathogenesis of the MHV OBLV60 strain (Lane *et al.,* 1997). Nevertheless, we can conclude from our study that the antiviral effects of IFN-γ are not mediated by down-regulation of MHVR. The precise mechanism of the antiviral effects of IFN-γ will require additional studies, as there appears to be discordance between the antiviral effects of NO *in vivo* and its effects *in vitro* (Lane *et al.,* 1997).

The alteration of A59 neuropathogenesis by DE-IFN-γ provides further support for the significance of IFN-γ in MHV infection. Inhibition of IFN-γ action by passive transfer of antibody (Smith *et al.,* 1991) enhanced virus replication and increased mortality, suggesting that local production of IFN-γ by infiltrating leukocytes is a critical component of the host response to MHV infection. In our experiments, the production of IFN-γ by DE-IFN-γ resulted in an exaggeration of the host response with more prominent encephalitis, improved viral clearance, and decreased mortality. The increased encephalitis may, in turn, induce local cytokine production and CTL

activity. Altogether, these data demonstrated that IFN-γ plays a critical role at least early in A59 infection. The longer-term consequences of DE-IFN-γ expression, however, cannot be definitively determined from this study because most of the DE-CAT-infected mice died. Examination of the single DE-CAT survivor and the four surviving DE-IFN-γ mice at 21 days postinfection showed no apparent differences, with both groups exhibiting mild encephalitis, moderate demyelination, and focally residual viral antigen (data not shown). The absence of differences was probably due to the fact that IFN-γ was expressed from the DI vector for only a brief period of time. The current studies confirm the validity of using the DI vector system for studying MHV pathogenesis *in vivo*. Expressing immune regulatory proteins at the site of viral infection may provide insights into the pathogenesis of MHV infection.

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