

Differential Effect of Murine Alpha/Beta Interferon Transgenes on Antagonization of Herpes Simplex Virus Type 1 Replication

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Alpha/beta interferons (IFN- α/β) are potent, endogenous antiviral cytokines that suppress the replication of RNA and DNA viruses, including herpes simplex virus type 1 (HSV-1). The present study compared the efficacies of IFN- α/β transgenes, including IFN- α 1, - α 4, - α 5, - α 6, - α 9, and - β , against HSV-1 infection. L929 cells transfected with the IFN- α/β transgenes produced similar levels of IFN, as measured by bioassay and enzyme-linked immunosorbent assay. In addition, transfected cells were less susceptible to HSV-1 infection than were cells transfected with a plasmid vector control. The murine IFN- β plasmid construct exhibited the greatest reduction, while the murine IFN- α 5 transgene showed a modest inhibitory effect in viral titers recovered from the supernatants of transfected, infected L929 cultures. Consistent with this observation, the IFN- β transgene antagonized viral transcript levels, including infected cell protein 27, thymidine kinase, and glycoprotein B, to a greater extent than did the IFN- α transgenes at 6 to 10 h postinfection as determined by real-time PCR. Cells transfected with the IFN- α 4, IFN- α 9, or IFN- β transgenes showed the greatest reduction in viral protein expression relative to the other transfected cells, which was associated with increased STAT1 expression. The absence of the IFN-responsive protein kinase R (PKR) gene completely abrogated the antiviral induction by all IFN- α/β against HSV-1. In the absence of RNase L, viral yields were increased 10-fold, but the antiviral effect of IFN was either unaffected or enhanced. These results suggest that the predominant IFN-mediated, antiviral pathway during HSV-1 infection taken by IFN- α/β in L929 cells utilizes PKR.

Herpes simplex virus type 1 (HSV-1) is an important neurotropic virus with a worldwide distribution that seems to have coevolved with the vertebrate immune system (44). Upon infection, HSV-1 genes are expressed in a sequential cascade, resulting in progeny viruses that are eventually cleared from the host or alternatively establish a latent infection in the neurons of the sensory ganglia. During the acute infection, the host immune response initially consists of neutrophils, macrophages, and natural killer cells as well as the proinflammatory cytokines (tumor necrosis factor alpha, interleukins 1 and 6) and the alpha/beta interferons (IFN- α/β), including IFN- α and IFN- β (1, 6, 15, 27, 36, 62, 71). Typically, following this initial onslaught, CD4⁺ and CD8⁺ T cells infiltrate the infected area (41, 61) and appear to play a dominant role in clearing the acute infection through direct or indirect means (7, 42, 64). To circumvent the hostile environment, HSV-1 has evolved mechanisms to evade immune surveillance, including antagonizing antigen processing and presentation (2, 28, 32, 70), ultimately hindering virus-infected cell recognition by CD8⁺ cytotoxic T lymphocytes (19), blocking complement activation (17, 72), and encoding a glycoprotein that works as a decoy receptor for immunoglobulin G (51). Furthermore, HSV-1 reportedly induces apoptosis of T cells (31, 56) and renders infected target cells resistant to the lethal hit of cytotoxic T lymphocytes (33). In addition, HSV-1-infected peripheral blood mononuclear

cells produce transforming growth factor β 1 (45), which is known to suppress major histocompatibility complex class II expression (37). Collectively, there is strong evidence indicating that the virus has adopted several strategies to elude the adaptive immune response and to promote the establishment of a lifelong infection in the host.

IFN- α/β are endogenous, antiviral cytokines that have previously been found to antagonize HSV-1 replication in vitro at the transcriptional and translational levels (40, 47, 50, 65). Likewise, in vivo studies have determined that IFN- α/β play a dominant role in controlling acute HSV-1 infection (22, 26, 66, 67) and facilitate the adaptive immune response to infection (16). Although it was originally thought that the target of IFN- α/β might include viral protein 16 (13), it has subsequently been suggested that the level of antagonism might be more generalized (52, 60). Similar to other antiviral pathways, HSV-1 has developed mechanisms to obstruct the action of IFN. For example, HSV-1-infected cell protein 0 (ICP0) and ICP34.5 have been demonstrated to confer resistance to the effects of IFN- α/β (24, 25, 38, 49). Resistance by ICP34.5 seems to reside in antagonizing the antiviral effects of IFN-inducible, double-stranded-RNA-dependent protein kinase R (PKR) (9), whereas the level by which ICP0 antagonizes IFN is presently unknown.

Most investigations evaluating the antiviral effects of IFN- α/β use hybrid or recombinant proteins at seemingly high concentrations (>100 U). Little attention has focused on relative differences between or within the endogenous IFN subtypes as they relate to antiviral activity. In fact, murine IFN- α subtypes as well as IFN- β act through the same receptor (59) yet evoke

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different antiviral or antiproliferative efficacies (5, 10, 68, 73). Within the human system, these functional differences reside at the receptor level (18, 55) and the activation of downstream signaling pathways (3, 29) and impact on specific gene induction (12). The present study was undertaken to evaluate the antiviral potency of five different murine IFN- α subtypes and IFN- β in transfected L929 cells as well as in wild-type and double-stranded RNA-activated kinase (PKR) or 2',5'-oligoadenylate synthetase (OAS)/RNase L-deficient cell lines infected with HSV-1 (McKrae strain). The results of this study show that IFN- β is the dominant antiviral IFN- α/β against HSV-1 infection, antagonizing viral replication at the transcriptional and protein level to a greater degree than do the IFN- α subtypes. They also show that PKR is essential for the anti-HSV-1 activity of IFN.

MATERIALS AND METHODS

Cells and virus. The murine fibroblast cell line L929 and the African green monkey kidney cell line, Vero, were obtained from the American Type Culture Collection (Manassas, Va.). Propagation and experiments were carried out in Dulbecco's modified Eagle medium (DMEM) (American Type Culture Collection) for L929 cells or RPMI medium (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum and an antibiotic-antimycotic mixture (Gibco), which is referred to as complete medium. Wild-type (mouse embryo fibroblasts, MEF-6) and PKR-gene-disrupted cells (PKR KO) (35) were propagated in complete DMEM. Likewise, wild-type (RL+/+) and RNase L-gene-disrupted cells (RL-/-) (75) were propagated in complete DMEM. HSV-1 (McKrae strain) and vesicular stomatitis virus (a gift from Robert Fleischmann, University of Texas Medical Branch, Galveston, Tex.) were propagated in Vero cells as previously described (21). The cells were incubated at 37°C, 5% CO₂, and 95% humidity at all times.

Plasmid DNA constructs. All murine IFN transgenes were cloned into the eukaryotic expression vector PKCMVintPolylinker (5,087 bp, Vical Inc., San Diego, Calif.) containing a simian virus 40 polyadenylation signal and a kanamycin resistance gene. The IFN genes (575 to 626 bp) are expressed under the control of a human cytomegalovirus immediate-early enhancer/promoter. Cloning sites within the vector are as follows: IFN- α 1-*Bam*HI/*Bgl*II, IFN- α 4-*Pst*I/*Sal*I, IFN- α 5-*Sal*I/*Bgl*II, IFN- α 6-*Pst*I/*Bgl*II, IFN- α 9-*Xba*I/*Bgl*II, and IFN- β -*Sal*I/*Xba*I (10). The plasmid constructs were transformed into the *Escherichia coli* strain INV α F' (Invitrogen, Carlsbad, Calif.) and grown in Terrific broth, containing 50 μ g of kanamycin/ml, followed by purification using Qiagen Maxi kits (Qiagen, Inc., Valencia, Calif.). After each plasmid isolation, restriction enzyme digestion assays were conducted and the products were analyzed by agarose gel electrophoresis.

Transfection and infection of L929 cells. Prior to the transfection, 3×10^5 L929 cells were plated in duplicate in six-well tissue cultures. Following overnight incubation, the cells were transfected with 3 μ g of plasmid DNA and 45 μ l of Superfect (Qiagen) in 0.5 ml of complete DMEM for 6 h followed by a change of fresh, prewarmed complete DMEM. Twenty-four hours posttransfection, supernatants were collected and cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.5. The viral inoculum was removed 1 h postinfection (p.i.) and replaced with fresh complete DMEM. After 24 h, the supernatants were collected and the HSV-1 titer was determined in microtiter plate plaque assays using Vero cells.

IFN bioassay. To quantitate biologically active amounts of IFN secreted at the end of the 24-h transfection period, pooled supernatants from two samples were assayed as previously described (22).

Incubation of cells with biologically active IFN- α/β . To determine the 50% inhibitory concentration (IC₅₀) for each IFN- α/β subtype, supernatants from the transfected L929 cells containing equivalent concentrations of biologically active IFN (1,000 U/ml) were diluted (half-log dilutions) and added to fresh L929 cells (500,000 cells/well). Following an overnight incubation (18 h), the supernatant was removed and cells were infected with HSV-1 (McKrae strain, MOI = 0.5). After 60 min, the virus-containing supernatant was removed and fresh complete DMEM was added. Following an additional 28- to 36-h incubation period, the cells were frozen and thawed and the clarified supernatant (10,000 \times g, 1 min) was assayed for infectious virus by plaque assay using Vero cells. The IC₅₀ was determined by linear regression analysis for each sample. The correlation coef-

ficient for each sample analyzed over 6 half-log dilutions ranged from 0.90 to 0.99.

To determine the susceptibility of wild-type or gene-disrupted cells to HSV-1 infection following exposure to IFN- α/β , supernatants from transfected L929 cells containing equivalent amounts of IFN- α/β subtypes (50 to 100 U/ml) were added to the MEF/6, PKR KO, RL+/+, or RL-/- cells (250,000 cells/well). Following an overnight incubation, the supernatant was removed and the cells were infected with HSV-1 (McKrae strain, MOI = 1.0). After 60 min, the virus-containing supernatant was removed and fresh complete DMEM was added. Following an additional 28- to 36-h incubation period, the cells were frozen and thawed and the clarified supernatant (10,000 \times g, 1 min) was assayed for infectious virus by plaque assay using Vero cells.

IFN-ELISA. To quantitate the amounts of IFN protein produced at the end of the 24-h transfection period, pooled supernatants from two samples were assayed in triplicate using an enzyme-linked immunosorbent assay (ELISA) specific for mouse IFN- α (PBL Biomedical Laboratories). The ELISA was carried out according to the manufacturer's protocol and analyzed at an absorbance of 450 nm using a FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, Vt.). The lower end of the sensitivity range of the IFN- α ELISA was 12.5 pg/ml.

Semiquantitative real-time PCR for viral genes and IFN-inducible genes.

Total cell RNA was isolated at 6 and 10 h p.i. in Ultraspect RNA isolation reagent (Biotex Inc., Houston, Tex.) according to the manufacturer's protocol. Before the reverse transcription step, DNA contamination was removed using DNase I according to the manufacturer's protocol (Gibco-BRL). First-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) and an oligo(dT) primer (Promega). Real-time PCR was carried out in 96-well PCR plates (Bio-Rad, Hercules, Calif.) using a Bio-Rad iCycler. Real-time PCR conditions for all primers included an initial denaturing step for 3 min at 95°C, followed by 30 cycles at 95°C for 10 s and annealing/elongation at 61°C for 35 s. Each reaction contained 45 μ l of PCR Platinum SuperMix (Gibco-BRL) and CYBRgreen I (Molecular Probes, Eugene, Oreg.) at a final dilution of 1:100,000. MgCl₂ was supplemented as indicated by the primer sequences. During the optimization procedures of the primers, 1% agarose gel analysis verified the amplification of one product of the predicted size with no primer-dimer bands. The absence of primer-dimer formation for each oligonucleotide set was also validated by establishing the melting curve profile. The PCR results were analyzed on the iCycler Software (version 2.3), and threshold cycles were determined as follows: after subtraction of the background fluorescence for each sample, the threshold fluorescence for each gene was determined at that point where the relative light units reached a level of more than 10 standard deviations above the baseline relative light units. At 40 cycles, the primers-only control did not give a signal above the threshold. At 35 cycles the primers with uninfected cells did not give a signal above the threshold. The semiquantitative comparison between samples was calculated as follows: the data were normalized by subtracting the difference of the threshold cycles (C_T) between the gene of interest's C_T and the "housekeeping" gene GAPDH's C_T (gene of interest C_T - GAPDH C_T = ΔC_T) for each sample. The ΔC_T was then compared to the expression levels of the vector control sample (sample ΔC_T - vector ΔC_T). To determine the relative enhanced expression of the gene of interest, the following calculation was made: fold change = $2^{-(\text{sample } \Delta C_T - \text{vector } \Delta C_T)}$. If the difference (sample ΔC_T - vector ΔC_T) was a positive value, then it was calculated as follows: fold change = $-1/2^{(\text{sample } \Delta C_T - \text{vector } \Delta C_T)}$ in order to get a negative value expressing the relative suppression of the gene of interest. Oligonucleotide sequences for the targeted genes include the following: *GAPDH*, 5'-GAATCTACTGGC GTCTTCAACC-3' and 5'-GTCATGAGCCCTTCCACGATGC-3' (2 mM MgCl₂); *icp27*, 5'-TGACGCCGAGACCAGAC-3' and 5'-GGCAAAAGTGC GATAGAGG-3' (3 mM MgCl₂); thymidine kinase (*tk*), 5'-AAACCACCAC CACGCAAC-3' and 5'-ACACCCGCCAGTAAGTCATC-3' (3 mM MgCl₂); and *gB*, 5'-CGTTCGCAGGTGTGTTTC-3' and 5'-ATGTCGGTCTCGT GTCG-3' (3 mM MgCl₂).

Western blot analysis. Twenty-four hours posttransfection or at various times p.i., total cell protein was harvested following disruption of cells in sodium dodecyl sulfate (SDS) containing SDS lysis buffer supplemented with a cocktail of EDTA-free protease inhibitors (Boehringer Mannheim, Mannheim, Germany) and 1 mM sodium orthovanadate (Sigma, St. Louis, Mo.). In experiments assessing viral protein expression, cells that had been transfected for 24 h were subsequently infected with HSV-1 (MOI = 0.5) and total protein was obtained from lysed cells at 12 or 24 h p.i. Protein was quantitated with the BCA Protein Assay Reagent (Pierce, Rockford, Ill.) using the FL600 fluorescence plate reader (Bio-Tek). Total cell protein (10 to 30 μ g) was electrophoresed on SDS-7.5% polyacrylamide gels (criterion precast gels; Bio-Rad) and transferred by semidry

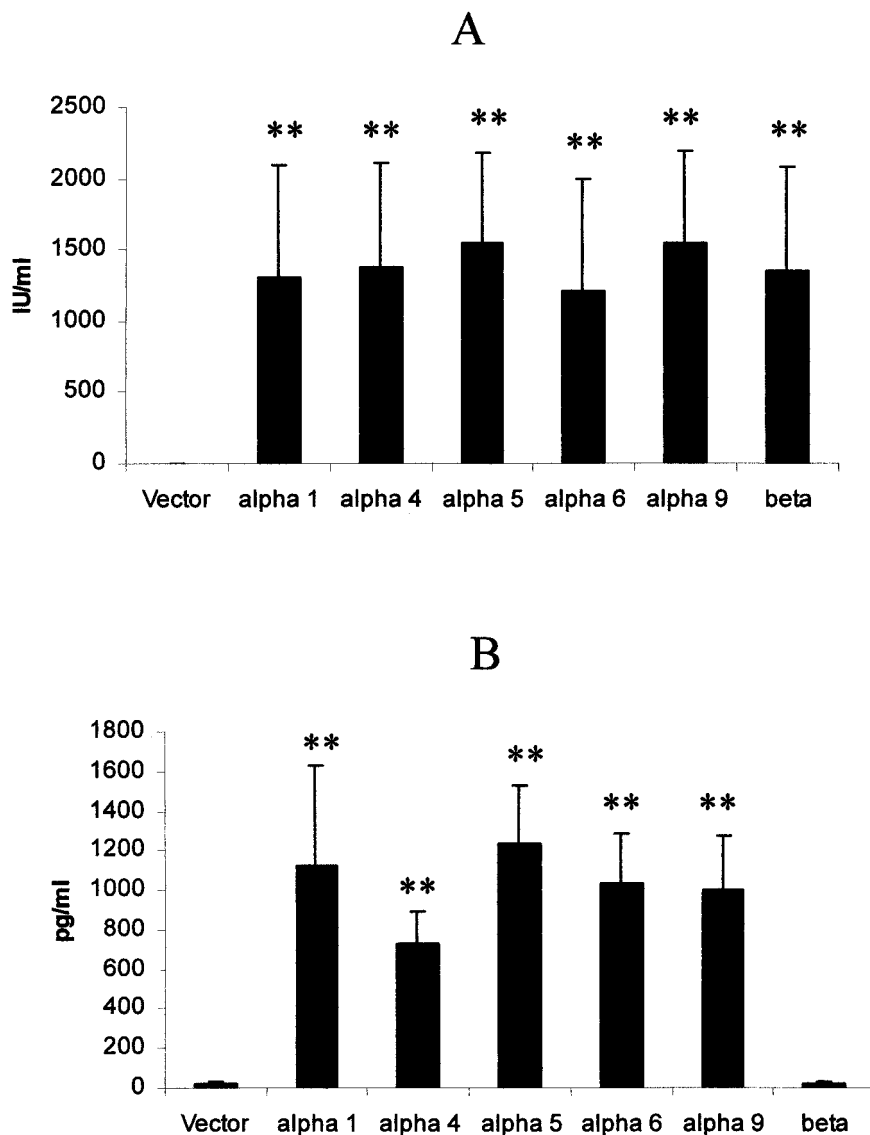


FIG. 1. Transfected L cells secrete biologically active IFN. L929 cells (500,000 cells/well) were transfected with 3 μ g of plasmid DNA alone (Vector) or plasmid containing the indicated IFN- α or - β transgene. Following a 24-h incubation, the supernatants were harvested and assayed for biologically active IFN- α or quantitated for IFN- α by ELISA (B). The figure is a summary of three experiments for each transgene or vector. Bars represent the mean \pm standard deviation. **, $P < 0.01$ in comparison of cells transfected with the IFN- α/β transgene to the vector control (A) or in comparison of cells transfected with the IFN- α transgenes to the IFN- β transgene or plasmid vector groups (B) as determined by ANOVA and Scheffe multiple-comparison test.

blotting onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 5% dry milk–2.5% bovine serum albumin in TTBS (0.1% Tween, 0.1 M Tris, 0.4 M NaCl [pH 7.4]) overnight at 4°C and incubated with a polyclonal rabbit anti-HSV antibody (Dako, Carpinteria, Calif.), a polyclonal anti-JAK1 p130 antibody (Biosource, Camarillo, Calif.), a polyclonal anti-STAT1 p91/p84 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), a polyclonal phospho-JAK1 (Y^{1022/1023}) antibody (Biosource), or a polyclonal phospho-STAT1 (Y701) antibody (New England Biolabs, Beverly, Mass.) for 2 h at room temperature on a shaking platform. The primary antibody was detected via an anti-rabbit-antibody coupled with horseradish peroxidase (Santa Cruz) followed by detection using chemiluminescence detection reagents (Pierce). The resultant blots were digitalized using the Bio-Rad 1000 image documentation system and software (Quantity One 4.0.3; Bio-Rad).

Statistics. Analysis of data was carried out by one-way analysis of variance (ANOVA) and Scheffe multiple-comparison test or the nonparametric Wilcoxon signed-rank test to determine statistically significant differences ($P < 0.05$) between the vector and IFN- α/β transgene groups.

RESULTS

Production of biologically active IFN by transfected L929 cells. In order to compare the potency of IFN- α/β against HSV-1 infection, transfected L929 cells were initially assayed for the production of biologically active IFN. Twenty-four hours posttransfection, culture supernatants were collected and assayed for IFN content. The results show that, with the exception of cells transfected with the plasmid vector alone, all transfected cells secreted equivalent amounts of biologically active IFN- α/β (Fig. 1A). For those cells transfected with the IFN transgenes, quantitative comparisons of IFN- α protein expression were conducted by ELISA (Fig. 1B). Cells transfected with the IFN- α subtype transgenes secreted similar

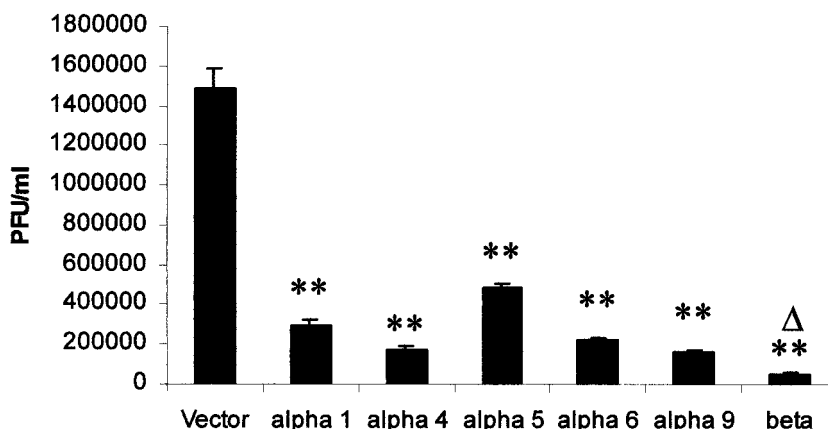


FIG. 2. Cells transfected with IFN- α/β transgenes are less sensitive to HSV-1 infection. L929 cells (500,000 cells/well) were transfected with 3 μ g of plasmid DNA alone (Vector) or plasmid containing the indicated IFN- α or - β transgene. Following an overnight incubation, the supernatant was removed and the cells were infected with HSV-1 (McKrae strain) at an MOI of 0.5. Twenty-four hours p.i., the cultures were frozen and thawed. Clarified supernatants were assayed for viral titer by plaque assay using Vero cells. This figure is a representative of two experiments, each conducted in duplicate. Bars represent the mean \pm standard deviation. **, $P < 0.01$ in comparisons of the IFN- α/β transgene-transfected cells to cells transfected with the plasmid vector alone, as determined by ANOVA and Scheffe multiple-comparison test. Δ , $P < 0.05$ when IFN- β transgene-transfected cells are compared to IFN- α transgene-transfected cells.

quantities of IFN- α , whereas cells transfected with the plasmid vector alone or the plasmid containing the IFN- β transgene secreted nominal amounts of IFN- α (Fig. 1B). Although transfection efficiencies were not determined using the plasmid construct, a previous study employing a similar construct (pCMV- β) found that transduction efficiencies ranged from 10 to 25% of cells between assays (data not shown).

Comparison of the efficacy of IFN- α/β transgenes against HSV-1 infection. Since the transfection of target cells with the various murine IFN- α/β transgenes generated similar quantities of biologically active IFN, the cells were assayed for their sensitivity to HSV-1 infection. Viral titers were determined in transfected cells infected with a highly neurovirulent strain (McKrae) of HSV-1. The results show a reduction in infectious virions recovered in the supernatants from cells transfected with the IFN- α or IFN- β transgenes in comparison to the vector control (Fig. 2). However, cells transfected with the murine IFN- β transgene consistently showed significantly lower viral titers than did cells transfected with the IFN- α transgene subtypes (Fig. 2). The viability of cells did not differ between transgene-transfected groups (97.0% \pm 0.8%) and nontransfected cells (97.8% \pm 0.5%). To further investigate this difference, the IC₅₀ for each IFN- α/β subtype was determined. Consistent with the above observation, IFN- β was found to have the lowest IC₅₀ in comparisons with the IFN- α subtypes, having statistically significant differences with IFN- α 1, IFN- α 5, and IFN- α 6 (Table 1).

IFN- α/β are known to suppress viral replication at the transcriptional and translational levels (50, 65). To address the degree and level of viral suppression, HSV-1 gene and protein expression was measured at times p.i. Regarding gene expression, HSV-1 immediate-early (*icp27*), early (*tk*), and late (*gB*) transcript levels were evaluated by real-time PCR. The results show that, similar to what is found in viral titers, cells transfected with the murine IFN- β transgene showed the greatest reduction in viral gene expression (immediate early, early, and

late HSV-1 genes) in comparison to cells transfected with the IFN- α transgenes, determined at 6 to 10 h p.i. (Fig. 3). However, IFN- α 9 and IFN- β suppressed HSV-1 *gB* gene expression to a similar degree. Consistent with the above findings, HSV-1 antigen expression was reduced in cells transfected with some IFN- α/β transgenes, compared to expression in plasmid vector-transfected cells (Fig. 4). Specifically, while the IFN- β , IFN- α 4, and IFN- α 9 transgene-transfected cells showed the greatest reduction in viral antigen expression (evident at proteins migrating at an apparent molecular mass of 120 to 125 kDa), there was no significant reduction in those cells transfected with the IFN- α 1, IFN- α 5, or IFN- α 6 transgenes (Fig. 4; Table 2).

IFN- α 4 and IFN- β transgenes augment STAT1 expression in transfected L929 cells. IFN- α/β utilize the JAK/STAT transduction pathway in inducing IFN-responsive gene expression (11). Therefore, it was of interest to determine if changes in

TABLE 1. IC₅₀ of IFN- α/β against HSV-1^a

Group	IC ₅₀
IFN- α 1	69 \pm 32 ^b
IFN- α 4	26 \pm 18
IFN- α 5	84 \pm 34
IFN- α 6	70 \pm 40
IFN- α 9	28 \pm 12
IFN- β	13 \pm 6*

^a Supernatants from transfected cells were diluted (half-log) and added to L929 cells (500,000/well) for overnight incubation. The following day, the supernatant was removed and HSV-1 (McKrae strain) was added to the cells at an MOI of 0.5. Following a 28- to 36-h period, the cultures were frozen and thawed, and the clarified supernatants were assayed for infectious virus by plaque assay using Vero cells. The IC₅₀ was determined by linear regression analysis for each sample. The correlation coefficient for each sample analyzed over 6 half-log dilutions ranged from 0.9 to 0.99.

^b Numbers represents means \pm standard deviations. This table is a summary of three experiments. *, $P < 0.05$ in comparisons of the IC₅₀ of IFN- β with IC₅₀s of IFN- α 1, IFN- α 5, and IFN- α 6 as determined by the Wilcoxon signed-rank test.

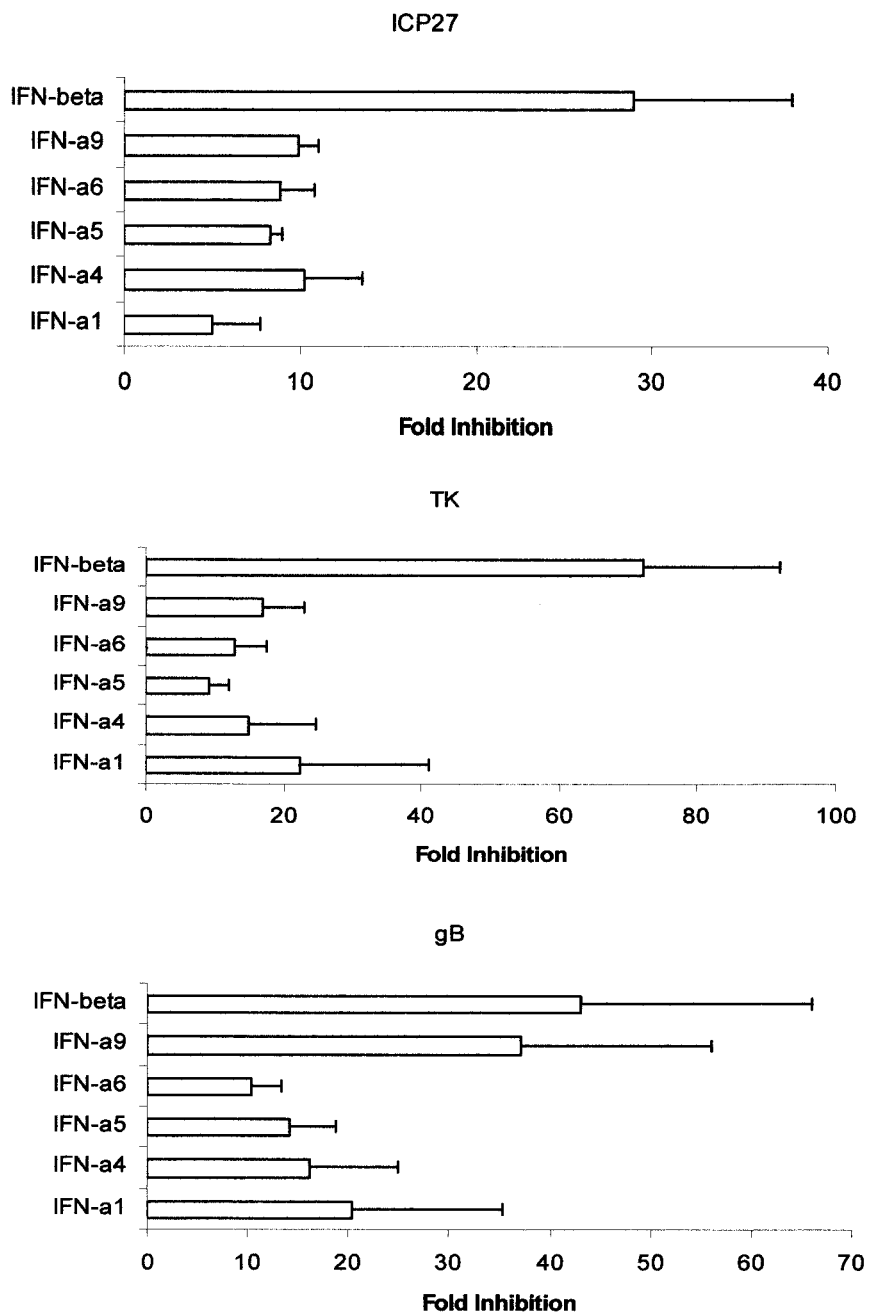


FIG. 3. Expression of HSV-1 α , β , and γ genes in infected cells transfected with IFN- α/β plasmid constructs. L929 cells (500,000 cells/well) were transfected with 3 μ g of plasmid DNA alone (Vector) or plasmid containing the indicated IFN- α or - β transgene. Following an overnight incubation, the supernatant was removed and the cells were infected with HSV-1 (McKrae strain) at an MOI of 0.5. At 6 to 10 h p.i., the cells were collected. Then RNA was harvested and used to generate cDNA template with reverse transcriptase. Using oligonucleotide primers specific for *icp27*, *tk*, and *gB*, the relative amount of each HSV-1 gene was determined by real-time PCR. The change (fold decrease) in viral gene expression was calculated from the relative level of expression for each IFN- α/β transgene group in comparison to that for the plasmid vector control. Bars represent mean \pm standard error of the mean. This figure is a summary of four experiments.

JAK1 and STAT1 expression following transfection of cells with the IFN- α/β plasmid constructs correlated with resistance to HSV-1 infection. To this end, L929 cells transfected with plasmid vector control or plasmids containing the IFN- α/β transgenes were assessed for JAK1 and STAT1 expression prior to and/or after infection with HSV-1. Similar levels of the

housekeeping gene β -actin and JAK1 were expressed by all transfected cells (Fig. 5). In addition, all IFN- α/β transgene-transfected cells showed increased levels of STAT1 expression prior to infection, compared to the nontransfected controls (Fig. 5). However, cells transfected with the IFN- α 4 or IFN- β transgenes consistently expressed the greatest amount of

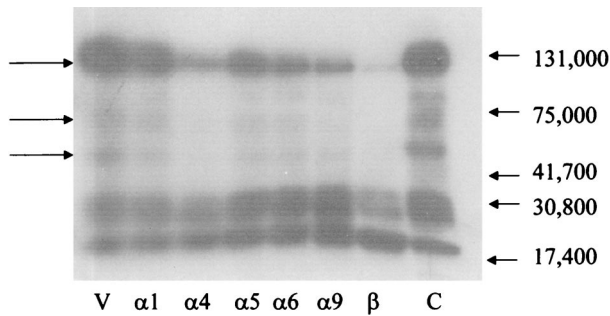


FIG. 4. IFN- α/β transgenes antagonize HSV-1 protein synthesis in transfected cells. Total cell lysates (10 μ g/lane) from transfected L929 cells previously infected with HSV-1 (24 h earlier) were electrophoresed on polyacrylamide gels, transferred onto PVDF membranes, and used for Western blot analysis with a polyclonal anti-HSV antibody. This figure is a representative of four experiments with identical outcomes. Western blot for HSV-1 antigen. Note the diminished expression of proteins migrating at approximately 50, 75, and 125 kDa (arrows) in comparisons of the plasmid vector (V) to the IFN- α or - β transgene groups. A corresponding Coomassie blue-stained polyacrylamide gel showed an equivalent level of protein loaded in each lane (data not shown). C, control.

STAT1, compared to the other IFN- α/β transgene-transfected cells (Fig. 5). By 24 h p.i., STAT1 expression was similar in all transfected cells but had declined from noninfected (0 h) cells. Since it is phosphorylated STAT1 (p-STAT1) that ultimately complexes with p-STAT2 and p48 to form ISGF3, p-STAT1 levels were measured in the transfected cells. Only in those cells transfected with the IFN- β transgene were the p-STAT1 levels consistently detected at very low levels (data not shown). Occasionally, cells transfected with the IFN- α 4 and IFN- α 9 transgenes expressed detectable p-STAT1. On the other hand, cells transfected with the IFN- α 9 or IFN- β transgene showed an increase in the expression of p-JAK1 (Fig. 5).

The antiviral effects elicited by IFN- α/β require PKR. IFN- α/β have been shown to activate a number of genes, including the IFN-stimulated genes OAS/RNase L and PKR (12). By infecting cells in which either the PKR or OAS/RNase L gene

TABLE 2. IFN- α 4- and IFN- β -transgene-transfected cells express less HSV-1 antigen^a

Plasmid construct or control	HSV-1 expression in 120- to 125-kDa protein
PKCMV alone	9.8 \pm 1.9 ^b
PKCMV-IFN- α 1	6.9 \pm 2.2
PKCMV-IFN- α 4	1.8 \pm 0.4*
PKCMV-IFN- α 5	8.4 \pm 2.5
PKCMV-IFN- α 6	7.9 \pm 1.3
PKCMV-IFN- α 9	4.3 \pm 0.9*
PKCMV-IFN- β	1.7 \pm 0.7*
Nontransfected control	14.3 \pm 3.8

^a Protein from cell lysates (10 μ g) of transfected L929 cells infected with HSV-1 (MOI = 0.5) obtained 24 h p.i. were resolved on SDS-7.5% polyacrylamide gels, transferred onto PVDF membranes, and probed for HSV-1 antigen using a polyclonal rabbit anti-HSV antibody. This table is a summary of four experiments using densitometric scanning software (ONE-Dscan software; Scanalytics, Billerica, Mass.) for image analysis of the protein migrating at 120 to 125 kDa in each of the transfected and nontransfected cells. A representative example is shown in Fig. 4.

^b Numbers represent the mean pixel equivalent \pm standard error of the mean. * $P < 0.05$ in comparisons of IFN- α/β transgene-transfected cells to the plasmid vector-transfected control as determined by Wilcoxon signed-rank test.

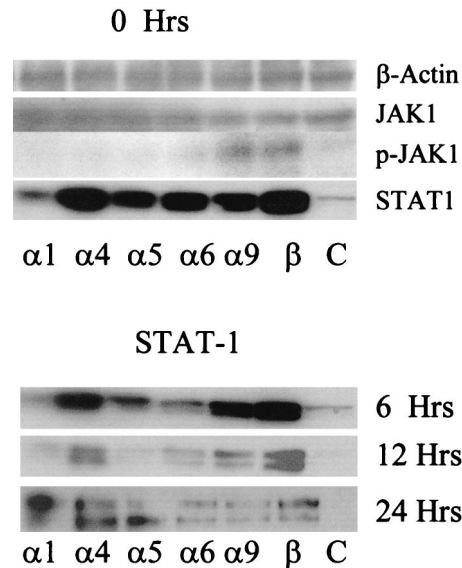


FIG. 5. STAT1 and p-JAK1 are elevated in cells transfected with the IFN- β transgene. Shown are total cell lysates (10 to 30 μ g/lane) from transfected or nontransfected (C) L929 cells that were noninfected (0 h) or infected with HSV-1 (MOI = 0.5) and were electrophoresed on polyacrylamide gels, transferred to PVDF membranes, and used for Western blot analysis with polyclonal antibody specific for the designated protein. This figure is a representative of two to eight experiments/time point with similar outcomes.

is disrupted, it might be possible to distinguish the antiviral efficacies of the different murine IFN- α and IFN- β subtypes. Consistent with previous observations (35), cells deficient in either PKR or OAS/RNase L were highly susceptible to HSV-1 infection, with a significant increase in virus yield compared to that of the wild-type counterparts, suggesting that PKR and OAS/RNase L are central in constitutive resistance to HSV-1. Specifically, in the absence of PKR, viral titers rose fivefold, as opposed to a 14-fold rise in viral titers in the absence of the OAS/RNase L gene compared to results for wild-type cells (data not shown). Pretreating cells deficient in PKR with IFN- α/β conferred no resistance to HSV-1 infection, whereas pretreatment of the corresponding wild-type cells with most IFN- α subtypes or IFN- β (50 to 100 U/ml) resulted in a two- to sixfold reduction in viral titers (Fig. 6a). Wild-type cells treated with IFN- α 6 did not show a significant reduction in viral titers. By comparison, the absence of OAS/RNase L had no significant impact on the antiviral effect of IFN- α/β s with the exception of IFN- α 6 (Fig. 6b). Specifically, the treatment of wild-type cells with IFN- α 6 did not reduce viral titers, whereas exposure of RL-/- cells to IFN- α 6 resulted in a significant reduction (Fig. 6b). We interpret these results to suggest that the absence of OAS/RNase L does not impact on resistance to HSV-1 conferred by most IFN- α/β (with the exception being IFN- α 6) under these experimental conditions.

DISCUSSION

In the present study, the efficacies of different types of IFN- α/β against HSV-1 were compared using cells transfected with plasmid constructs containing IFN- α/β transgenes. We have

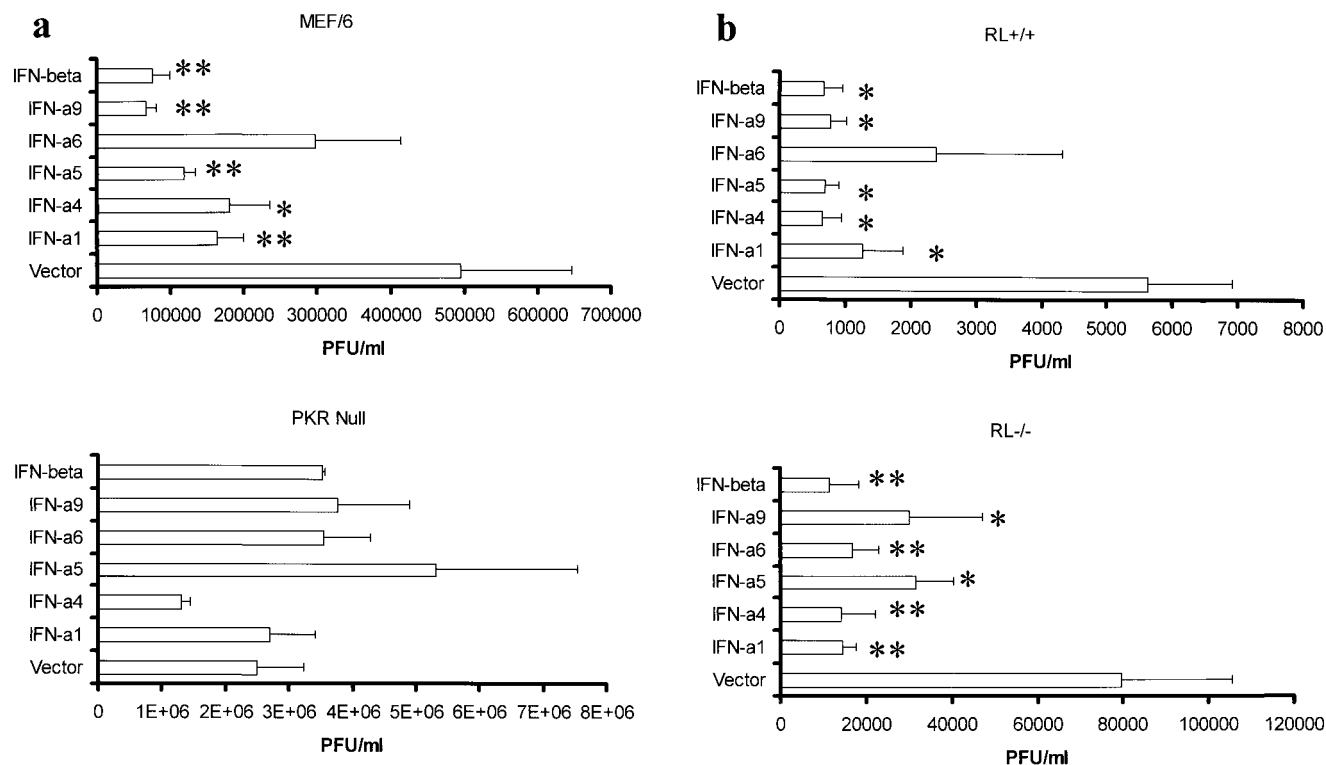


FIG. 6. IFN- α/β antagonizes HSV-1 replication through PKR. (a) Wild-type (MEF/6) or PKR-null cells (2.5×10^5 cells/well) were incubated with equivalent amounts of the indicated IFN-containing supernatants from transfected L929 cells (50 to 100 U/ml based on bioassay). Following an overnight incubation, the cells were infected with HSV-1 (MOI = 1.0) and the cultures were assayed for viral titers 28 h p.i. This figure is a summary of three experiments. Bars represent mean \pm standard error of the mean. *, $P < 0.05$; **, $P < 0.01$ in comparisons of the cells incubated with IFN- α/β -containing supernatants with the corresponding wild-type cells incubated with vector-transfected culture supernatants, as determined by ANOVA and Scheffé multiple-comparison test. (b) Wild-type (RL+/+) or OAS/RNase L-null cells (RL-/-) cells (2.5×10^5 cells/well) were incubated with equivalent amounts of the indicated IFN-containing supernatants from transfected L929 cells (50 to 100 U/ml based on bioassay). Following an overnight incubation, the cells were infected with HSV-1 (MOI = 1.0) and the cultures were assayed for viral titers 28 h p.i. This figure is a summary of four experiments. Bars represent mean \pm standard error of the mean. *, $P < 0.05$; **, $P < 0.01$ when the corresponding wild-type or knockout cells incubated with IFN- α/β -containing supernatants are compared to the cells incubated with vector-transfected culture supernatants as determined by ANOVA and Scheffé multiple-comparison test.

previously found that, following transfection, the IFN transgene product elicits an antiviral state following secretion into the culture milieu. Specifically, neutralizing antibody to IFN- α/β applied to the culture supernatant of the transfected cells blocks the antiviral effect (D. J. J. Carr, unpublished observation). These results suggest that the transgene product must bind extracellularly to the IFN- α/β receptor in order to confer resistance to viral infection through the induction of numerous IFN-responsive genes. Such results are consistent with a previous finding indicating that a functional IFN- α/β receptor is required to elicit antiviral effects through autocrine IFN- α/β production (58). IFN- β was found to suppress HSV-1 replication to the greatest extent, in comparison to IFN- α subtypes when viral titers from the cultures of infected, transfected L929 cells are measured. Viral titers in L929 cells transfected with the IFN- β transgene were reduced by 30-fold compared to results for the IFN- $\alpha 5$ transgene, which elicited the least favorable repression of viral replication with only a threefold reduction in viral titer. Since the L929 cells transfected with the IFN- α/β transgenes produced similar amounts of biologically active IFN- α/β , the disparity in efficacy between the IFN- β transgene and the IFN- α plasmid constructs cannot be based

on the quantity of the transgene product secreted. Unfortunately, the IFN- β product could only be measured by bioassay, so, therefore, a true quantitative measurement could not be determined. Nevertheless, similar amounts of IFN- α were generated as determined by ELISA and bioassay and yet differences in antiviral activity were observed between IFN- α subtypes, suggesting that, even within the same family of IFN- α/β , the efficacy against a viral pathogen can differ.

Cells transfected with the IFN- β transgene strongly suppressed HSV-1 immediate early, early, and late gene expression as represented by *icp27*, *tk*, and *gB*, respectively. In comparison, cells transfected with the IFN- α transgenes antagonized HSV-1 gene expression to similar degrees, with the notable exception of the IFN- $\alpha 9$ transgene, which suppressed HSV-1 *gB* gene expression to a level nearly equivalent to that of the IFN- β transgene. However, at the protein level, the major HSV-1 protein(s) identified (migrating at an apparent molecular mass of 120 to 125 kDa) was equally suppressed in cells transfected with either the IFN- $\alpha 4$ or IFN- β transgene. Likewise, cells transfected with the IFN- $\alpha 9$ transgene showed a significant reduction in the expression of the 120- to 125-kDa HSV-1 protein. These findings are in agreement with the out-

come of viral loads recovered from transfected L929 cells, in which fold reduction in viral titers by the IFN- β , IFN- $\alpha 9$, and IFN- $\alpha 4$ transgenes was 30, 9.0, and 8.6, respectively, compared to 6.7-, 5.0-, and 3.0-fold reduction by the IFN- $\alpha 6$, IFN- $\alpha 1$, and IFN- $\alpha 5$ transgenes, respectively. Taken together, the data clearly indicate that the IFN- β transgene shows the greatest degree of efficacy against HSV-1 replication *in vitro*, followed by IFN- $\alpha 4$ and IFN- $\alpha 9$, a finding supported by data establishing the IC₅₀ for each of the IFN- α/β subtypes. Of note, a previous study suggests that, following viral infection, either IFN- β or IFN- $\alpha 4$ is produced, which can subsequently stimulate the expression of other IFN- α species (including IFN- $\alpha 2$, - $\alpha 5$, - $\alpha 6$, and - $\alpha 8$) through the IFN- α/β receptor, ultimately conferring resistance to viral infection (43). In the present study, it is tempting to speculate that the transfection of cells with the IFN- $\alpha 4$ or IFN- β transgene prior to infection allows cells sufficient time to express other delayed IFN- α species to counter HSV-1 replication. Although other IFN- α transgenes (IFN- $\alpha 9$ notwithstanding) are capable of antagonizing HSV-1 replication, it is interesting that the degree of suppression is not as strong as that for IFN- $\alpha 4$, IFN- $\alpha 9$, or IFN- β , implying that either (i) additional IFN- α/β subtypes are not expressed in L929 cells following transfection with the IFN- $\alpha 1$, IFN- $\alpha 5$, or IFN- $\alpha 6$ transgene or that (ii) the time needed to express additional IFN- α/β subtypes is greater than the incubation period between transfection and infection (18 h).

IFN- α/β act through the same receptor (48), facilitating the transphosphorylation and activation of Tyk2 and JAK1 (54), ultimately leading to the phosphorylation of the STAT1 molecule (63). Consequently, the antiviral resistance induced by the IFN- α/β may, in part, reside with the level of JAK1 or STAT1 expression and/or phosphorylation (14). To this end, JAK1 and STAT1 expression was assessed in transfected L929 cells prior to and at various times *p.i.* Although JAK1 expression was equivalent in all cells analyzed, the induction of STAT1 expression prior to infection was greatest in cells transfected with the IFN- $\alpha 4$ or IFN- β transgene. Correlating with the elevated STAT1 expression in the IFN- β transgene-transfected cells, there was also the detection of p-STAT1 and p-JAK1 expression, indicating that the IFN- β transgene activates STAT1 and JAK1 within 18 h following transfection. At 6 to 12 h *p.i.*, STAT1 expression was still elevated in those cells transfected with the IFN- $\alpha 4$ or IFN- β transgenes, compared to expression in the other transfected groups. It is interesting that these cells showed the greatest degree of resistance to HSV-1 infection based on viral titers and viral protein expression. Although the relationship between STAT1 expression and HSV-1 infection has not been clearly defined, a recent article suggests that HSV-1 antagonizes the phosphorylation of STAT1 through the failure to activate JAK1 (74). Presumably then, the inhibition of p-STAT1 accumulation would prevent the formation of the transcription factor ISGF3 and reduce the activation of IFN-stimulatory genes driven by the IFN-stimulated response element. Although the present study did not assess p-STAT1 levels at times *p.i.*, our lab has found that Vero cells transfected with a plasmid containing the human IFN- β transgene express pSTAT1 levels 24 h *p.i.*, whereas expression of pSTAT1 in cells transfected with the human IFN- $\alpha 2$ transgene is absent, similar to expression in nontransfected, infected cells, suggesting that IFN- β may counter the action of HSV-1

in preventing the activation of JAK1 (23). This notion is supported with the increased expression of p-JAK1 expression in cells transfected with the IFN- β transgene. While the responsible agent antagonizing the activation of the IFN signaling cascade during HSV-1 infection is presently unknown, it may involve the immediate early gene product ICP0 (74).

Two prominent antiviral pathways activated following IFN- α/β receptor ligation are OAS and PKR (20). OAS leads to the activation of endo-RNase L, which degrades viral transcripts (53) and inactivates translational machinery (30). PKR phosphorylates the α subunit of eukaryotic initiation factor 2 α , resulting in the inhibition of protein synthesis (46, 57). HSV-1 has been found to prevent UV- or anti-Fas antibody-induced apoptosis associated with a marked reduction in caspase-3 and caspase-8 activity (34). By preventing apoptosis of infected cells, cellular machinery is preserved for efficient viral replication and packaging of progeny virus. However, IFN- α/β promote apoptosis (4) through a PKR-mediated pathway (69) and therefore would reduce the potential for viral replication. Although there are multiple IFN-responsive genes activated through the IFN- α/β receptor, it might be possible to segregate the antiviral efficacy for each IFN- α/β species by initially focusing on the dominant PKR and OAS/RNase L antiviral pathways known to impact on HSV-1 infection (35). In the absence of RNase L, the antiviral efficacy of IFN- $\alpha 6$ was significantly increased. In addition, the strength of the inhibition as measured by recoverable virus was increased in the RNase L knockout cells treated with the other IFN- α/β . A similar observation was found when these cells were transduced with an adenoviral construct expressing the murine IFN- β gene (unpublished observation). We interpret these results to indicate that the activation of the downstream effector molecule of OAS, RNase L, targets not only viral RNA but also IFN-inducible transcripts as well. In the absence of this pathway, the longevity of IFN-stimulatory cascades would be prolonged, increasing the antiviral environment induced by the IFN- α/β . In contrast to the results with the RL-/- cells, in the absence of PKR, the efficacy of IFN- α/β against HSV-1 is completely lost. These results substantiate the contributions of other investigators, illustrating the countering effect of the HSV-1 $\gamma_1 34.5$ protein on PKR action (8, 39). Since, under normal conditions, the virus devotes a considerable amount of energy to encoding the $\gamma_1 34.5$ protein, in order to circumvent the antiviral action of PKR by dephosphorylating eukaryotic initiation factor 2 α following PKR-induced phosphorylation (25), the PKR pathway is undoubtedly an important endogenous contributor to antagonizing HSV-1 replication. Future studies are required to evaluate the levels of other components of the ISGF3 complex in the transfected cells, in addition to the downstream effector molecules generated from the activation of the IFN-inducible genes, as a means to address differences in antiviral efficacy between different IFN- α/β (i.e., IFN- α and - β).

In summary, the present study has shown that cells transfected with an IFN- β plasmid construct are less sensitive to HSV-1 infection than are cells transfected with plasmid constructs containing five different IFN- α transgenes. Each of these transgenes, nearly equal in size, was cloned into the same plasmid and produced a similar amount of biologically active IFN. To our knowledge, this is the first report that has com-

pared murine IFN- α/β against HSV-1 infection and found murine IFN- β to provide superior protection against the HSV-1 infection, as measured by viral titers and viral gene and protein expression.

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