JOURNAL OF VIROLOGY, Jan. 2007, p. 332–348 Vol. 81, No. 1
0022-538X/07/$08.00+0 doi:10.1128/JVI.01592-06

Cytomegalovirus Induces Interferon-Stimulated Gene Expression and Is Attenuated by Interferon in the Developing Brain▽

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Received 25 July 2006/Accepted 12 October 2006

Cytomegalovirus (CMV) is considered the most common infectious agent causing permanent neurological dysfunction in the developing brain. We have previously shown that CMV infects developing brain cells more easily than it infects mature brain cells and that this preference is independent of the host B- and T-cell responses. In the present study, we examined the innate antiviral defenses against mouse (m) and human (h) CMVs in developing and mature brain and brain cells. mCMV infection induced interferon (IFN)-stimulated gene expression by 10- to 100-fold in both glia- and neuron-enriched cultures. Treatment of primary brain cultures with IFN-α, -β, and -γ or a synthetic RNA, poly(I:C), reduced the number of mCMV-infected cells, both in older cells and in fresh cultures from embryonic mouse brains. When a viral dose that killed almost all unprotected cells was used, IFN-protected cells had a natural appearance, and when they were tested with whole-cell patch clamp recording, they appeared physiologically normal with typical resting membrane potentials and action potentials. mCMV infection increased expression of representative IFN-stimulated genes (IFIT3, OAS, LMP2, TGTP, and USP18) in both neonatal and adult brains to similarly large degrees. The robust upregulation of gene expression in the neonatal brain was associated with a much higher degree of viral replication at this stage of development. In contrast to the case for downstream gene induction, CMV upregulated IFN-α/β expression to a greater degree in the adult brain than in the neonatal brain. Similar to the case with cultured brain cells, IFN treatment of the developing brain in vivo depressed mCMV replication. In parallel work with cultured primary human brain cells, IFN and poly(I:C) treatment reduced hCMV infection and prevented virus-mediated cell death. These results suggest that coupling IFN administration with current treatments may reduce CMV infections in the developing brain.

Cytomegalovirus (CMV), a double-stranded DNA virus of the betaherpesvirus family, is considered the most common infectious agent that causes permanent neurological dysfunction of the developing brain (2, 62). Human CMV (hCMV) infections in the developing brain can lead to mental retardation, epilepsy, microcephaly, microgyria, hydrocephalus, and deafness (4, 21, 37). The developing brain is particularly sensitive to CMV infection (6, 29, 54, 55, 58) due to both the immature state of the systemic immune system and a preference of CMV for developing glia and neurons (61). CMV in the mature brain is less of a concern, except in immunocompromised individuals (22, 23, 32).

Outside the brain, interferon (IFN) has been reported to limit some CMV infections (27, 30, 66). A number of reports have suggested that brain cells differ from cells of other organs relative to innate and systemic antiviral immune responses. The brain has some characteristics of an immunologically privileged region. For instance, although most cells outside the brain express major histocompatibility complex (MHC) class I molecules, neurons in the brain either do not express MHC class I or do so at a substantially reduced level (45). This may be beneficial due to the fact that neurons do not replicate, and so a vigorous attack by the immune system on virally infected postmitotic neurons may cause more problems than the virus itself. In the brain, functional receptors for IFN-γ are expressed in all cells, but they are expressed at different levels, and actions of IFN-α/β on microglia, astrocytes, and neurons have also been described (12, 13, 31, 43). Most cells, including astrocytes and microglia, are able to produce IFN-α/β as an initial nonspecific immune response against virus infection (52, 64); IFN-γ is released by activated T lymphocytes as part of the specific immune response. The intrinsic IFN system provides the first line of defense against viral infections, including systemic CMV infections, and can delay viral replication allowing the activation of the systemic adaptive immune responses.

One explanation for the increased pathogenesis in the developing brain compared to that in the mature brain is that the systemic immune system is too immature during development to fight CMV infection. T and B lymphocytes of the adaptive immune system, as well as other cells of the systemic immune system, including natural killer cells and macrophages/monocytes, are involved in combating CMV infections (2, 5, 8, 9, 10, 20, 46, 49), and the efficacies of these systems increase with development. Another mechanism that might underlie the susceptibility of developing brain cells to CMV infections is that the innate cellular immune response to viral infection may not yet be fully functional in the immature brain cells. In the present work, we tested the hypothesis that developing brain cells show an attenuated response to CMV infection by monitoring activation of IFN expression and genes downstream of

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▽ Published ahead of print on 25 October 2006.

IFN and found that CMV induced strong expression of IFN-stimulated genes in developing and mature brain cells. We also asked whether IFN treatment might ameliorate a CMV infection in developing brain cells in vitro and in vivo and determined that neurons and glia are protected from CMV infection by IFN and that protected cells appear to function normally. These results indicate a critical role for the cellular IFN response in the control of CMV replication in the brain.

MATERIALS AND METHODS

Brain inoculation. Adult mice were anesthetized with a mixture of ketamine and xylazine, and a Hamilton microsyringe was used to inject mouse CMV (mCMV) into the brain (1.75 μl). Neonatal mice were anesthetized with hypothermia and injected similarly. All mice were given 2×10^4 PFU/g body weight; weighing the brains of postnatal day 1 (P1) and adult mice showed that brain weight was roughly proportional to body weight. The use of mice for these experiments was approved by the Yale University Committee on Animal Use.

Tissue culture. The brains of embryonic day 15 (E15 to E18) mice were harvested, disaggregated with papain treatment, and plated on polylysine-coated glass or plastic dishes, as previously described (57). For mixed neuron and glia cultures, cells were maintained in Dulbecco's minimal essential medium with 10% fetal calf serum. Glia-enriched cultures were generated from brains of postnatal day 5 mice and grown under the same culture conditions. Neuron-enriched cultures were grown from E19 mouse brains by using Neurobasal medium supplemented with B27 (Gibco); this medium supports the growth of neurons but not glia. In all culture experiments used for RNA analysis, at least three independent cultures were used per group.

Human primary brain cells were obtained from tissue collected from patients undergoing treatment for temporal lobe epilepsy; these tissues were generously provided by D. Spencer (Yale University). Tissue blocks were cut into small cubes and plated onto Millipore filter insets or poly-L-lysine-coated coverslips for primary culture. Cells were harvested and propagated in minimal essential medium with 10% fetal bovine serum. Patient welfare was the sole rationale for surgery. Use of tissue after surgery was approved by the Yale University Human Investigation Committee. All cells used were primary cultures; no cell lines that may behave differently from primary cells were used in any experiment.

An mCMV that expressed a green fluorescent protein (GFP) reporter was used as described elsewhere (57); the sequence coding for GFP was inserted at the ie2 site, a position dispensable for growth and latency of mCMV (11). mCMV stocks were grown on NIH 3T3 cells. The hCMV (kindly provided by J. Vieira) used also had a GFP reporter that was expressed in infected cells; this hCMV has been described elsewhere (25). hCMV stocks were grown on normal human fibroblasts. Stocks of virus were kept frozen at −80°C; the titers of the CMVs were determined with plaque assays.

Whole-cell patch clamp electrophysiology. To determine whether IFN treatment protected neurons from mCMV infection and allowed neurons to maintain their normal electrophysiological characteristics, whole-cell recording was used to study mouse brain cultures that were inoculated with mCMV. Cells were selected with an Olympus upright BX51 microscope fitted with fluorescence and differential interference infrared illumination. Cells plated on polylysine-coated round coverslips were mounted in a perfusion bath chamber on an immovable stage, and the microscope itself was mounted on rails and moved by rotary micrometers to increase stability during whole-cell recording. The bath solution (pH 7.4, with NaOH) in most experiments contained the following chemicals at the indicated concentrations (in mM): NaCl, 124; KCl, 3; $MgCl_2$, 2; $CaCl_2$, 2; NaH_2PO_4, 1.23; $NaHCO_3$, 26; and glucose, 10. The pipette solution (pH 7.3, with KOH) in most experiments contained the following chemicals at the indicated concentrations (in mM): $KMeSO_4$, 130; $MgCl_2$, 1; HEPES, 10; EGTA, 1.1; Mg-ATP, 2; Na_2-GTP, 0.5; and Na_2-phosphocreatin, 10. Voltage and current clamp experiments were performed with an EPC9 amplifier controlled by Pulse acquisition software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Details of whole-cell recording can be found elsewhere (60, 63).

Quantitative real-time RT-PCR. One microgram of total RNA prepared from cells or snap-frozen mouse brain tissue was reverse transcribed by random hexamer priming using the TaqMan reverse transcription (RT) kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed on an Applied Biosystems 7500 real-time PCR system as follows. Duplicate 25-μl PCRs contained 100 ng reverse-transcribed RNA, 12.5 μl SYBR green reaction mix (Applied Biosystems), and 200 nM sense and antisense primers. After an initial incubation at 95°C for 5 min, PCR amplification was performed by cycling 50 times for 1 min at 95°C followed by 1 min at 60°C. Gene expression was normalized to expression of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and quantified using the 7500 System sequence detection software (Applied Biosystems). Representative results showing expression of the 2′5′-oligoadenylate synthetase gene (OAS) in four control brains and four mCMV-infected neonatal brains are shown in Fig. 6A. The primers used for PCR were as follows: for the GAPDH gene, 5′-TCTGGAAAGCTGTGCCGTG-3′ (sense) and 5′-CCAGTGAGCTTCCCGTTCAG-3′ (antisense); for ISG15, 5′-CAATG GCCTGGGACCTAAA-3′ (sense) and 5′-CTTCTTCAGTTCTGACACCGTC AT-3′ (antisense); for OAS, 5′-GATGTCAAATCAGCCGTCAA-3′ (sense) and 5′-AGTGTGGTGCCTTTGCCTGA-3′ (antisense); for TGTP, 5′-CAGCCCAC AAGCGTCA-3′ (sense) and 5′-TGGAATGGTGGCTAATGCTC-3′ (antisense); for IE1, 5′-GTTACACCAAGCCTTTCCTGGAT-3′ (sense) and 5′-TG TGTGGATACGCTCTCACTCTCTAT-3′ (antisense); for GFAP, 5′-AACAAC CTGGCTGCGTATAGA-3′ (sense) and 5′-ACTTCCTCCTCATAGATCTT C-3′ (antisense); for NSE, 5′-GATGGTGATCGGTATGGATG-3′(sense) and 5′-GACAAAGTCCTGGTAGAGTG-3′ (antisense); for the interferon-induced protein with tetratricopeptide repeats 3 gene (IFIT3), 5′-GGGAAACTACGC CTGGATCTACT-3′ (sense) and 5′-CATGCTGTAAGGATTCGCAAAC-3′ (antisense); for LMP2, 5′-GAGCTACACGGGTTGGA-3′ (sense) and 5′-ATG AGATGCGCTAACAAGTC-3′ (antisense); for USP18, 5′-CTCCGGCTTGTG TAGACTCT-3′ (sense) and 5′-GGGACTGGCGGTGACT-3′ (antisense); for IFN-β, 5′-CGCCTGGATGGTGGTC-3′ (sense) and 5′-AGTCCGCCTCTGAT GCTTA-3′ (antisense); and for IFN-α1, 5′-GCTACTGGCCAACCTGCTC-3′ (sense) and 5′-CTGCGGGAATCCAAAGTC-3′ (antisense).

For a further confirmation of our quantitative PCR data described above, we also ran a Northern blot analysis to examine the induction of mRNA expression by IFN. Ten micrograms of total RNA was run in each lane in a 1% formaldehyde-agarose gel along with RNA molecular weight markers. A blot from this gel was hybridized sequentially overnight first with a ^{32}P-labeled OAS probe and subsequently with a radiolabeled mouse β-actin probe. Further details relating to the Northern blotting methods are found elsewhere (56).

Cell viability. Normal human astrocytes (Sciencell, San Diego, CA) were plated at a density of 5,000 cells per well in 96-well dishes and incubated overnight before medium (0.1 ml per well) was replaced for pretreatment with IFN-α, poly(I:C), or ganciclovir. Viability was assessed using an MTT assay (Molecular Probes) according to the manufacturer's instructions. Optical density was read at 570 nm by using a Dynatech MR500 enzyme-linked immunosorbent assay plate reader (Dynatech Lab Inc., Alexandria, VA) and corrected from background control. Each condition was tested in quadruplicate.

Immunocytochemistry for IRF3 and OAS. For interferon regulatory factor 3 (IRF3) and OAS immunocytochemistry, monolayer human brain cultures on glass coverslips were incubated with a 1:100 dilution of polyclonal rabbit anti-IRF3 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:100 dilution of rabbit anti-OAS antiserum (Abgent Inc., San Diego, CA), followed by detection with a 1:200 dilution of donkey anti-rabbit immunoglobulin–Alexa Fluor 594 (Invitrogen-Molecular Probes, Eugene, OR).

Time-lapse microscopy of hCMV infections. Primary cultures from normal human brains were plated onto 35-mm culture dishes with thin glass bottoms (MatTek Co., Ashland, MA). Cultures were grown in Dulbecco's minimal essential medium containing 10% fetal bovine serum. Before reaching confluence, medium was changed to Leibovitz's L-15 medium (Gibco) containing 10% fetal bovine serum and pretreatment drugs consisting of human IFN-α, poly(I:C), or ganciclovir. Recombinant GFP-expressing human CMV was added to reach a multiplicity of infection (MOI) of 1. The dish was sealed and placed onto a heated (37°C) stage of an Olympus IX-81 fluorescence microscope. Focus, field coordinates, shutters, filters, and Hamamatsu digital camera were all computer controlled by using Slidebook software (Intelligent Imaging Innovations Inc., Denver, CO). Images were recorded for GFP (hCMV) and phase contrast at 6-min intervals. Total recording periods ranged between 50 and 70 h.

Drugs and chemicals. Human IFN-αA/D (universal type I IFN hybrid, catalog no. I4401), mouse IFN-β (catalog no. I9032), mouse IFN-γ (catalog no. I4777), and ganciclovir (catalog no. G2536) were obtained from Sigma-Aldrich (St. Louis, MO); poly(I:C) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Statistical data analysis. If not otherwise noted, Student's *t* test using the two-tailed distribution was used to determine significant differences in virus replication and gene expression. *P* values of <0.05 were considered statistically significant.

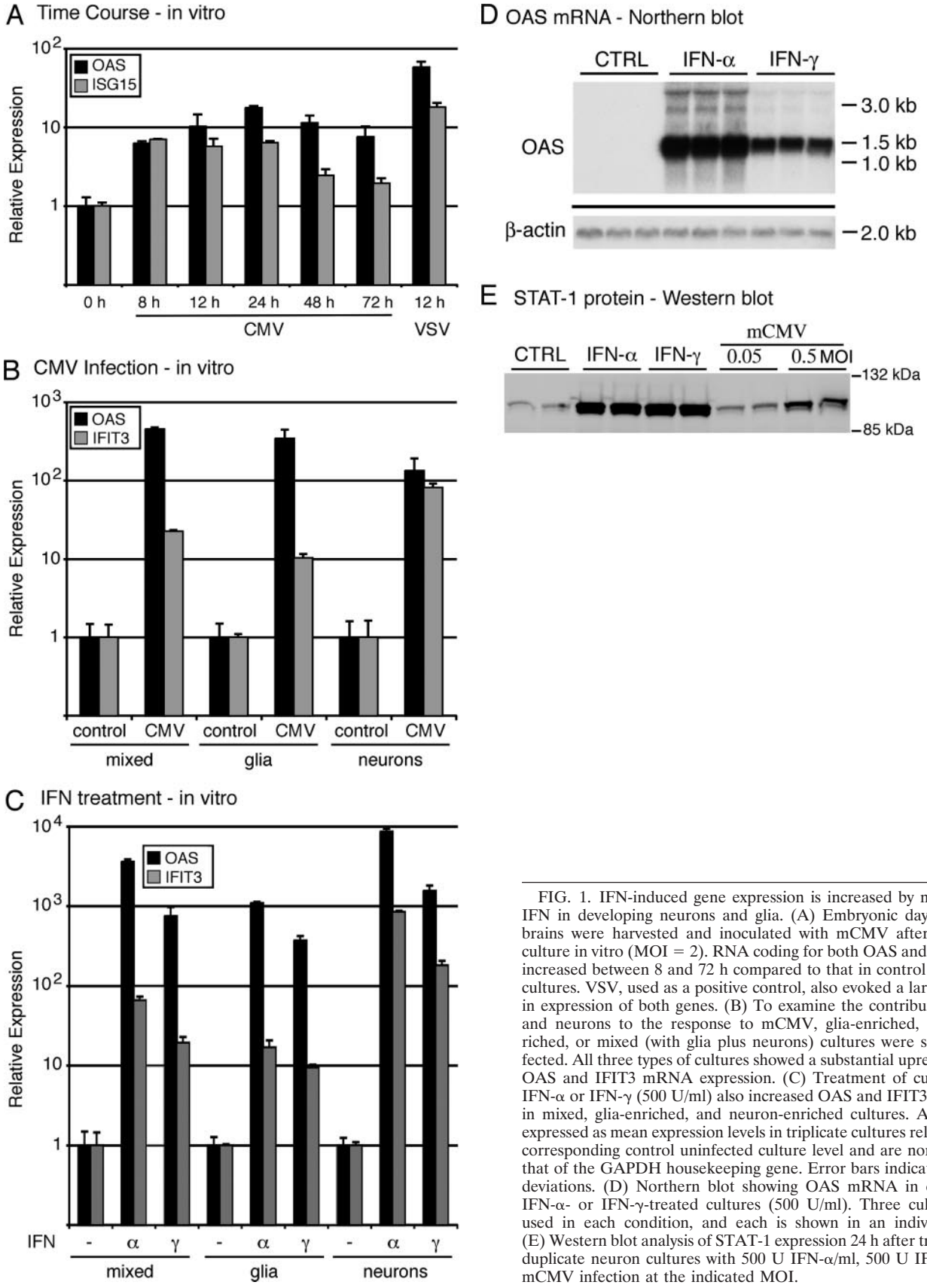


FIG. 1. IFN-induced gene expression is increased by mCMV and IFN in developing neurons and glia. (A) Embryonic day 18 mouse brains were harvested and inoculated with mCMV after 6 days of culture in vitro (MOI = 2). RNA coding for both OAS and ISG15 was increased between 8 and 72 h compared to that in control uninfected cultures. VSV, used as a positive control, also evoked a large increase in expression of both genes. (B) To examine the contribution of glia and neurons to the response to mCMV, glia-enriched, neuron-enriched, or mixed (with glia plus neurons) cultures were similarly infected. All three types of cultures showed a substantial upregulation of OAS and IFIT3 mRNA expression. (C) Treatment of cultures with IFN-α or IFN-γ (500 U/ml) also increased OAS and IFIT3 expression in mixed, glia-enriched, and neuron-enriched cultures. All data are expressed as mean expression levels in triplicate cultures relative to the corresponding control uninfected culture level and are normalized to that of the GAPDH housekeeping gene. Error bars indicate standard deviations. (D) Northern blot showing OAS mRNA in controls or IFN-α- or IFN-γ-treated cultures (500 U/ml). Three cultures were used in each condition, and each is shown in an individual lane. (E) Western blot analysis of STAT-1 expression 24 h after treatment of duplicate neuron cultures with 500 U IFN-α/ml, 500 U IFN-γ/ml, or mCMV infection at the indicated MOI.

RESULTS

Induction of intrinsic antiviral response by neurons and glia in vitro. To study the responses of brain cells to either mCMV infection or IFN stimulation, we employed three types of cultures: a mixture of cells consisting of primary glia and neurons, enriched cultures consisting primarily of glia, and enriched cultures of mostly neurons. We used quantitative RT-PCR to study mRNA levels of representative genes as a measure of IFN-stimulated gene expression. To determine the time course of gene induction, we started by harvesting mixed cultures at different times after inoculation with mCMV. Compared to the case with control mock infections (no virus), the expression of two representative IFN-stimulated genes (OAS and ISG15) was increased at all times examined from 8 h to 72 h after infection and on average was maximal at 24 h (Fig. 1A). A different virus (the single-stranded RNA virus vesicular stomatitis virus [VSV]) was used as a control at 12 h, as it has previously been shown to stimulate a robust expression of IFN and downstream genes (50). As expected, the induction levels of OAS and ISG15 were greater after VSV infection than after mCMV infection (Fig. 1A).

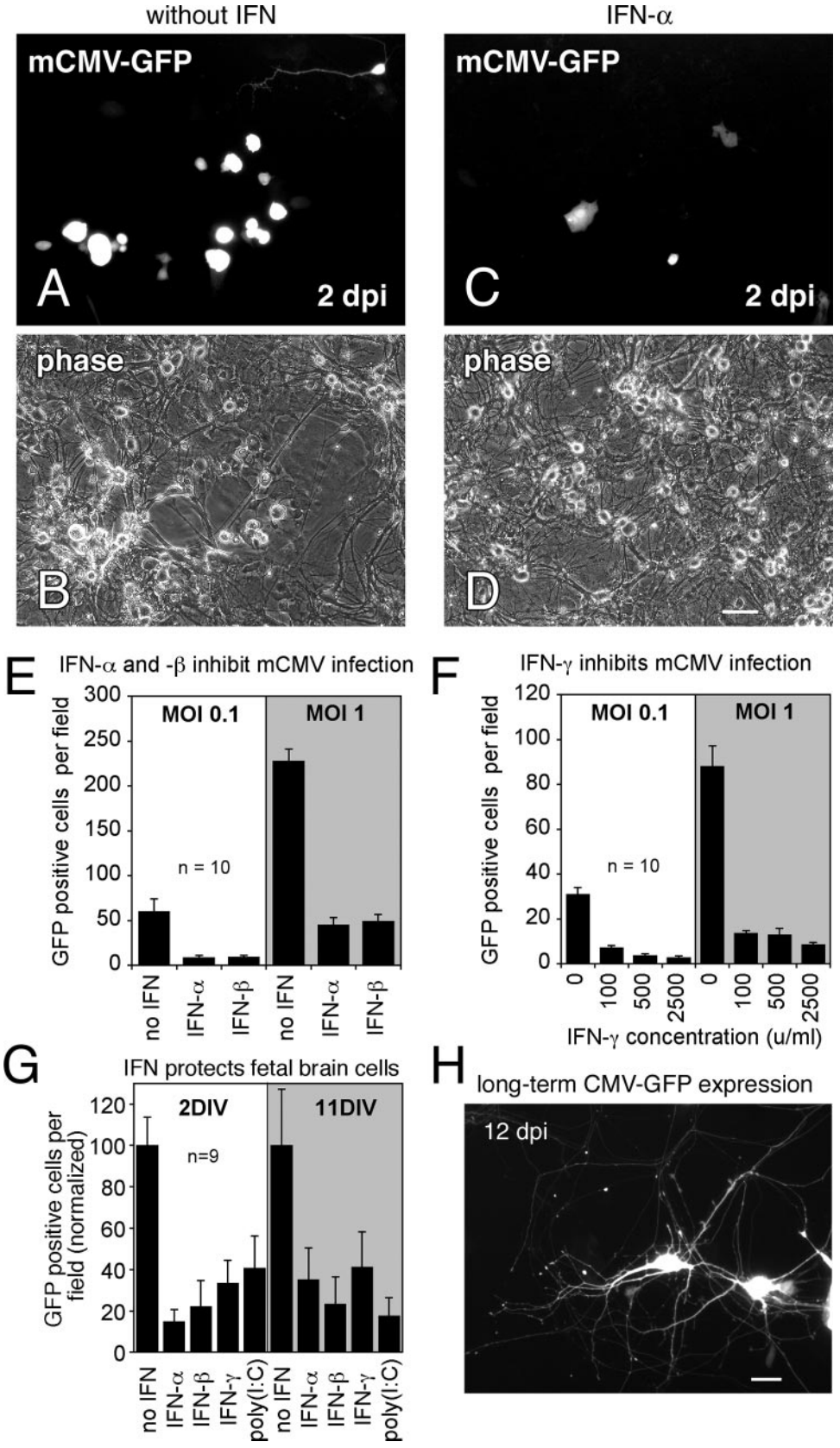
We then compared the IFN responses in mixed, neuron, and glial cultures 24 h after mCMV inoculation by examining expression of OAS and IFIT3. These genes were chosen because OAS is strongly induced by IFN, whereas IFIT3 expression is more moderately induced. In all three types of cultures, OAS expression was increased by more than 100-fold, and IFIT3 expression was increased by at least 10-fold after mCMV infection. In the neuron-enriched cultures, IFIT3 expression appeared to be increased to an even higher degree than in the other two types of cultures (Fig. 1B). As a test for the relative number of glia and neurons in enriched cultures, we used quantitative RT-PCR to compare two mRNAs selectively expressed by neurons or glia, NeuN for neurons and glial fibrillary acidic protein (GFAP) for glia. In a comparison of the ratio of NeuN and GFAP expression, neuron-enriched cultures had an approximately 100-fold greater NeuN-to-GFAP expression ratio than glia-enriched cultures (data not shown). These data verify the enrichment of neurons in the neuron-enriched cultures and suggest that the neurons possess the ability to respond to mCMV infection through induction of IFN-stimulated gene expression and are not solely dependent on glia for protection.

To determine if IFN-α or -γ would also induce IFN-stimulated gene expression in these cells, IFN-α or IFN-γ (500 U/ml) was added to the cells, and cultures were harvested after 24 h. Both IFN-α and -γ strongly enhanced expression of OAS and IFIT3 in mixed, glia-enriched, and neuron-enriched cultures (Fig. 1C). These results confirm that, in addition to being competent for IFN-stimulated gene expression after virus infection, these cells are also capable of responding to exogenously added cytokines and would therefore be sensitive to local cellular release of IFNs. To confirm further that IFN increased mRNA expression of an IFN-sensitive gene, we performed a Northern blot analysis of total RNA purified from IFN-treated neurons by using an OAS-specific probe. As we found with quantitative RT-PCR, OAS expression was induced by both IFN-α and IFN-γ but to a greater extent by IFN-α than IFN-γ (Fig. 1D).

To examine further the effects of IFN treatment and mCMV infection on the intrinsic cellular antiviral response in neurons, we also measured the expression of a representative IFN-induced protein, STAT-1. IFN-treated or mCMV-infected neuron-enriched cultures were lysed 24 h posttreatment in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and Western blot analysis was performed using a STAT-1-specific antibody (Cell Signaling Technologies, Beverly, MA). A low level of STAT-1 protein was detected in nontreated control neuronal cultures (Fig. 1E). However, both IFN-α and IFN-γ induced upregulation of STAT-1 expression (Fig. 1E). mCMV at a low MOI of 0.05 induced a little increase in STAT-1; raising the MOI to 0.5 caused an increase in the level of STAT-1 protein but an increase less than that seen after IFN-α or IFN-γ treatment.

Interferons protect brain cells from mCMV in vitro. To determine whether mouse brain cultures were protected by IFN, we inoculated embryonic day 17 mouse cultures after 10 days in vitro with an MOI of either 0.1 or 1. As different IFNs may exert different antiviral effects, we tested the hypothesis that all three IFNs (IFN-α, -β, and -γ) would reduce mCMV infections of young brain cells. Expression of GFP was used as a reporter for mCMV infection. Many cells in the untreated cultures showed virally expressed GFP two days postinoculation (Fig. 2A, E, and F). In contrast, all three IFNs dramatically reduced the number of virus-infected cells (Fig. 2C, E, and F). IFN-α/β reduced the number of infected cells by 80% or more (Fig. 2E). Treatment with 100, 500, and 2,500 U/ml IFN-γ was also effective at protecting cells, with the higher concentrations again providing greater protection and resulting in fewer infected brain cells (Fig. 2F).

As CMV is a particularly damaging virus for the developing brain, we next asked whether less-mature embryonic brain cells show any sign of protection from mCMV infection after IFN treatment. Here we used cells from embryonic day 15/16 mice and maintained them in vitro for only 48 h before adding IFN. At this stage of early brain development, most of the cultured cells are neurons, as determined by morphological characteristics of dendrites and axons and by immunostaining for the neural antigen NeuN. Remarkably, IFN-α, -β, and -γ all reduced mCMV infection (Fig. 2G). In parallel, treatment with poly(I:C), a synthetic double-stranded RNA that induces IFN in a Toll-like receptor 3 (TLR3)-dependent manner (1), also reduced mCMV infection of cultured brain cells. Similar results were found with E16 mouse cultures after 11 days of culture in vitro (Fig. 2G). This set of experiments was repeated five times using E15 to E19 mouse cultures, with testing performed after 1 to 12 days in vitro. In all five replicates, IFN treatment reduced mCMV infections (data not shown). Thus, even in embryonic brain cells in vitro, IFN reduces mCMV infection. Furthermore, this protective effect is long lasting. In mCMV-infected brain cultures not treated with IFN, most cells were dead within 3 days. In contrast, a single IFN application at the beginning of the experiment provided substantial protection for the duration of a 12-day experiment, a time during which most cells still showed no GFP expression, and some cells that were infected retained morphological characteristics of neurons (Fig. 2H). Experiments were not continued after 12 days postinfection (dpi), but as most of the cells were still


without IFN
IFN-α
mCMV-GFP
mCMV-GFP
A
2 dpi
C
2 dpi
phase
phase
B
D
E
IFN-α and -β inhibit mCMV infection
GFP positive cells per field
MOI 0.1
MOI 1
n = 10
no IFN
IFN-α
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F
IFN-γ inhibits mCMV infection
IFN-γ concentration (u/ml)
G
IFN protects fetal brain cells
GFP positive cells per field (normalized)
2DIV
11DIV
n=9
poly(I:C)
H
long-term CMV-GFP expression
12 dpi

healthy at that stage, it is likely that the IFN-mediated protection would have lasted substantially longer.

Postinfection treatment with interferon attenuates virus spread. In the experiments described above, IFNs were added prior to inoculation with mCMV. To determine whether IFN would attenuate an existing infection, we added IFN 18 h after inoculation of mCMV in mouse brain culture. CMV was added to one corner of a 22- by 22-mm coverslip containing cells over the entire coverslip. After 18 h, a number of GFP-positive cells infected with mCMV were detected in the inoculated corner. We compared IFN-α- and IFN-γ-treated cells to control cultures not protected by IFN. All cultures were infected with mCMV. Both IFN-α (1,000 U/ml) and IFN-γ (1,000 U/ml) depressed the number of infected cells over an observation period of 5 days, with the greatest attenuation of infection at the points farthest from the initial infection (Fig. 3).

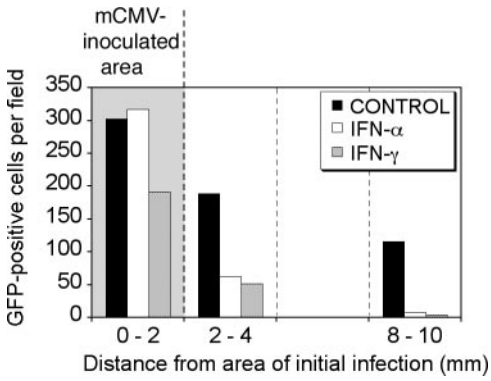

FIG. 3. Interferon exposure after mCMV infection attenuates virus spread. Embryonic day 18 mouse brain cultures were grown on a 22- by 22-mm coverslip. Two microliters of mCMV solution containing 2,000 PFU was placed in one corner of the coverslip and given 30 min for viral attachment before medium was changed several times. Eighteen hours after viral inoculation, 20 to 50 GFP-positive cells were detectable in the initially infected area. Medium was removed and replaced by medium containing IFN-α (1,000 U/ml) or IFN-γ (1,000 U/ml) or control medium. This figure shows the mean number of GFP-positive cells in neighboring microscopic fields with increasing distance to the initially infected area. Note the significantly impaired spread of CMV in postinfection IFN-treated cultures.

Physiological properties of CMV-infected cultures. The experiments described above show that, while mCMV kills most unprotected cells, IFN-treated cells appear morphologically normal. However, the question remains whether the morphologically normal cells would also show normal neuronal physiological characteristics. Using whole-cell patch clamp recording, we first examined the physiological alterations in neurons after mCMV infection, something that has not been previously reported. CMV-infected neurons show impaired physiology even at early stages of mCMV infection before morphological changes became evident. In a series of experiments, we examined changes of electrophysiological properties that occurred as early as 1 or 2 dpi (Table 1). At 1 dpi, neurons showed a significant shift of their resting potential in a positive direction. At 2 dpi, the resting potential was further depolarized, and in addition, cells showed significant changes in their action potential characteristics. Kinetics of action potentials can be described by amplitude and duration at half amplitude (Fig. 4A). Both amplitude and half-width duration were significantly changed 2 days after infection (Fig. 4B; Table 1). Action potentials were evoked by a 10-ms depolarizing-current step and mean amplitude decreased from ~97 mV in control neurons to 78 mV in infected cells, and the mean spike duration increased from ~1.9 ms to ~2.3 ms. We next analyzed neuronal excitability of control and mCMV-infected neurons. Increasing current steps were applied through the recording pipette, and the number of evoked action potentials (Fig. 4C) was measured and plotted in an output-input curve (Fig. 4D). Control cells robustly responded with action potentials upon positive-current steps, whereas mCMV-infected neurons at both 1 dpi and 2 dpi showed highly significant attenuation in their ability to generate action potentials ($P < 0.0001$; Kolmogorov-Smirnov test). In addition, the number of spontaneously occurring action potentials decreased over time in infected neurons, reaching significance at 48 h postinfection (Table 1).

For control purposes, we also recorded data from neurons infected with a GFP-expressing adeno-associated virus 2 (AAV-GFP) (10^8 infectious units/dish; 5 dpi) to rule out the possibility that changes in physiological properties of the neurons are due to the expression of GFP. In accordance with previous reports about the inert nature of GFP on neuronal activity (18, 60), green AAV-GFP-infected neurons resembled control cells in their electrophysiological characteristic, indicating that the change of neuronal function in CMV-infected neurons is attributable to the virus and not due to the expression of GFP.

Together, our data given above indicate that even in the absence of clear morphological evidence of impaired neuronal viability, infected neurons show significant impairment in their function.

Therefore, in the next set of experiments, we addressed the question whether IFN could prevent functional impairment induced by mCMV infection. Here, we compared the protective effect of IFN on neurons at a late time point when unprotected mCMV-infected neurons (MOI = 1) showed clear signs

FIG. 2. IFN-α/β and -γ protect mouse neuronal cultures from mCMV infection. Mouse neuronal cultures (E18) were infected with mCMV at an MOI of 0.1 or 1 in the absence or presence of IFN. The number of GFP-expressing cells in IFN-treated dishes was significantly lower than that for nontreated cultures. A representative photomicrograph of mCMV-infected cultures (A) and the corresponding phase-contrast image (B) are shown. (C and D) Typical image of cultures pretreated with IFN-α (1,000 U/ml) and infected with the same virus concentration as that used for the control cultures. Pictures were taken at 2 dpi. Scale bar, 25 μm. Bar graphs summarizing the protective effects of IFN-α and -β (E) and of IFN-γ (F) on mCMV infection of neuronal cultures are also shown. (G) mCMV infection is reduced by IFN-α, -β, or -γ and poly(I:C) in embryonic day 16 mouse brain cells after 2 and 11 days in vitro (DIV). Each bar represents the mean data of nine samples. In panels E through G, each of the three IFNs caused a statistically significant (ANOVA; P values were <0.001 for E and F and <0.05 for G) reduction in the number of GFP-positive cells. (H) A representative photomicrograph of a field of many cells with two GFP-expressing CMV-positive neurons treated with IFN with normal neuronal morphology 12 days after infection. Scale bar, 12 μm.

TABLE 1. Effect of early mCMV infection on the physiology of mouse neurons[a]

Treatment group	Mean value ± SD (no. of neurons)			
	V-rest (mV)	AP amplitude (mV)	AP duration (ms)	AP frequency (no./100 s)
Control	−59 ± 9.1 (16)	97 ± 7.5 (16)	1.86 ± 0.4 (16)	56 ± 103 (16)
CMV				
1 dpi	**−53 ± 8.1 (17)**	93 ± 8.6 (18)	2.04 ± 0.5 (18)	22 ± 26 (17)
2 dpi	***−36 ± 9.6 (6)***	***78 ± 11.0 (6)***	**2.3 ± 0.6 (6)**	**9 ± 18 (6)**
AAV, 5 dpi (GFP control)	−59 ± 5.4 (4)	99 ± 5.3 (4)	2.15 ± 0.8 (4)	34 ± 26 (4)

[a] Summary of membrane and action potential (AP) characteristics of control neurons, mCMV-infected neurons at 1 and 2 dpi, and AAV-2-infected control neurons (5 dpi) as a GFP control. Resting membrane potential (V-rest) was averaged from a 100-second recording trace. Action potential duration was measured at half amplitude as shown in Fig. 4A. Frequency was determined at resting conditions over a 100-s window. Bold indicates significant differences from the control value ($P < 0.05$; Mann-Whitney test). Bold with italics also indicates significant differences ($P < 0.01$; Mann-Whitney test).

of deterioration with a swelled or granular appearance, an increase in phase brightness due to the viral cytopathic effects and to cell cytomegaly, and a retraction of processes (Fig. 5A). In contrast, neurons and astroglia in IFN-β (500 U/ml)-treated cultures exhibited normal morphology and, even 6 days after mCMV inoculation, showed only modest GFP expression in some cells. In contrast, in cultures not protected by IFN, most cells showed signs of infection and GFP expression.

Establishing a stable seal formation was significantly more difficult in mCMV-infected non-IFN-treated cultures than in IFN-treated cells, suggestive of fragile membrane characteristics exhibited by cells not protected by IFN. In this context, the mean seal resistance ± standard error of the mean was 592 ± 266 MΩ ($n = 8$) in cells without IFN treatment, compared to 2,600 ± 224 MΩ ($n = 13$) in cells with IFN treatment, which was not different from that of uninfected controls (2,392 ± 327 MΩ; $n = 12$). In addition, only 3 out of 10 cells in the non-IFN-treated group presented a detectable but depolarized

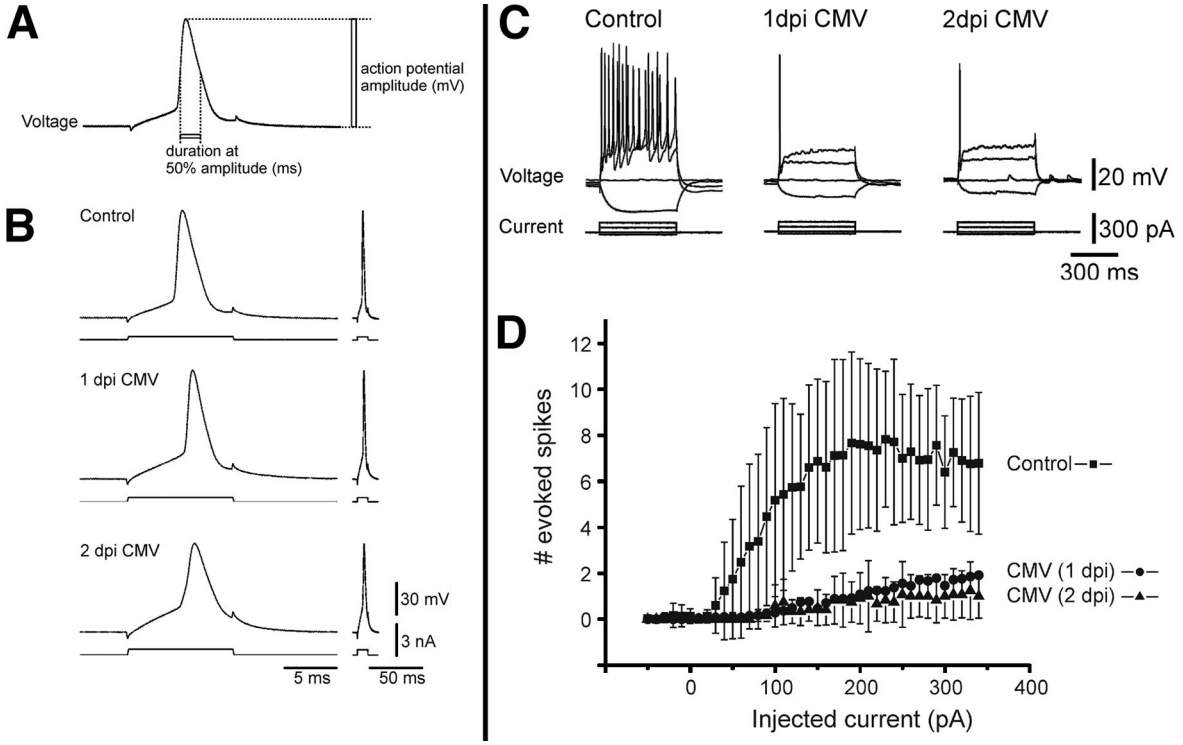

FIG. 4. Early CMV-induced electrophysiological changes preceding morphological signs of infection. Mouse neuronal cultures (E18) were infected with mCMV at an MOI of 1 and analyzed for their electrophysiological characteristics at early stages of infection, 1 and 2 dpi, before cytopathic changes became evident. (A) Experimental protocol for measurement of the action potential amplitude and duration. (B) Exemplary action potential traces evoked by a 10-ms depolarizing-current step from representative control and CMV-infected neurons. Note the widening of the action potential and decreased amplitude in infected cells. (C) Representative examples showing the suppression of excitability 1 and 2 days after infection. Shown are the responses to 500-ms injections of −40, 0, 100, and 200 pA. (D) Group data indicating that 1 and 2 days after infection with CMV, the number of action potentials evoked by depolarizing-current steps (500-ms duration) is substantially reduced ($n \geq 6$; $P < 0.0001$ [Kolmogorov-Smirnov test]). Values are means ± standard deviations; error bars are shown for control and CMV 2 dpi data.

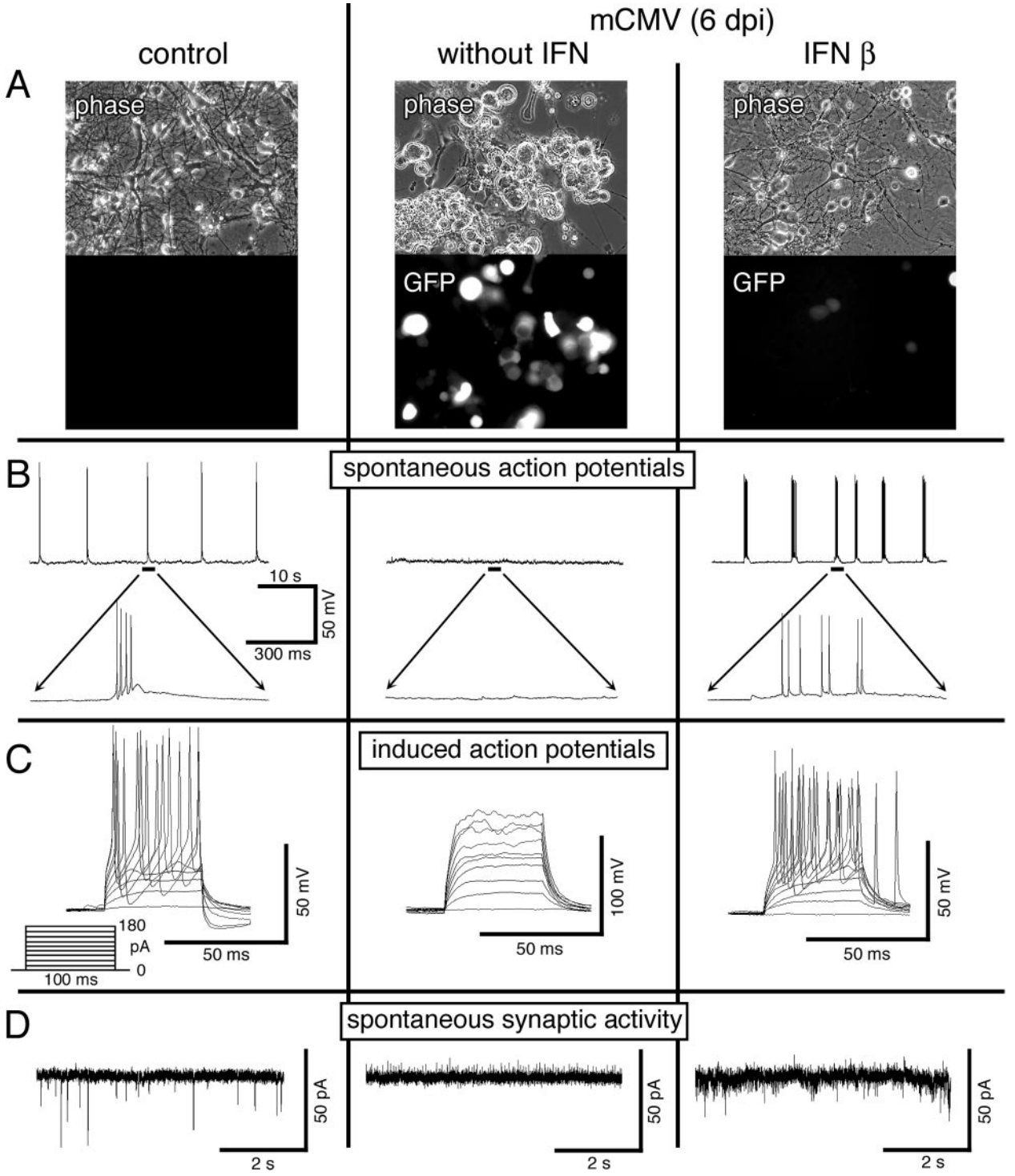

FIG. 5. Normal electrophysiological activity of neurons protected by IFN-β from mCMV infection. Mouse neuronal cultures (E17) were infected with mCMV at an MOI of 1 and analyzed for their electrophysiological characteristics at 6 dpi using whole-cell patch-clamp. (A) Representative display of uninfected control (left) or cultures infected with mCMV without (center) or with (right) IFN-β (1,000 U/ml) pretreatment. (B) Typical examples of recordings in current-clamp mode to assess spontaneous action potential events. Both CMV example cells expressed GFP, indicating CMV infection. No activity in infected cells (center column); characteristic spike periods with normal action potential configuration in control and IFN-β-protected cultures. The lower traces magnify horizontal line-marked sections from upper traces. (C) Superimposed traces of membrane potential in response to a series of current steps (100 ms, 20-pA increment). No induction of action potentials despite membrane depolarization in infected nontreated neurons (center). In contrast, in IFN-protected cultures (right), neurons responded with action potentials to membrane depolarization similar to those of control cells (left). (D) Recorded in voltage clamp at −60 mV, spontaneous synaptic currents were absent in nontreated mCMV-infected cultures (center) but present in control (left) and IFN-protected (right) cultures, as indicated by the downward deflections of the current trace.

resting membrane potential (-39 ± 8.2 mV; $n = 3$), compared to a resting potential of -50 ± 7.3 mV ($n = 6$) in IFN-treated cells. The resting membrane potential in uninfected control cells was in the same range, -51.4 ± 2.8 mV ($n = 9$). Since maintaining an adequate membrane potential is an active and energy-requiring process, the depolarized membrane potential in the nonprotected cultures is an indicator of general deterioration of cellular physiology. In current clamp, no spontaneous action potentials could be detected in any cells ($n = 10$) after mCMV inoculation in the absence of IFN. When depolarizing current up to 180 pA was injected through the recording pipette, no spikes could be induced. Furthermore, no synaptic activity could be detected in any of the nonprotected recorded neurons (center column of Fig. 5).

In striking contrast, in cultures treated with IFN-β and inoculated with mCMV, neurons appeared normal morphologically and exhibited typical features of healthy neurons, including spontaneous action potential activity or action potential induction after moderate current injections in neurons that showed no spontaneous spikes. mCMV-infected neurons in the presence of IFN-β (Fig. 5, right column) displayed the same neuronal characteristic as noninfected control neurons (Fig. 5, left column). Example traces from a neuron that expressed GFP are shown on the right side of Fig. 5B and C. In addition, voltage clamp experiments (-60 mV holding potential) revealed the presence of synaptic currents in those IFN-treated cells, as shown in Fig. 5D (right panel). The corresponding control traces are presented on the left. Together, these physiological experiments demonstrate that at a period when mCMV has killed most nonprotected cells, IFN-protected neurons are alive and show electrical behavior consistent with normal physiological activity, including action potentials and synaptic currents.

Induction of antiviral response in adult and developing brain in vivo. CMV is a particular problem in the developing brain but less so in the mature brain. We previously demonstrated that mCMV shows a strong infection of both the brain in vivo and brain slices in vitro at postnatal day 1, compared with a relatively low level of infection in the adult brain (61). One mechanism that might underlie the differential susceptibility to CMV infection is that developing brain cells have a reduced ability to mount an IFN response to CMV infection.

We first asked whether baseline expression of IFN-related genes was greater in the adult brain than in the embryonic or neonatal brain, which might in part explain the attenuated CMV infection in the adult brain. To examine the basal level of IFN-related gene expression, the brains of mice at three developmental stages, embryonic day 15, postnatal day 1, and adult mice, were compared (means for three to six brains for each age group). The basal expression levels of four representative genes were similar for the three age groups. When standardized for the adult level, defined as 1.0, the expression levels of the IFIT3, OAS, IFN-α, and IFN-β genes were, respectively, 1.0, 0.62, 0.9, and 0.75 for E15 mice, and 1.6, 0.4, 0.3, and 0.5 for P1 mice.

To compare IFN responses to viral infection in adult and developing brains, P1 and adult mice were inoculated intracerebrally with mCMV and harvested 24 or 72 h after infection. mCMV caused a substantial increase in expression of the IFN-stimulated genes (IFIT3 and OAS) studied after 24 and 72 h. Interestingly, the response to mCMV was just as strong in the P1 brain as the response in the adult brain. In fact, the expression of the IFN-induced gene OAS was increased by a greater degree in the developing brain than in the adult brain at the 72-h interval (Fig. 6B). In contrast, expression of the IFN-β gene was induced to a low but consistent level in the brains of adult mice (mean increase of 5.4-fold at 24 hours postinfection [hpi]; $P = 0.0004$) but was not induced in the neonates (mean increase of 1.5-fold at 24 hpi; $P = 0.17$) (Fig. 6B). As in the in vitro cell culture experiment, the cellular IFN response was also efficiently activated in the brains of positive-control VSV-infected mature and developing mice (Fig. 6B). mCMV infection therefore induced robust IFN-stimulated gene expression in the brains of both adult and neonatal mice, but IFN was more strongly induced in the adult brain than in the neonatal brain.

We also studied the levels of expression of additional IFN-stimulated genes after mCMV infection to rule out the possibility that our previous observations were limited to OAS and IFIT3 expression. The IFN-stimulated genes LMP2, TGTP, and USP18 at 24 hpi all showed increased expression after mCMV infection of the brain (Fig. 6C). Like OAS expression, expression of TGTP and USP18 was induced to a slightly greater degree in the P1 brain than in the adult brain. Similar to the case for IFN-β expression, there was a significant ($P = 0.03$) difference in induction of IFN-α expression in the adult mice (mean increase of 4.1-fold) compared to that in neonates (1.7-fold increase). Therefore, induction of IFN-stimulated gene expression in the brains of adult and neonatal mice after mCMV infection appears to be a property of most, if not all, IFN-stimulated genes.

The expression of IFN-stimulated genes was surprisingly large in the neonate compared to that in the adult. One possibility that might explain this unexpected finding is that mCMV replication occurs at an increased rate in the developing brain compared to the mature brain, so more virus may be infecting a greater number of cells in the developing brain, thus leading to an increase in IFN-related gene expression. To test this hypothesis, we first measured the relative level of mCMV-coded RNA, focusing on the viral mCMV IE1 gene and the reporter gene for GFP expressed by the virus. At 24 h postinfection, IE1 and GFP mRNA were expressed on average 4.5-fold more in the adult than in the neonate. Remarkably, at 72 hpi, the level of mCMV IE1 RNA increased approximately 300-fold in the neonatal brain, while it increased less than 3-fold in the adult brain (Fig. 6D). A similar pattern was also found for GFP expression, consistent with a substantial difference in the rate of replication of the virus in the mature and developing brain.

In a separate experiment, virus (2 μl; 2×10^4 PFU) was injected into the brains of postnatal day 1 ($n = 5$) and adult ($n = 6$) (7-week-old) mice. At 72 h postinoculation, the brains were harvested and the number of mCMV genomes was determined by quantitative PCR analysis of mCMV IE1 and GFP DNA (rather than RNA, as done previously). Based on threshold PCR cycles, neonates showed 17-fold-higher levels of IE1 DNA and 22-fold greater expression of GFP DNA than adult mice. These differences are significant (P values of 0.004 for IE1 DNA and 0.005 for GFP DNA) and suggest a level of virus replication in the neonatal brain substantially greater (approx-

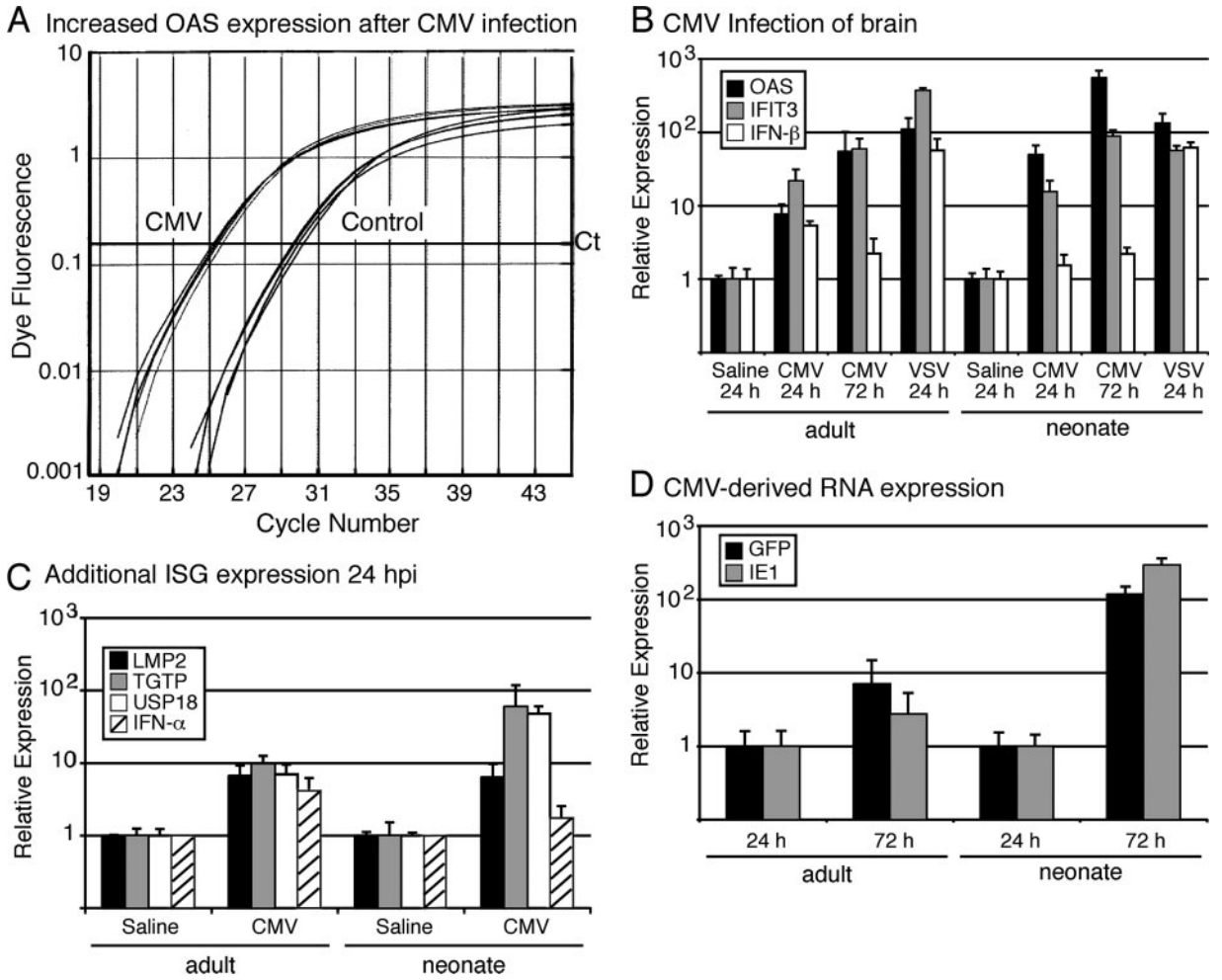

FIG. 6. mCMV infection of developing and mature brain activates an IFN response. (A) Representative quantitative RT-PCR data showing OAS expression 24 hpi in the brains of four saline-injected (control) and four CMV-infected neonatal mice. Note the tight clustering of individual mice in each group. Ct, cycle threshold. (B) Expression of OAS, IFIT3, and IFN-β genes in adult and P1 neonate mice 24 to 72 h after mCMV or VSV infection. The expression levels of all three genes are enhanced by mCMV infection in both P1 neonate and adult mice. (C) Expression levels of four additional IFN-stimulated genes (ISG) were examined 24 h after mCMV infection. All four were induced by mCMV, and the response in neonatal mice was at least as strong as the adult response. (D) Expression of CMV-derived IE1 and GFP RNA in adult and neonatal mice. All data are expressed as mean expression levels in four mice relative to those of the control saline-injected mice of each group (adult and neonate) and are normalized to the expression level of the GAPDH housekeeping gene. Error bars indicate standard deviations.

imately 20-fold) than that in the adult brain, consistent with the viral RNA data. As the total DNA in the adult brain was 1.4 times greater than that of the neonates at the time of tissue harvest, the total mCMV genome number can be estimated as being about 14-fold greater in neonates than in adults. Thus, if we view the IFN-related gene induction as a function of viral load and we compare IFN-related gene induction per unit virus, then the IFN-related induction per unit virus is less in the neonatal brain, particularly at the 72-h time point, a period sufficient for at least two rounds of virus replication (32). Particularly striking is the fact that expression of IFN-β was not enhanced in neonates compared to adults at 72 hpi, despite much greater levels of virus RNA and DNA at this time point.

Interferon protects the developing brain from mCMV infection in vivo. In the culture experiments described above, we found that IFN protected cultured mouse brain cells from infection by mCMV. Direct mCMV injection into the brains of adult mice does not result in substantial neurological dysfunction or viral spread, and the infection resolves within approximately one week. In contrast, CMV infection of the developing brain results in substantial CMV replication and damage to infected brain cells and can be lethal. To determine if IFN might similarly protect the developing brain from mCMV infection, postnatal day 1 mice were given a preinoculation intracerebral injection of IFN-β (10^4 U in 1.5 μl) 12 h prior to virus inoculation. mCMV (10^5 PFU in 1 μl), together with a second dose of IFN-β (5,000 U in 0.7 μl), was then injected by using a Hamilton microsyringe. Animals were euthanized 48 h later, brains were harvested, and DNA from the entire brain was purified. At the time of euthanasia, neither infected controls (CMV only; $n = 9$) nor IFN-treated CMV-infected mice ($n = 10$) showed detectable behavioral symptoms of viral infection, suggesting that the brain infection had not reached a state where neurological dysfunction had occurred. The relative amount of mCMV DNA in each brain was measured by

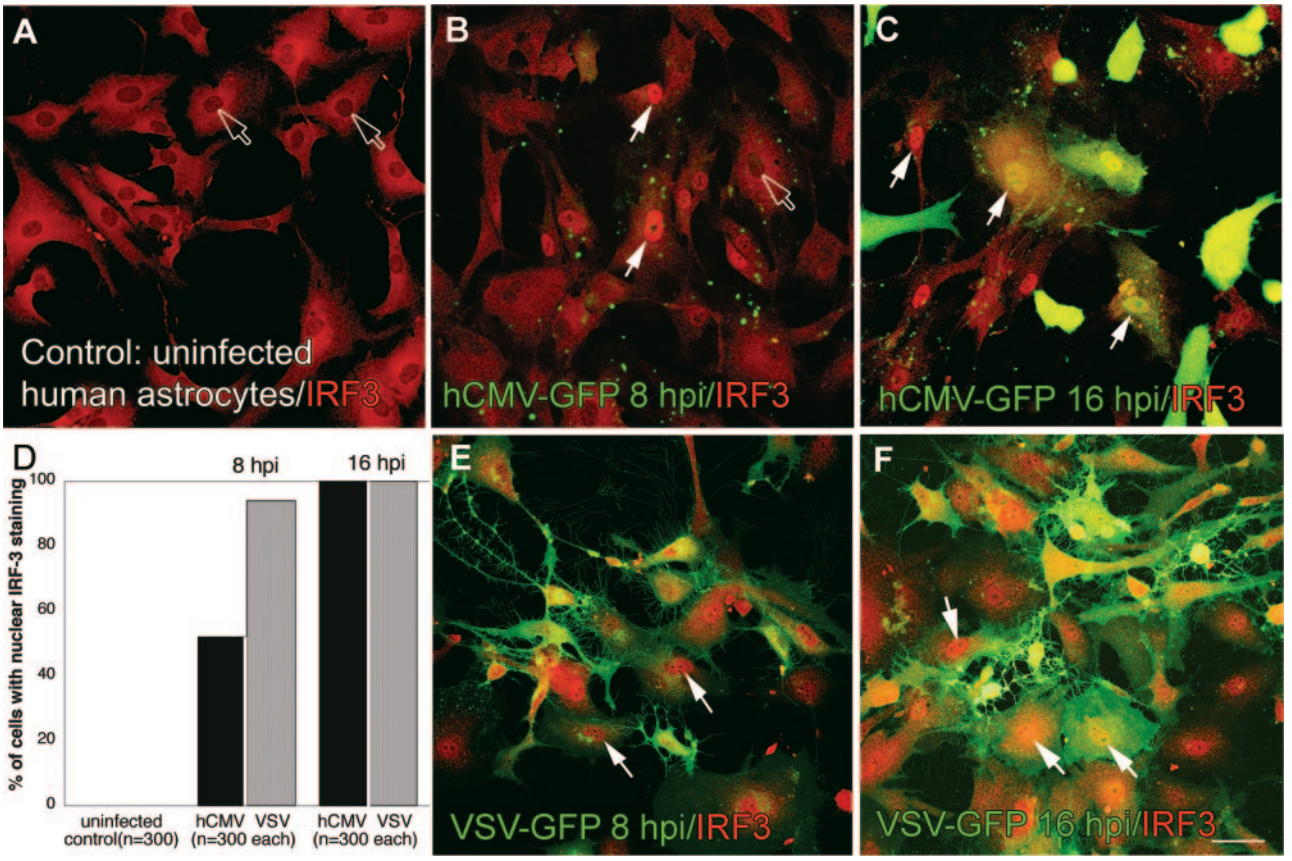

FIG. 7. hCMV infection induces IRF3 translocation to the nucleus. (A) In control noninfected human astrocytes, red IRF3 immunoreactivity is particularly strong in the cytoplasm and weak in the nucleus (open arrows). (B) At 8 hpi, a substantial increase in red IRF3 immunoreactivity in the nucleus is found (solid arrows in panels B, C, E, and F). The green GFP is used as a reporter for the presence of hCMV (MOI = 1). (C) At 16 hpi, almost all astrocytes show IRF3 immunoreactivity in the nucleus and viral GFP expression has increased. (D) The bar graph shows the percent of astrocytes showing nuclear immunoreactivity for IRF3 after infection by hCMV and VSV. A control virus, VSV-GFP (MOI = 1), shows a more rapid IRF3 response at 8 hpi (E), but by 16 h, VSV and hCMV showed similar percentages of responding cells (F). Scale bar, 20 μm.

quantitative PCR. IFN treatment reduced the number of detectable mCMV genomes by a statistically significant amount (39-fold depression; $P = 0.02$). This experiment provides in vivo confirmation for our earlier in vitro data, indicating that IFN treatment substantially attenuates CMV infection in the developing mouse brain.

Interferon protects human brain cells from hCMV infection. The experiments described above focus on the protection of mouse brain cells from mouse CMV infection. We next tested the hypothesis that human brain cells are similarly protected from hCMV by IFN and that hCMV infection induces an antiviral response from human brain cells. Primary human brain cells were cultured and then infected with an hCMV that contains a gene coding for GFP (25, 57). Two types of human brain cells were used. The first were astrocytes obtained from Sciencell. The second were primary cells harvested from human cortex and consisted of multiple brain cell types.

Virus infection initiates multiple signaling pathways leading to activation of IRF3 and NF-κB (47). IRF3 phosphorylation promotes dimerization, nuclear localization, and binding to IRF3 consensus binding sites, which induces the expression of certain type I IFNs and specific IFN-stimulated genes. Infection by hCMV caused IRF3 to be translocated from the cytoplasm to the nucleus, with many of the infected astrocytes showing a clear nuclear immunoreactivity 8 hpi (Fig. 7). By 16 hpi, almost all cells showed a nuclear localization of IRF3 (Fig. 7). As VSV causes a strong increase in IRF3 nuclear localization, we used VSV that expressed GFP in infected cells (59) as a positive control. Activation by hCMV was delayed relative to that by VSV, but by the 16-h time point, both viruses had caused nuclear translocation of IRF3 in almost all astrocytes (Fig. 7). In contrast, noninfected control astrocytes showed predominantly a nonnuclear cytoplasmic localization of immunoreactivity for IRF3.

We also examined the expression of OAS as a response to viral infection. Consistent with the mCMV-induced expression of OAS mRNA (Fig. 1), hCMV infection resulted in a very strong induction of OAS protein expression (Fig. 8). These data indicate that hCMV initiates IRF3 activation and robust OAS expression, demonstrating that hCMV induces an antiviral response in astrocytes.

At both day 1 and day 4 postinoculation, IFN (100 or 500 U/ml)-treated human brain or astrocyte cultures showed attenuated hCMV infections based on the relative number of cells showing the viral GFP reporter gene-mediated green fluores-

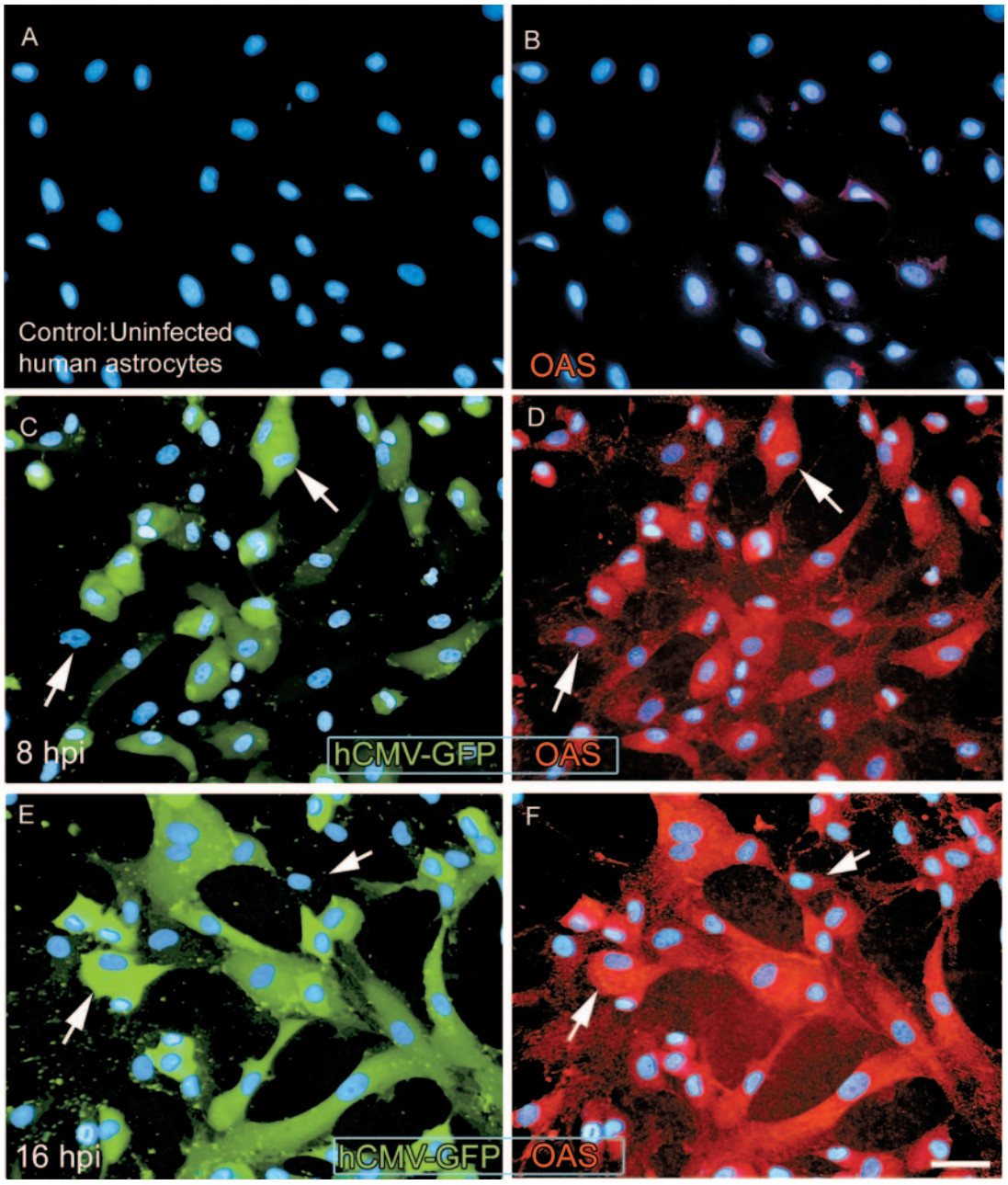

FIG. 8. OAS immunoreactivity is upregulated by hCMV infection. (A and B) Control uninfected human astrocytes showing little OAS immunoreactivity. (C and D) At 8 hpi, viral gene expression (GFP gene) is observed in astrocytes, and a substantial increase in OAS expression is found. Panels C and D show the same field, and arrows indicate the same cell. (E and F) At 16 hpi, more astrocytes are infected (as shown by the GFP reporter), and strong OAS expression is detected. Arrows show the same cell in panels E and F. Scale bar, 30 μm. Blue, nuclear DAPI (4′,6′-diamidino-2-phenylindole) stain; green, hCMV-GFP; red, OAS Alexa Fluor 594. Scale bar, 20 μm.

cence (Fig. 9A through H) ($P < 0.05$; analysis of variance [ANOVA]). The IFN protection was dose dependent, with higher doses of IFN conferring a greater degree of protection against hCMV infection. In addition, the synthetic double-stranded RNA poly(I:C) was also tested and found to provide a substantial level of protection against hCMV, even greater than the protection afforded by the doses of IFN used here. In parallel with the GFP reporter expression, cells not treated with IFN or poly(I:C) showed substantially greater cytopathic effects, as seen by the pathological structure of the untreated cells (Fig. 9A and D) compared with the IFN- or poly(I:C)-protected cells (Fig. 9B, C, E, and F).

We tested further whether the decreased GFP expression in treated cultures reflected overall viability of the cultures by using an MTT cell viability assay. Two days postinfection, hCMV infection (MOI = 5) reduced the viability of human glia cells to 52.5 ± 1.3% (optical density) of the level of the mock-infected (no-hCMV) controls ($n = 4$); controls levels

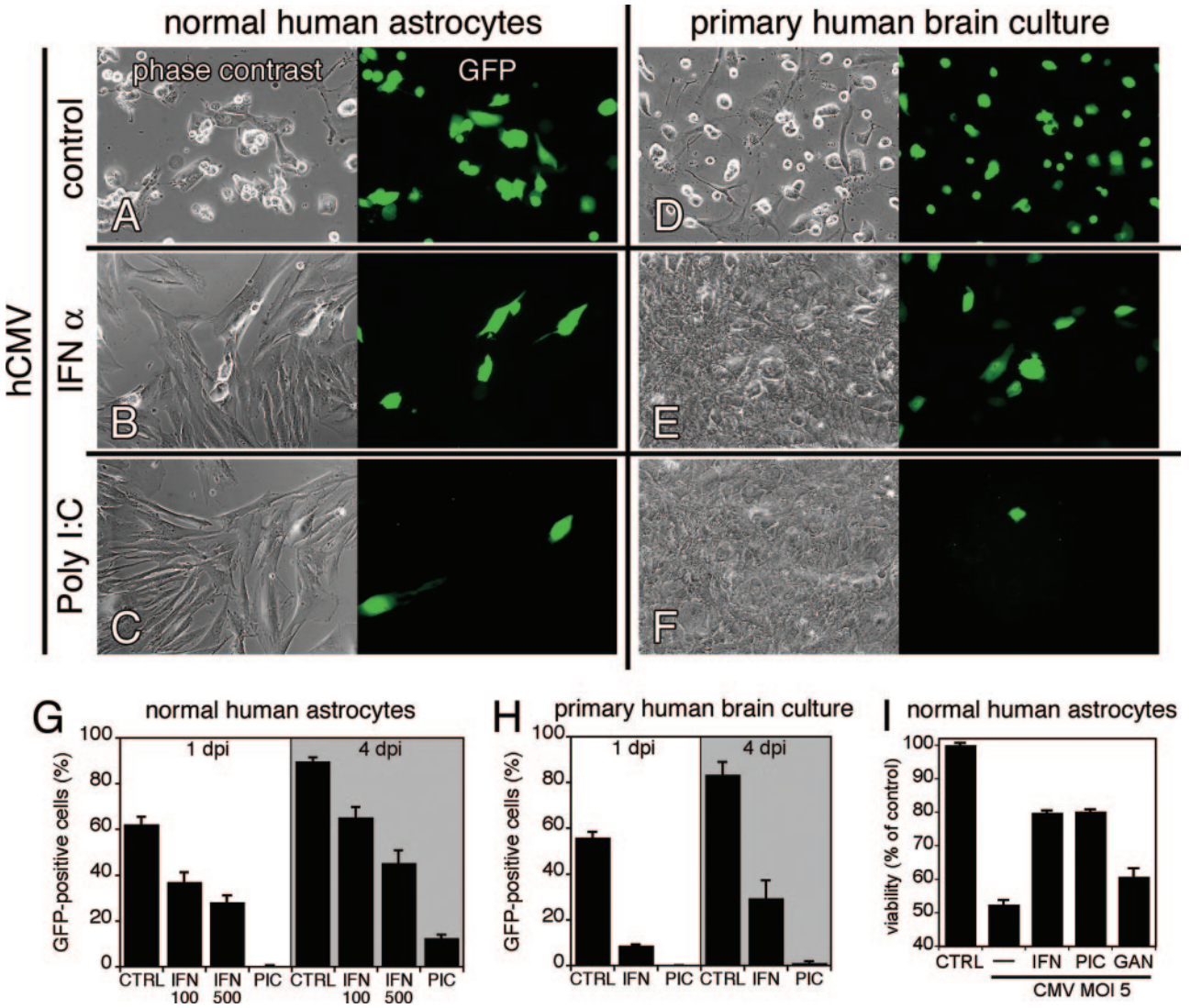

FIG. 9. IFN-α and poly(I:C) protect human astrocytes from hCMV infection. Cultured human astrocytes and mixed-cell explant cultures from human brain tissue were cultured and infected with hCMV at an MOI of 1. Representative images were taken at 4 days postinoculation in control conditions (A and D), after IFN-α pretreatment (500 U/ml) (B and E), and after poly(I:C) pretreatment (20 μg/ml) (C and F). (G and H) Bar graphs summarize the protective effects of IFN-α and poly(I:C) at different concentrations and time points (1 and 4 dpi). Notably, poly(I:C) (PIC) provided the strongest protection from hCMV infection. CTRL, control. (I) An MTT cell viability assay was used to assess hCMV (MOI = 5)-induced cytotoxicity. Pretreatment with IFN and poly(I:C) (PIC) had a significant protective effect and increased cell viability compared to no treatment (−) or ganciclovir (20 μM) treatment (GAN) ($n = 4$). CTRL, control.

were defined as 100%. In contrast, pretreatment with IFN or poly(I:C) significantly ($P < 0.05$; ANOVA) protected the cultures from hCMV infection with a viability value of 79.7 ± 0.8% or 80.2 ± 0.6% ($n = 4$), respectively, of the control value (Fig. 9I).

Time-lapse imaging of IFN protection of human brain cells. To examine further the protection of human brain cells from hCMV infection by IFN, time-lapse digital video imaging was used to compare hCMV infection of brain cultures in the presence and absence of IFN, poly(I:C), or ganciclovir, an anti-herpesvirus-family drug that is clinically used to treat peripheral CMV infections (Fig. 10). Time-lapse recording provides insight into the progression of cytopathic effects of hCMV on unprotected human brain cells compared with its effects on the IFN-treated brain cells and would be able to detect transient morphological changes or reporter gene expression. At 20 hpi at an MOI of 1, an increase in phase-bright cells was found in the nontreated and ganciclovir-treated cultures, along with an increase in the number of human brain cells showing GFP expression, as a reporter for hCMV infection. By 50 hpi, most of the brain cells were GFP positive and phase bright, indicating a serious cytopathic effect of the virus. In striking contrast, at 50 hpi and even at a longer total postinoculation interval (70 hpi), very few GFP-positive cells could be detected at any time point in IFN-α- or poly(I:C)-treated cells, and there was little evidence of a cytopathic effect and few or no phase-bright cells (Fig. 10).

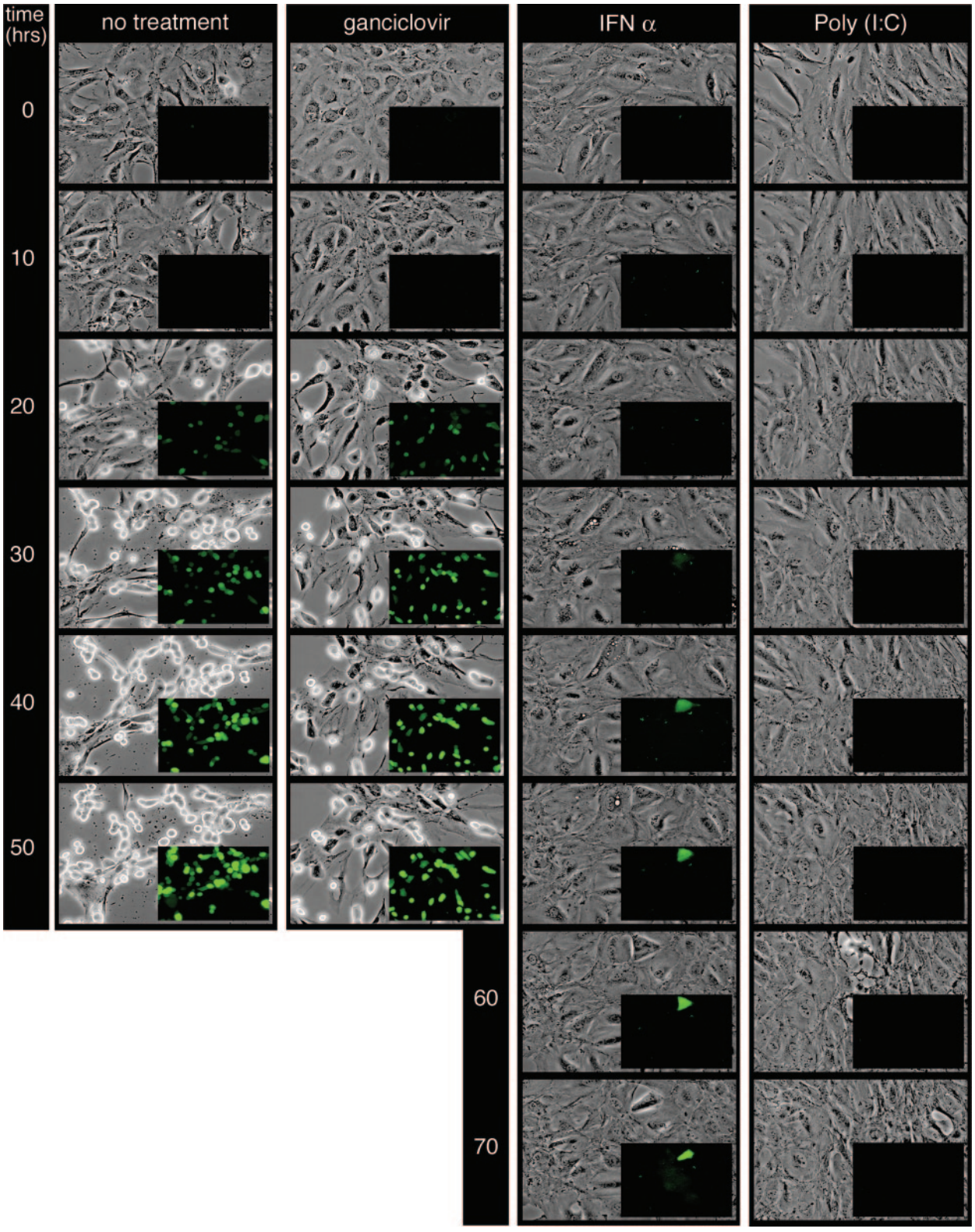

FIG. 10. Time-lapse recording of human primary brain cultures infected with hCMV. Explant cultures from brain tissue specimens were infected with mCMV at an MOI of 1. Pictures for time-lapse recording were automatically taken every 6 min over a period of 50 or 70 h. For the displayed panels, every 100th frame was used, corresponding to 10-hour-intervals. GFP-fluorescent images are inserted in the corresponding phase-contrast fields. The first column shows a typical experiment of hCMV infection without treatment, the second column displays an experiment in the presence of ganciclovir (20 μM) with no protection from infection, the third column demonstrates the protection by IFN-α, and the fourth column demonstrates protection by poly(I:C) (25 μg/ml). The last two were observed for a prolonged period.

DISCUSSION

Although CMV has been suggested as the virus most commonly associated with neurological deficits, there is relatively little information available on the innate immunity of developing brain cells, particularly related to CMV infection. Here we show that CMV infection of neonatal and embryonic brain cells both in vitro and in vivo induces a robust expression of IFN-stimulated genes. Importantly, IFN treatment substantially reduces infection of both mouse and human CMV in brain cells.

Neurons play a role in their antiviral defense. There is an ongoing question about the role neurons take in their own defense against viruses. Neurons show little or no MHC expression (45). Remarkably, here we showed that fetal cultures enriched 100-fold for neurons show just as strong an IFN response as glial cultures. This suggests that neurons can generate an intrinsic antiviral response to CMV infection. An important feature of neurons is that unlike many other cell types, there is generally no ongoing turnover after birth. Thus, virally infected neurons that are lost are generally not replaced, increasing the probability of long-lasting neurological deficits. The low level of MHC molecules may be important for neurons to avoid being destroyed by the adaptive immune system. Thus, an innate IFN response may be a more critical feature in protection against CMV by neurons than by other cell types. Recent work has shown that neurons also show a response to infections by other viruses, including Theiler's murine encephalomyelitis virus, La Crosse virus, and West Nile virus (17, 42). One report showed that only a small percent of neurons (3%) show IFN expression after viral infection in the brain (17). In the present experiments, it is unlikely that such a small percentage of neurons could account for the several-hundredfold increase in expression of antiviral mRNAs found in the neuron-enriched cultures. The more robust response in our experiments could be due to a difference in the type of virus or to the increased probability of encountering a virus or IFN in culture compared with in vivo experiments in which neuronal infection could be a more rare or random event.

Electrophysiological disturbances in mCMV-infected neurons. In our whole-cell patch clamp electrophysiological tests described here, we found that even before substantive deterioration of cell morphology, infected neurons expressing GFP showed aberrant electrical behavior. The shape of the action potential changed, spontaneous spikes decreased, current injections failed to induce spike propagation, the resting membrane potential shifted in a positive direction, and synaptic activity was reduced. Neurons infected with a control recombinant virus, AAV-GFP, showed normal electrophysiology, indicating that the dysfunctional electrophysiology was due to mCMV infection and not to the GFP reporter gene. The electrophysiology of CMV-infected neurons has not been previously studied. Studies of other nonbrain cells, particularly fibroblasts, have suggested that CMV causes disturbances in Na^+ and Ca^{2+} ion levels (3, 14, 40). The characteristic cytomegaly of CMV-infected cells has been suggested to be due to changes in intracellular Na^+ (34).

Weak antiviral action in developing brain. The adult brain is protected from CMV by a number of mechanisms, including a CD4 (39) and peripheral CD8 (36) T-cell response. We have previously shown that developing brain cells in vivo and in vitro are more likely to show mCMV infections than mature brain cells, and that this preference is independent of the host B- and T-cell responses (57, 61). In the present study, we compared the innate IFN-mediated antiviral responses to mCMV in mature and developing brains. Baseline (uninfected) levels of IFN-related gene expression were similar in embryonic day 15, postnatal day 1, and adult mouse brains, suggesting that the higher sensitivity of developing brain cells to mCMV infection was probably not a consequence of low baseline antiviral gene expression. Both embryonic and adult brain tissues also showed robust expression of IFN-stimulated genes after mCMV infection. However, when mCMV infections in adult and neonatal brains were compared by quantitative PCR and RT-PCR, the rate of virus replication in neonates was 10- to 100-fold greater than that in adults, consistent with previous observations made by counting brain cells expressing mCMV-regulated reporter genes (61).

One possible explanation for this apparent discrepancy between IFN induction and virus replication is that developing brain cells show an overall IFN response similar to that of adult brain cells but that a distinct, specific antiviral gene(s) necessary for inhibition of mCMV replication is not expressed by neonatal cells. However, this may be unlikely based on the fact that virus replication is inhibited in neonatal mice and neuronal cultures after exogenous administration of IFN. We therefore favor an alternate interpretation: because the rate of replication in the neonatal brain is more than an order of magnitude greater than that in the adult brain, the strong IFN response observed in neonatal brains may in part be due to the greater viral load, which results in a greater number of infected cells at the time of tissue harvest. Thus, the overall IFN response we observe in the adult brain likely corresponds to a strong response in relatively few cells that effectively inhibits virus replication, while the overall response seen in the neonatal brain derives from a much weaker response in a large number of cells that does not effectively control the virus.

mCMV infection elicits an IFN response in infected animals through TLR3 and TLR9 by both MyD88-dependent and -independent mechanisms (16, 51). Furthermore, infection of human cells with hCMV has been shown to induce IFN-stimulated gene expression, and this activation appears to occur by both IRF3-dependent and -independent mechanisms (15, 33, 65). A significant amount of antiviral gene expression is induced in hCMV-infected cells early after infection (6 to 8 hpi) and in the presence of the protein synthesis inhibitor cycloheximide, indicating that some gene expression is directly induced by hCMV rather than as a secondary consequence of IFN-β expression (7, 15). Thus, the activation of antiviral gene expression by betaherpesviruses is a complex process that involves multiple cellular pathways.

We found that IFN-stimulated gene expression was induced to similar degrees in the brains of adult and neonatal mice after mCMV infection. However, expression of IFN-α and IFN-β appeared to be induced only in the adult brain, albeit to a low level. Because gene expression was monitored using the entire brain as the source of RNA, the low induction of these genes in adult mice may reflect a relatively high level of expression in few infected cells. The difference observed between IFN-stimulated gene expression and the expression of the IFN genes themselves likely reflects the multiple mechanisms by which mCMV may activate these pathways. These findings raise the hypothesis that whereas IFN-stimulated gene expres-

sion may limit CMV replication in infected cells of both developing and mature brains, IFN-α and IFN-β released by infected adult cells may protect other uninfected cells in the brain. The presence of a substantive induction of IFN-α and -β in the adult brain, but not in the developing brain, may be one factor that contributes to the normal protection against CMV in the adult brain but is lacking in the developing brain. The precise mCMV-sensing pathway that appears to be absent in the brains of neonatal mice remains to be determined.

Interferon protects developing brain. The time-lapse digital image recording showed that cytopathic effects were seen clearly at 20 hpi in the absence of IFN protection, but little cytopathic effect or GFP reporter gene expression was found at any time point in the cells protected by IFN or poly(I:C). Although ganciclovir prevents herpesviruses from spreading, this is done partially on the basis of killing infected cells. Whereas this cell death may not be a problem in tissue that regenerates (e.g., liver), in tissue such as the brain that shows relatively little cellular regeneration, loss of crucial cells could generate permanent neurological debilitation. It is striking, therefore, that in both mouse and human cells, IFN and poly(I:C) provide substantial protection against the virus without resulting in the cell death found with ganciclovir.

IFN treatment resulted in a robust protection of embryonic, neonatal, and adult mouse brain cells in vitro, human brain cells in vitro, and developing mouse brains tested with in vivo experiments. All three IFNs tested, IFN-α, -β, and -γ, were effective at reducing CMV infection. Thus, IFN administration might be considered as an additional therapy for the treatment of hCMV infections in the brain. IFN is already in use for the treatment of brain disease, including multiple sclerosis or tumors in humans, by either peripheral (19) or central IFN administration (24, 41), and for West Nile virus and Japanese encephalitis virus (26, 48), and it should not produce unexpected side effects with use in the brain.

We also found that, similar to IFN-α/β and IFN-γ treatments, treatment of mouse neuronal cultures, human astrocytes, and human primary brain cultures with the double-stranded RNA poly(I:C) protects cells from mCMV or hCMV infection. Extracellular poly(I:C) induces expression of IFN-β in cells, including microglia through activation of TLR3, a Toll-like receptor which recognizes double-stranded RNA (1, 53). Multiple cell types in the central nervous system (CNS) (particularly astrocytes and microglia) are known to express a variety of TLRs, including TLR2, TLR3, TLR4, and TLR9 (28). These receptors have been shown to initiate antiviral programs and activate innate immune responses in the CNS (35, 44). Recently, human neuron-like NT2 cells were shown to also express TLR3 and to respond to poly(I:C) stimulation by producing IFN-β (38). Interestingly, we found that protection against CMV infection after poly(I:C) treatment was greater than that conferred by treatment with IFN-α in human astrocytes and primary brain cultures, indicating the efficiency of this signaling pathway in inducing an antiviral response in cells of the CNS. These results indicate that the use of TLR3 agonists in treating CMV infections of the brain merits further study. The protection of developing brains cells by IFNs shown here supports the view that IFNs may be a useful addition to the treatment of CMV infections of the developing brain.

ACKNOWLEDGMENTS

We thank Y. Yang for helpful technical support.

NIH AI48854 provided grant support for this work. M.D.R. was supported by NIH grant K22 AI064757.

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