ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 313 (2003) 126–135

www.elsevier.com/locate/yviro

The murine double-stranded RNA-dependent protein kinase PKR and the murine 2′,5′-oligoadenylate synthetase-dependent RNase L are required for IFN-β-mediated resistance against herpes simplex virus type 1 in primary trigeminal ganglion culture

Khaldun Al-khatib,[a] Bryan R.G. Williams,[b] Robert H. Silverman,[b] William Halford,[c] and Daniel J.J. Carr[a,*]

[a] *Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA*
[b] *Department of Cancer Biology/NB40, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA*
[c] *Tulane University Medical Center, Program in Molecular Pathogenesis and Immunity, New Orleans, LA 70112, USA*

Received 9 December 2002; accepted 13 March 2003

Abstract

A study was undertaken to evaluate the efficacy of an adenoviral construct expressing the murine interferon-β (IFN-β) transgene (Ad:IFN-β) against herpes simplex virus type 1 (HSV-1) infection in a primary trigeminal ganglion (TG) cell culture. The transduction efficiency ranged from 0.2 to 11.0% depending on the multiplicity of infection (m.o.i.) of the adenoviral vector (0.5–50.0). Moreover, neurons were the main target of the adenoviral transduction. TG cultures transduced with Ad:IFN-β displayed up to a 19-fold reduction in viral titers compared with cells transduced with an Ad:Null or nontransduced TG culture controls. Transduction with Ad:IFN-β up-regulated two critical antiviral genes, double-stranded RNA-dependent protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (OAS). The absence of PKR or RNase L (downstream effector molecule of OAS) attenuated Ad:IFN-β efficacy against HSV-1 replication, implicating a critical role for PKR and OAS/RNase systems in the establishment of IFN-induced resistance against HSV-1 in TG cells.

Keywords: HSV-1; Adenoviral vector; Neuron; IFN-β; Trigeminal ganglia; PKR; OAS

Introduction

Herpes simplex virus type 1 (HSV-1) corneal infection represents a significant clinical problem. The virus enters the host by direct inoculation of mucous membranes, where it establishes a lytic infection of epithelial cells (Krummenacher et al., 1998). During the primary infection, HSV-1 is able to spread to the neurons of the sensory ganglion [e.g., trigeminal ganglion (TG)] and establish a latent infection (Cook and Stevens, 1973). It is within the peripheral nervous system that the virus must overcome immune detection, evade innate immunity, and alter expression of lytic genes in establishing a life-long persistent infection capable of reactivation either spontaneously or following environmental cues.

Type I interferons (IFN-α/β) are crucial antiviral proteins induced in the early innate immune response to viral infection. These regulatory cytokines antagonize synthesis of viral mRNA and translation of viral proteins (Pestka et al., 1987). Type I IFNs not only enhance immunologic targeting of virally infected cells by activating natural killer cell cytotoxicity and stimulating T_H1 cell development (Gutterman, 1994), but they also induce the synthesis of intracellular proteins that directly inhibit viral replication. IFN-responsive, antiviral proteins include double-stranded RNA-dependent protein kinase R (PKR) and 2′,5′-oligoadenylate synthetases (OAS) that function through RNase L (Foster, 1997). OAS is a group of enzymes that catalyze the synthesis of 5′-triphosphorylated, 2′ to 5′ linked oligoad-

* Corresponding author.
E-mail address: don-carr@ouhsc.edu (D.J.J. Carr).

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(03)00298-8

enylates, typically three or four nucleotides in length (2-5A) (Kerr and Brown, 1978). The 2–5A molecules bind with high affinity to RNase L, an endoribonuclease that catalyzes the cleavage of single-stranded mRNA and rRNA, thereby leading to inhibition of protein synthesis (Silverman, 1997). Relative to HSV-1 infection, disruption of the RNase L gene has been found to increase the susceptibility of mice to HSV-1 keratitis and viral-mediated mortality through an ill-defined mechanism (Zheng et al., 2001). Another IFN-responsive protein, PKR, is a serine/threonine kinase that is normally inactive, but is activated by binding to dsRNA or other polyanions (Meurs et al., 1990; Katze et al., 1991). Activated PKR suppresses translation initiation via phosphorylation of eukaryotic initiation factor 2 (Meurs et al., 1992) and acts as a signal transducer for proinflammatory gene expression (Williams, 2001). Recent results demonstrate that the absence of PKR greatly increases HSV-1 replication in mouse embryo fibroblasts (Khabar et al., 2000).

The present study was undertaken to test the hypothesis that an adenoviral vector that contains the murine IFN-β transgene (Ad:IFN-β) is proficient in delivery and expression of the transgene to primary TG cell cultures. Adenoviral vectors are powerful tools in vivo because of the wide range of cells and tissues they can transduce with high efficiency. The use of such vectors would provide efficient means to deliver IFN-β to the TG, which might subsequently interfere with reactivation in vivo. The present study aimed to test whether RNase L or PKR plays a role in mediating the antiviral effects of IFN-β. The use of primary TG cell cultures is advantageous over the use of cell lines. Specifically, the TG is the natural site in the establishment of latency during acute infection as well as reactivation from latency in quiescent HSV-1 infection. In addition, cell lines are transformed cells typically undergoing major changes in their pro- and antiapoptotic pathways (Farassati et al., 2001; Malkinson et al., 1997; Roninson, 2002). In some instances, these same pathways are involved as a defense against viral infection. Both RNase L and PKR control cell growth, cell differentiation, viral clearance, and apoptosis (Castelli et al., 1998; Gil et al., 2002; Zhou et al.,1997). Therefore, IFN-signaling is more likely to be altered in these cell lines as compared to primary cells. The present study found primary TG cells transduced with Ad:IFN-β secreted biologically active IFN and repressed viral replication in a dose-dependent fashion that was dependent on PKR and to a lesser extent, OAS/RNase L.

Results

Transduction efficiency and production of IFN-β

A replication-deficient adenovirus construct expressing green fluorescent protein (Ad:GFP) was employed to determine the efficacy of transduction. Transduction of primary TG cells resulted in green fluorescence expressed in cultured cells in a dose-dependent fashion (Fig. 1A). TG cells represent a heterogeneous population composed of neurons, fibroblasts, and supporting cells (e.g., Schwann cells). Based on morphological characteristics of the neurons (i.e., large, single cells that contain a darkly pigmented cytoplasm and a prominent nucleus), the cells most receptive to transduction (m.o.i. = 5.0) were most likely neurons (Fig. 1B). To validate this observation, Ad:GFP transduced TG cell cultures were fixed and stained for neuron-specific enolase. A disproportionably high number of cells that were positive for GFP costained with neuron-specific enolase (Fig. 2). In contrast, fibroblasts and most of the supporting cells that normally predominate these cultures expressed low to undetectable levels of GFP. However, upon increasing the m.o.i. (m.o.i. = 50.0) of the Ad:GFP, a more heterogeneous population of cells expressed GFP (data not shown). The results suggested that Ad:GFP preferentially transduced neuronal cells when a low m.o.i. was used. On average, each primary TG cell culture contains 341 $\pm$ 29 neurons/well with each well containing between 5 and 10 $\times$ 10^4 cells at the time of transduction.

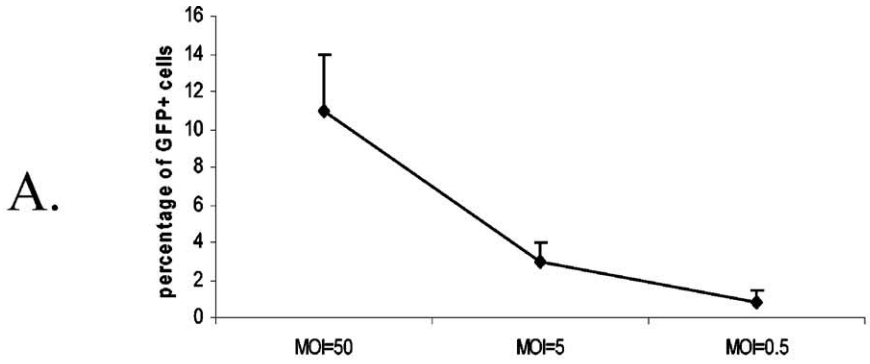
Since targeted cells were efficiently transduced with the adenoviral construct expressing the reporter gene product GFP, cells were subsequently targeted using the replicative-deficient adenoviral construct containing the murine IFN-β gene. Cells that were transduced with Ad:IFN-β secreted biologically active IFN-β in a dose-dependent fashion (Table 1). Nontransduced cells or cells transduced with the Ad:Null construct secreted no measurable level of biologically active IFN-β.

Ad:IFN-β inhibits HSV-1 replication

To determine the efficacy of Ad:IFN-β in antagonizing HSV-1 replication in primary TG cell cultures, dissociated TG cells were transduced with Ad:IFN-β and assayed for resistance to the highly neurovirulent strain of HSV-1, McKrae. Virus titers were significantly less in the Ad:IFN-β transduced cells compared with the nontransduced cells or cells transduced with Ad:Null vector (Fig. 3A). The reduction in infectious viral titer was dependent on the m.o.i. of the Ad:IFN-β used for transduction (Fig. 3B). In the presence of neutralizing antibody to IFN-β, no antiviral activity was observed in the Ad:IFN-β transduced cultures, suggesting the IFN-β must be secreted to confer resistance to HSV-1 infection (data not shown).

Absence of a functional PKR or OAS/RNase L pathway attenuates the antiviral state of the Ad:IFN-β construct

In an attempt to identify contributing factors to the antiviral nature of the IFN-β transgene, levels of two critical IFN responsive, antiviral genes (PKR and OAS) were assessed in the transduced TG cells using real-time PCR. PKR and OAS transcript levels were 13- and 26-fold higher, respectively, in the TG cultures transduced with Ad:IFN-β compared



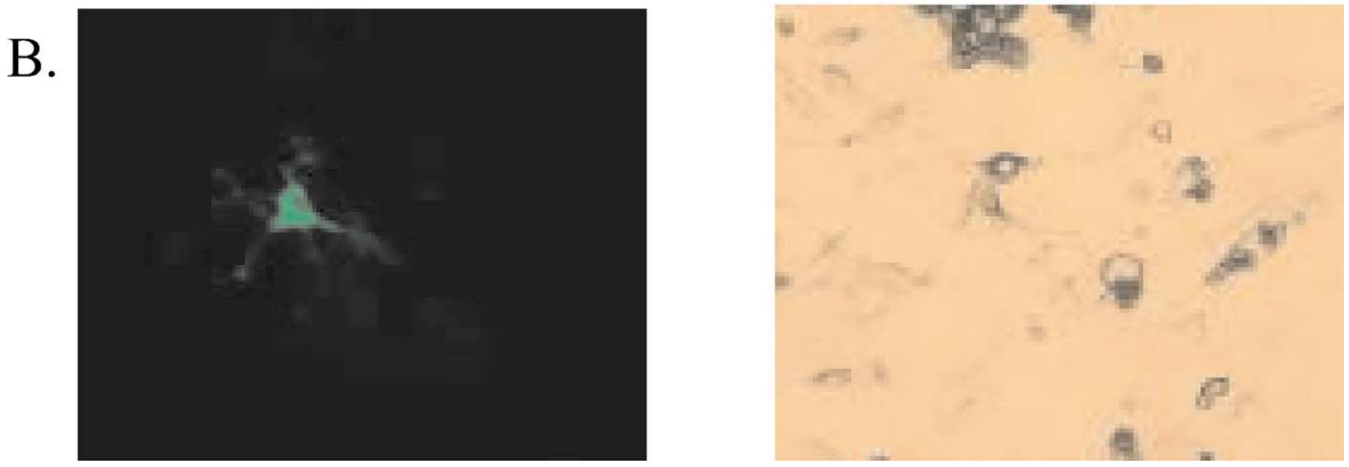


Fig. 1. Transduction of mouse trigeminal ganglion cell cultures. (A) TG cells isolated from ICR mice were plated in 24-well microtiter plates in 1 ml of TG media. After 7 days of culturing, the medium was removed and Ad:GFP was added at the indicated m.o.i. Forty-eight hours posttransduction, three random fields were counted for total and GFP expressing cells by fluorescence microscopy (Zeiss Axiovert 135) under × 200 magnifications. The graph represents the percentage of cells expressing GFP (number of cells expressing GFP/total number of cells in the field). The experiment was conducted twice, each in triplicate. (B) Representative photomicrographs of a neuron in a TG cell culture 48 h after transduction with Ad:GFP at an m.o.i. of 5, as seen when illuminated with visible light (right panel) or the 360- to 400-nm spectrum of light (left panel).

to the Ad:Null- or nontransduced controls (Fig. 4). To delineate the potential relevance of the PKR and OAS/RNase L pathways in the establishment of an antiviral state following Ad:IFN-β transduction, dissociated TG cells from mice deficient in the RNase L enzyme (RL−/−) or in PKR (PKR−/−) were employed. RNase L is the downstream effector molecule induced following OAS activation, and its deletion produces a functional knockout of the OAS pathway (Zhou et al., 1997). There was no difference in the viral titers recovered from wild-type (wt), PKR−/−, and RL−/− in the nontransduced or Ad:Null-transduced TG cell cultures (Fig. 5A). Although transduction with Ad:IFN-β reduced the viral yield from the TG cell cultures taken from the knockouts or wild-type mice, the sensitivity of the knockouts to IFN was dependent on the m.o.i. of HSV-1. At an m.o.i. = 1.0 only the absence of PKR adversely affected the Ad:IFN-β efficacy compared to the wt or the RL−/− TG cell cultures (Fig. 5A). Specifically, in the wt or RL−/− TG cultures, Ad:IFN-β transduction reduced the viral titer 17-fold, whereas the reduction in PKR−/− TG cell cultures was only seven-fold (Fig. 5D). However, at a lower m.o.i. (m.o.i. = 0.1 or 0.02) the absence of either PKR or OAS adversely affected the Ad:IFN-β efficacy (Figs. 5B and C). Specifically, at an m.o.i. of 0.02, in the wt cultures, Ad:IFN-β transduction reduced the viral yield 129-fold, whereas the reduction in RNase L−/− cultures was 25-fold and the PKR cultures was only 15-fold (Fig. 5D).

Discussion

The present study demonstrates the feasibility of gene transfer using a replication-deficient, recombinant adenovi-

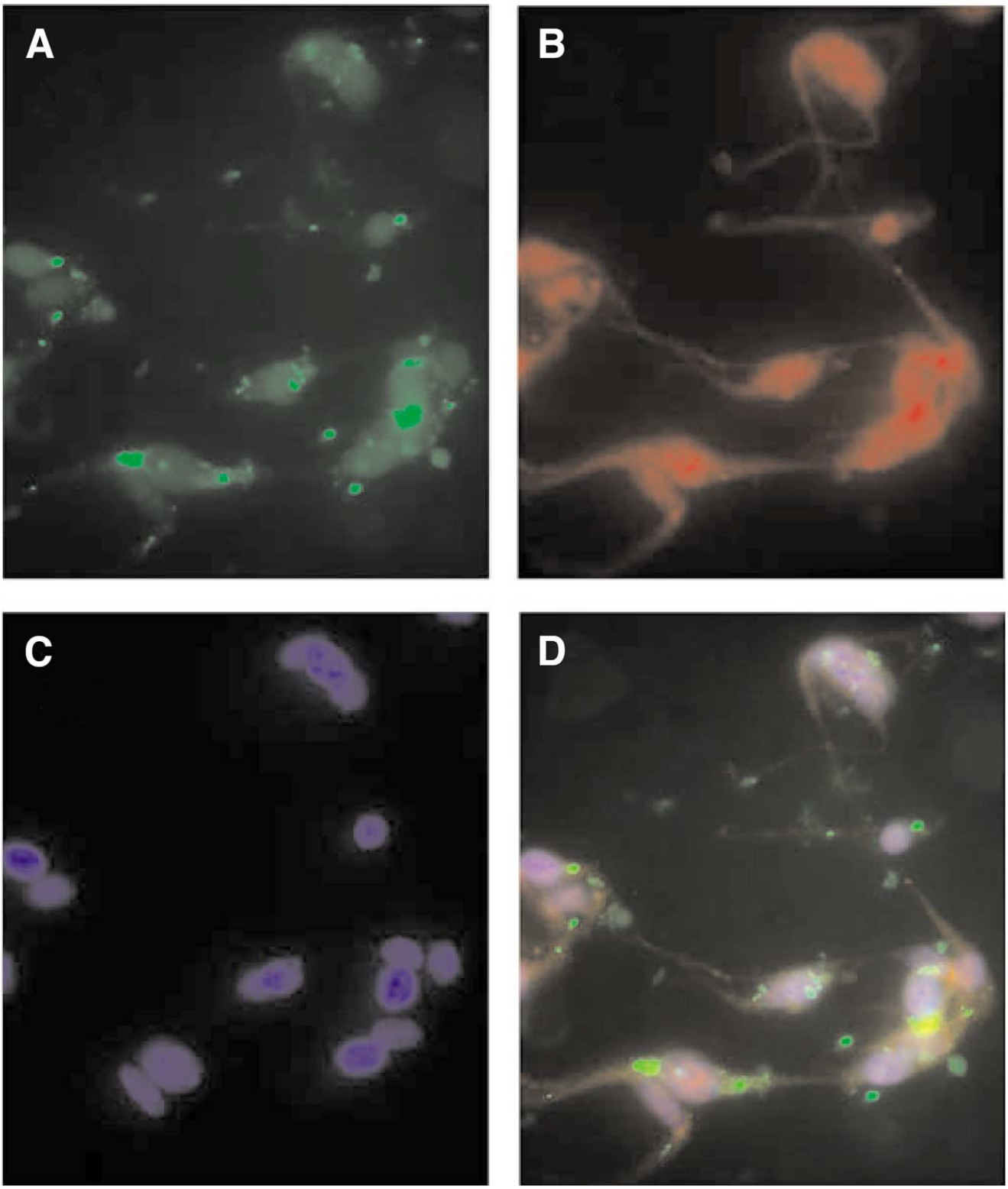


Fig. 2. Adenoviral vectors preferentially transduce neurons in primary TG cell cultures. Dissociated TG cells isolated from ICR mice were grown in culture on coverslips and were transduced with Ad:GFP (m.o.i. = 5.0) 7 days after establishment of the culture. Two days later, the cells where fixed and stained as described under materials and methods. GFP expression in Ad:GFP transduced cells (A) colocalized with neuron-specific enolase (B); nuclei were counterstained with DAPI (C). (D) represents the merge image.

ral vector to deliver an exogenous murine IFN-β transgene to primary TG cell cultures. Adenoviral transduction in the peripheral nervous system is rarely investigated. Previous data showed that adenoviral vectors injected in the posterior horn of the spinal cord could deliver genes to motor neurons and to a lesser degree, other types of neurons and supporting

Table 1
Cells transduced with Ad:IFN-β secrete biologically active IFN-β[a]

m.o.i. of the Ad:IFN-β used (IU/ml)[b]	Amount of biologically active IFN-β
50	100 ± 29
5	20 ± 4.5
0.5	1.5 ± 0.6
0	0.0 ± 0.0
Ad:Null (m.o.i. = 5)	0.0 ± 0.0

[a] ICR Mouse dissociated TGs were plated at a density of 8×10^4 (equivalent to 1 TG) in 24-well plates. After 7 days in culture, the media was removed and Ad:IFN-β was added at the designated m.o.i. Ad:Null (viral vector control) was used to transduce cultures as well. Nontransduced cultures are indicated as m.o.i. = 0. Thirty-six hours posttransduction, the supernatant was collected and the concentration of IFN-β was measured by plaque reduction assay using vesicular stomatitis virus. Results are representative of two independent experiments (n = 3/group/experiment). The level of detection is 1.0 units of IFN/ml.

[b] Numbers represent the concentration of IFN-β expressed as IU/ml ± SEM.

cells (Huber et al., 2000). The present study demonstrates that adenoviral vector is a successful means to deliver desired genes preferentially to the neurons in the TG in a dose-dependent fashion. One possible mechanism for this tropism is the selective presence of the Coxsackie adenovirus receptor (CAR) on neuronal cell surfaces but not other supporting cells. Adenovirus binds to CAR to successfully transduce neuronal cells in vitro (Caillaud et al., 1993). Previous data demonstrated that CAR was primarily located on neurons derived from the CNS and that glia and other supporting cells did not express the receptor on their surface (Soudais et al., 2001). However, the results of this study show that if the m.o.i. of the adenoviral vector is increased, other cell populations in the TG culture can be transduced. It is likely that at a relatively high m.o.i., the adenoviral vector loses cellular specificity through utilizing less efficient receptors to enter the cell (Chiu et al., 2001; Nemerow and Stewart, 1999).

IFN-β has previously been found to up-regulate OAS in primary neuronal cultures through ISGF3 (Massa et al., 1999). In the present study using primary TG cell cultures, the two prominent antiviral, IFN-regulatory genes, OAS and PKR, were elevated 13-fold and 26-fold, respectively, following transduction with the Ad:IFN-β vector. In contrast with previous data using embryonic fibroblast cell lines (Al-Khatib et al., 2002), the present study found the absence of either the RNase L or the PKR pathway did not enhance HSV-1 replication. The disparity between these results may reside with those pathways modified in the cell lines controlling cell growth and apoptosis that ultimately mediate one form of resistance to viral infection (Farassati et al., 2001; Roninson, 2002). The absence of either PKR or RNase L in primary cells is compensated by the presence of other intact antiviral pathways as previously suggested (Zhou et al., 1999) with a number of candidate genes identified (de Veer et al., 2001). Taken together, the use of primary cell cultures provides a more credible system to illustrate the complexities and redundancies associated with the antiviral state elicited by type I IFNs.

Lowering the m.o.i. of HSV-1 resulted in an m.o.i.-dependent decrease in viral yield in the wild-type, RNase L−/−, and PKR−/− cells. However, the importance of PKR or OAS/RNase L systems in mediating IFN antiviral action became more evident with a lower viral inoculum. Specifically, the absence of either PKR or RNase L adversely reduced the antiviral efficacy of IFN-β when a low m.o.i. of HSV-1 was used (m.o.i. = 0.02). However at higher m.o.i. (m.o.i. = 1.0), only the absence of PKR proved detrimental to IFN action. Consistent with our results, other investigators have reported IFN-induced antiviral pathways become more critical and their absence becomes more evident when changing the concentration of IFN exposure or the virus used to infect the IFN-treated cells (Der and Lau, 1995; Zhou et al., 1997; Stojdl et al., 2000).

ICP34.5 is a γ1 HSV viral protein that activates the endogenous cellular protein phosphatase 1α (PP1) to counter the effects of activated PKR (He et al., 1997; Leib et al., 2000). Furthermore, HSV also encodes Us11 (a γ_2 protein) that inhibits PKR activity (Poppers et al., 2000) by preventing a conformational change normally induced by PACT, a protein activator of PKR (Peters et al., 2002). Since Us11 is an abundant tegument protein brought into the cells upon infection, it may act early to block phosphorylation of eIF2α (Leib, 2002; Poppers et al., 2000). On yet another level, HSV-1 reportedly blocks the JAK1/STAT1 signaling cascade by preventing phosphorylation of STAT1 and thus preventing the accumulation of ISGF3 and subsequent induction of IFN stimulatory genes (Yokota et al., 2001). In fact, HSV infection decreases the level of 2-5A synthetase mRNA in IFN-α-treated cells (Taylor et al., 1995). In addition, HSV-1 infection activates ppp(A2′p)nA system in interferon-treated cells, resulting in accumulation of ppp(A2′p)2A and ppp(A2′p)3A species. These compounds are only weak activators of the ppp(A2′p)nA-dependent RNase and under appropriate conditions are capable of inhibiting the activation of this RNase by authentic ppp(A2′p)nA (Cayley et al., 1982, 1984). These findings illustrate the diverse strategies HSV-1 has developed to counter PKR as well as OAS pathways.

Multiple, unique, as well as redundant pathways likely evolved by the host upon encountering HSV-1 and the viral strategies for neutralizing host defenses (Stark et al., 1998). The redundancy in the antiviral state of Ad:IFN-β-transduced cells was evident in the present TG cell-culture model using a relatively high m.o.i. of HSV-1. In the absence of PKR, cells transduced with the Ad:IFN-β were still resistant to HSV-1 infection compared to the control-transduced cells, although the level of resistance had dropped appreciably. We interpret these results to suggest that while the PKR pathway is central to controlling HSV-1, additional IFN-inducible pathways are also involved, evident at the

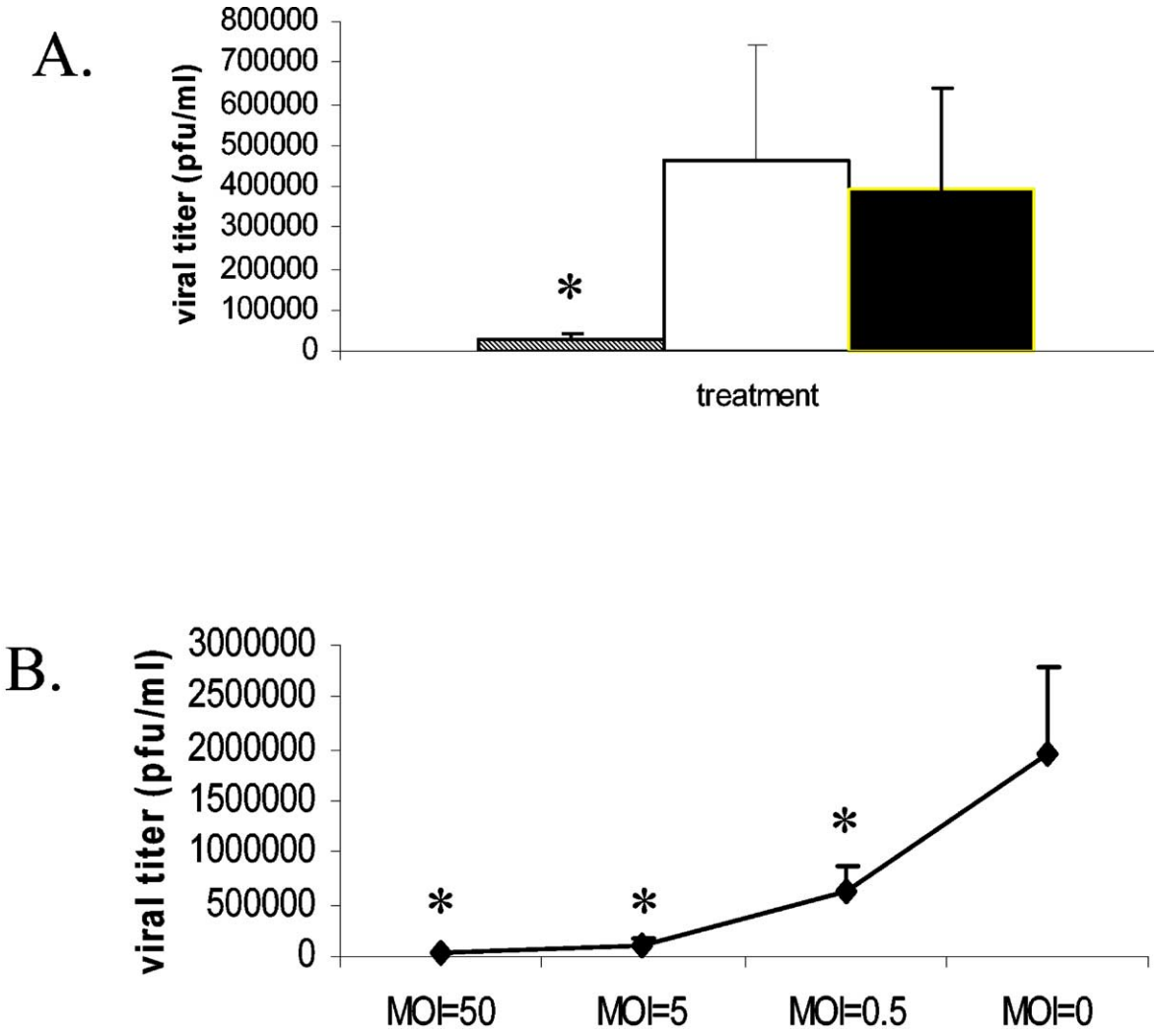


Fig. 3. Mouse TG cell cultures transduced with Ad:IFN-β show resistance to HSV-1 infection. (A) Primary TG cells isolated from ICR mice were transduced with the Ad:IFN-β vector (hatched column) or the Ad:Null vector (white column) at an m.o.i. = 5. The black column represents the nontransduced group. After 36 h the medium was removed and the cells were then infected with HSV-1 (m.o.i. = 1.0). Viral titers (PFU/ml) were measured 24 h postinfection (p.i.). Results are representative of three experiments (n = 3/group/experiment) (*$P < 0.05$ comparing Ad:IFN-β-transduced group with Ad:Null-transduced or to nontransduced groups). (B) Primary TG cells were transduced with Ad:IFN-β at the indicated m.o.i. Cells were subsequently infected with HSV-1 at an m.o.i. of 1 and the viral titer was measured 24 h p.i. Results are the average of two experiments each conducted in triplicate. *$P < 0.05$ comparing the viral titers between cultures transduced at the designated m.o.i. to nontransduced cultures.

higher m.o.i. of HSV-1. At the lower level of infection (m.o.i. = 0.02–0.1), it would appear that OAS/RNase L and PKR are the prominent anti-HSV-1 pathways induced by IFN-β in the TG cells. Consequently, within the confines of our experimental model it would appear that a threshold of virus or viral-derived proteins must be achieved to antagonize the antiviral effects of OAS and PKR. For HSV-1, the level required must be between 0.1 and 1.0 PFU/cell. At or above the level of 1.0 PFU/cell, additional IFN-inducible pathways independent of OAS or PKR appear to be functionally active in hindering HSV-1 replication. The characterization of the relative contributions of these IFN stimulatory pathways will be a challenge due to the large number of IFN-regulated genes (Der et al., 1998). However, only by investigating the specific functions of the proteins encoded in the interferon-stimulated genes will the biological effects of IFNs be understood. Elucidating viral and enzyme-specific mechanisms that mediate the antiviral actions of IFN may also facilitate the design of additional therapeutic strategies to overcome viral pathogenesis.

Materials and methods

Mice, cells, and viruses

Female 8- to 12- week-old ICR mice (Harlan Sprague–Dawley, Indianapolis, IN) as well as C57BL/6 mice (Jackson Labs, Bar Harbor, ME), RNase L-null mice (Zhou et al., 1997), and PKR-null mice (Yang et al., 1995) were used in

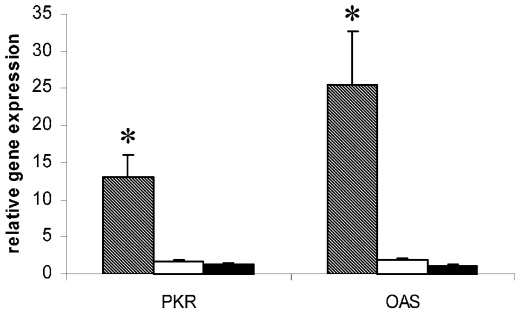


Fig. 4. Ad:IFN-β transduction up-regulates PKR and OAS. Primary TG cells isolated from ICR mice were transduced with either Ad:IFN-β (hatched column) or Ad:Null (white column) at an m.o.i. of 5. A third group was left nontransduced (black column). RNA isolation and reverse transcription was performed with the cells 36 h posttransduction. cDNA templates were amplified using primers specific for OAS and PKR via real time PCR. The data are expressed in terms of relative value representing the relative gene transcript level normalized to the GAPDH level. Results are the average of three independent experiments (n = 2/group/experiment). *$P < 0.01$ comparing Ad:IFN-β-transduced group with Ad:Null-transduced or nontransduced groups.

these experiments. All animals were handled in accordance with the National Institutes of Health guidelines on the care and use of laboratory animals (Publication no. 85-23, revised 1996). All procedures were approved by the University of Oklahoma Health Sciences Center institutional animal care and use committee. L929 and Vero cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and propagated in DMEM or RPMI-1640 medium, respectively, containing 0.375% HCO_3 supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic solution (GIBCO, Gaithersburg, MD) (referred to as complete DMEM or RPMI, respectively).

The viruses used in this study were McKrae strain of HSV-1 and vesicular stomatitis virus (VSV) (a gift from Dr. Robert Fleischmann, UTMB). HSV-1 and VSV were propagated in Vero cells and aliquots were stored at -80°C. The three following replication-defective adenoviral vectors (ΔE1–ΔE3) were used in this study: Ad:GFP, Ad:IFN-β, and Ad:Null (empty vector). The adenovirus vectors were constructed as previously described (Al-Khatib et al., 2002).

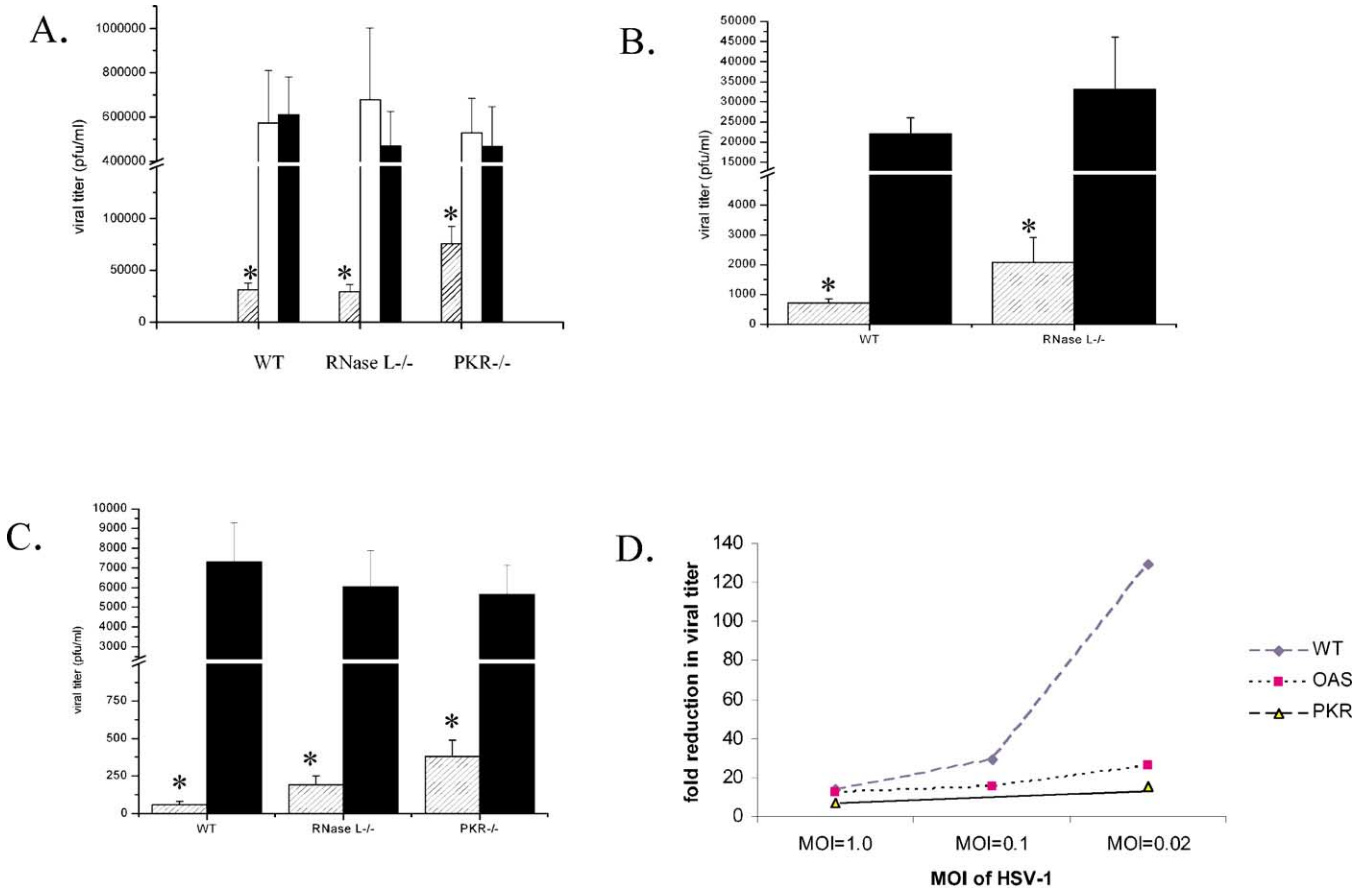


Fig. 5. The absence of a functional PKR or OAS pathway attenuates the antiviral state of the Ad:IFN-β construct. TG were obtained from wild-type C57BL/6, PKR−/−, and RL−/− mice. Dissociated TG cells were plated and 7 days later, the media were removed and either Ad:IFN-β or Ad:Null was added to the cultures at an m.o.i. of 5. A third group was left nontransduced. Thirty-six hours later, the cultures were infected with HSV-1 at an m.o.i. of 1.0 (A), 0.1 (B), or 0.02 (C), and viral titers were measured 24 h p.i. Results are an average of two independent experiments (n = 3 per group per experiment). (*$P < 0.01$ comparing Ad:IFN-β-transduced group with Ad:Null-transduced or to nontransduced groups). (D) Data represented as the fold-reduction in viral titer observed following transduction with Ad:IFN-β relative to Ad:Null-transduced cells.

The adenoviral vectors were all propagated in E293 cells (ATCC) and the E1-complementing cell line under the following conditions: complete DMEM, 37°C, 5% CO_2, and 95% humidity.

Establishment of TG cell cultures

TG cell cultures were prepared according to a modified version of the protocol described by Moriya et al. (1994). TG cells were cultured in complete DMEM medium with 10 ng of nerve growth factor 2.5s (Collaborative Biomedical Products, Bedford, MA) per milliliter (TG medium). TGs were aseptically removed from mice and placed in TG medium on ice. TGs were pooled in 1.5 ml of calcium- and magnesium-free Hank's balanced saline solution containing collagenase type XI (1 mg/ml; Sigma Chemical Co., St. Louis, MO) and collagenase type IV (1 mg/ml; Sigma) and incubated at 37°C for 75 to 90 min. To facilitate dissociation, ganglia were triturated every 15 min with a 1-ml serological pipette. Next, 8.5 ml of TG medium was added to the dissociated cells, which were then pelleted by centrifugation (4°C, 5 min, 200 *g*), and the collagenase-containing supernatant was discarded. The cells were rinsed twice with DMEM medium and finally resuspended in TG medium. The cells were distributed into two 24-well culture plates (1 ml of cell suspension per well) which had been thin coated with rat tail collagen type I (50 mg/ml; Collaborative Biomedical Products) and recombinant mouse laminin (2.5 mg/ml; Collaborative Biomedical Products) according to the vendor's instructions. Cultures were incubated in a 37°C tissue culture incubator (5% CO_2, 95% humidity).

Immunocytochemistry

Dissociated TG cells obtained from ICR mice were grown in culture on coverslips coated with laminin and collagen. Cells were transduced with Ad:GFP (m.o.i. = 5) 7 days after establishment of the culture. Two days following transduction, cells were fixed for 30 min in 3% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (pH 7.5) and then rinsed three times with 1.0 ml of PBS containing 0.1% Triton X-100 (pH 7.5). Nonspecific binding sites were blocked by 10% normal goat serum for 30 min at room temperature. Cells were incubated overnight with polyclonal anti-neuron-specific enolase antibody (Chemicon, Temecula, CA) (1:100 dilution) in PBS (pH 7.5) containing 1% normal goat serum at 4°C. Following the overnight incubation, the cells were then rinsed three times with 1.0 ml of PBS containing 0.1% Triton X (pH 7.5). Subsequently, the cells were incubated with Texas red conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) (1:200 dilution) in PBS (pH 7.5) containing 10% normal goat serum for 1 h and washed three times for 5 min each with PBS (pH 7.5). The cells were then mounted and nuclei were counterstained with DAPI (Vector Laboratories). Flourescently labeled cells were viewed using a Nikon E800 fluorescent microscope. Cells treated without primary antibody served as controls and were unlabeled. All the steps of fixation and staining were done in a dark room to reduce bleaching of GFP.

Transduction studies

Dissociated TG cells were plated at a density of 4×10^4 cells/ml (1/2 TG) in 24-well plates. At day 7 postplating, the medium was removed and either the Ad:IFN-β or the Ad:Null vector was added at the indicated m.o.i. A third group was left nontransduced as a negative control. Twenty-four hours posttransduction, the medium was collected and HSV-1 was added at the indicated m.o.i. The medium was replaced with HSV-1-free medium 1 h postinfection (p.i.). Twenty four hours p.i., cells were lysed by rapid freeze-thawing and viral titers were determined in duplicate by plaque assay using Vero cells. Experiments were carried out three times, each time in triplicate.

Determination of IFN-β concentration

Collected supernatants from transfected cell cultures were clarified by centrifugation (10,000 *g*, 6 min). L929 cells were incubated for 24 h with serial dilutions of supernatants and subsequently infected with VSV at an m.o.i. = 0.05. When the cytopathic effect in the control wells was maximal (between 32 and 36 h p.i), plates were stained with Crystal violet. In each assay, standards with recombinant murine IFN-β (PBL Biomedical Laboratories, New Brunswick, NJ) were employed. The standards themselves were calibrated against WHO international standard IFN-β (Braton Biotech, Inc., Gaithersburg, MD). Fifty percent inhibition of cytopathic effect was equivalent to 1 IU/ml for IFN-β. The incubation of the supernatant with anti-mouse (500 neutralizing units) IFN-β antibody (PBL Biomedical Laboratories) antibody at the time of transduction completely neutralized the protective effect.

Reverse transcription

TG culture cells were transduced (m.o.i. = 5.0) with either the Ad:IFN-β or the Ad:Null vector. A third control group was not transduced. RNA was harvested 36 h following transduction with the adenoviral construct. RNA was extracted in Ultraspect RNA isolation reagent (Biotecx Inc., Houston, TX). First-strand cDNA was synthesized using avian myoblastosis virus reverse transcriptase (Promega, Madison, WI).

Real-time PCR

Real-time PCR using the iCycler (Bio-Rad, Hercules, CA) was used to quantify cellular gene expression. SYBR Green (Molecular Probes, Eugene, OR) was used as the intercalating flourophore at a final dilution of 1:100,000.

Primers with the following sequences were used in these experiments:

GAPDH (3 mM $MgCl_2$)	F: 5′-GAATCTACTGGCGTCTTCACC-3′
	R: 5′-GTCATGAGCCCTTCCACGATGC-3′
PKR (4 mM $MgCl_2$)	F: 5′-GGAAAATCCCGAACAAGGAG-3′
	R: 5′-CCCAAAGCAAAGATGTCCAC-3′
OAS1a (4 mM $MgCl_2$)	F: 5′-ATTACCTCCTTCCCGACACC-3′
	R: 5′-CAAACTCCACCTCCTGATGC-3′

The $MgCl_2$ concentration is indicated next to the primer pair above. A melting curve showed only one peak with no existing primer-dimer product. To confirm the absence of primer dimers, 1% agarose gel analysis verified the amplification of one product of the predicted size with no primer-dimer bands. All reactions were run with 35 cycles at 95° C (30 s), followed by 60°C (50 s). Targeted gene expression data were analyzed quantitatively using the comparative C_T method. For each gene the threshold at which product was detected (10 standard deviations above the background signal) was compared to the endogenous (GAPDH) control. Prior to commencing studies, the comparative C_T method was validated by ensuring that the efficiencies of both the target gene and the GAPDH primers were approximately equal. Results were calculated as ΔC_T, i.e., the difference between the gene of interest and GAPDH mean thresholds and expressed in relative values calculated as previously described (Harle et al., 2002). Oligonucleotide primers used to detect OAS expression were targeted to that species encoding for a 43-kDa protein (Coccia et al., 1990).

Statistics

One-way analysis of variance (ANOVA) and Tukey's *t*-test were used to determine significant ($P < 0.05$) differences among the Ad:IFN-β-, Ad:Null-, and nontreated groups relative to IFN-β production, viral load, and cellular gene expression, using the Statistica program (Stat soft, Tulsa, OK).

Acknowledgments

We thank Dr. Wei Cao for assistance in capturing the fluorescent images. This work was supported by a grant from the Presbyterian Health Foundation and an RPB Stein research professorship (D.J.J.C.), AI 34039 (B.R.G.W.), CA 44059 (R.S.), an unrestricted grant from Research to Prevent Blindness Inc., and NEI Core Grant P30 EY 12190-01A1.

References

Al-Khatib, K., Williams, B.R., Silverman, R.H., Halford, W.P., Carr, D.J., 2002. Absence of PKR attenuates the anti-HSV-1 activity of an adenoviral vector expressing murine IFN-beta. J. Interferon Cytokine Res. 22 (8), 861–871.

Caillaud, C., Akli, S., Vigne, E., Koulakoff, A., Perricaudet, M., Poenaru, L., Kahn, A., Berwald-Netter, Y., 1993. Adenoviral vector as a gene delivery system into cultured rat neuronal and glial cells. Eur. J. Neurosci. 5 (10), 1287–1291.

Castelli, J.C., Hassel, B.A., Maran, A., Paranjape, J., Hewitt, J.A., Li, X.L., Hsu, Y.T., Silverman, R.H., Youle, R.J., 1998. The role of 2′-5′ oligoadenylate-activated ribonuclease L in apoptosis. Cell Death Differ. 5 (4), 313–320.

Cayley, P.J., Davies, J.A., McCullagh, K.G., Kerr, I.M., 1984. Activation of the ppp(A2′p)nA system in interferon-treated, herpes simplex virus-infected cells and evidence for novel inhibitors of the ppp(A2′p)nA-dependent RNase. Eur. J. Biochem. 143 (1), 165–174.

Cayley, P.J., Knight, M., Kerr, I.M., 1982. Virus-mediated inhibition of the ppp(A2′p)nA system and its prevention by interferon. Biochem. Biophys. Res. Commun. 104 (2), 376–382.

Chiu, C.Y., Wu, E., Brown, S.L., Von Seggern, D.J., Nemerow, G.R., Stewart, P.L., 2001. Structural analysis of a fiber-pseudotyped adenovirus with ocular tropism suggests differential modes of cell receptor interactions. J. Virol. 75 (11), 5375–5380.

Coccia, E.M., Romeo, G., Nissim, A., Marziali, G., Albertini, R., Affabris, E., Battistini, A., Fiorucci, G., Orsatti, R., Rossi, G.B., et al., 1990. A full-length murine 2–5A synthetase cDNA transfected in NIH-3T3 cells impairs EMCV but not VSV replication. Virology 179 (1), 228–233.

Cook, M.L., Stevens, J.G., 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect. Immunol. 7 (2), 272–288.

De Veer, M.J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J.M., Silverman, R.H., Williams, B.R., 2001. Functional classification of interferon-stimulated genes identified using microarrays. J. Leukoc. Biol. 69 (6), 912–920.

Der, S.D., Lau, A.S., 1995. Involvement of the double-stranded-RNA-dependent kinase PKR in interferon expression and interferon-mediated antiviral activity. Proc. Natl. Acad. Sci. USA 92 (19), 8841–8845.

Der, S.D., Zhou, A., Williams, B.R., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Natl. Acad. Sci. USA 95; 15623–15628.

Farassati, F., Yang, A.D., Lee, P.W., 2001. Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1. Nat. Cell Biol. 3 (8), 745–750.

Foster, G.R., 1997. Interferons in host defense. Semin. Liver Dis. 17 (4), 287–295.

Gil, J., Garcia, M.A., Esteban, M., 2002. Caspase 9 activation by the dsRNA-dependent protein kinase, PKR: molecular mechanism and relevance. FEBS Lett. 529 (2–3), 249–255.

Gutterman, J.U., 1994. Cytokine therapeutics: lessons from interferon alpha. Proc. Natl. Acad. Sci. USA 91 (4), 1198–1205.

Harle, P., Cull, V., Agbaga, M.P., Silverman, R., Williams, B.R., James, C., Carr, D.J., 2002. Differential effect of murine alpha/beta interferon transgenes on antagonization of herpes simplex virus type 1 replication. J. Virol. 76 (13), 6558–6567.

He, B., Gross, M., Roizman, B., 1997. The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA 94 (3), 843–848.

Huber, A.B., Ehrengruber, M.U., Schwab, M.E., Brosamle, C., 2000. Adenoviral gene transfer to the injured spinal cord of the adult rat. Eur. J. Neurosci. 12 (9), 3437–3442.

Katze, M.G., Wambach, M., Wong, M.L., Garfinkel, M., Meurs, E., Chong, K., Williams, B.R., Hovanessian, A.G., Barber, G.N., 1991. Functional expression and RNA binding analysis of the interferon-induced, double-stranded RNA-activated, 68,000-Mr protein kinase in a cell-free system. Mol. Cell Biol. 11 (11), 5497–5505.

Kerr, I.M., Brown, R.E., 1978. pppA2′p5′A2′p5′A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc. Natl. Acad. Sci. USA 75 (1), 256–260.

Khabar, K.S., Dhalla, M., Siddiqui, Y., Zhou, A., Al-Ahdal, M.N., Der, S.D., Silverman, R.H., Williams, B.R., 2000. Effect of deficiency of the double-stranded RNA-dependent protein kinase, PKR, on antiviral resistance in the presence or absence of ribonuclease L: HSV-1 replication is particularly sensitive to deficiency of the major IFN-mediated enzymes. J. Interferon Cytokine Res. 20 (7), 653–659.

Krummenacher, C., Nicola, A.V., Whitbeck, J.C., Lou, H., Hou, W., Lambris, J.D., Geraghty, R.J., Spear, P.G., Cohen, G.H., Eisenberg, R.J., 1998. Herpes simplex virus glycoprotein D can bind to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. J. Virol. 72 (9), 7064–7074.

Leib, D.A., 2002. Counteraction of interferon-induced antiviral responses by herpes simplex viruses. Curr. Top. Microbiol. Immunol. 269, 171–185.

Leib, D.A., Machalek, M.A., Williams, B.R., Silverman, R.H., Virgin, H.W., 2000. Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. Proc. Natl. Acad. Sci. USA 97 (11), 6097–6101.

Malkinson, A.M., Dwyer-Nield, L.D., Rice, P.L., Dinsdale, D., 1997. Mouse lung epithelial cell lines—tools for the study of differentiation and the neoplastic phenotype. Toxicology 123 (1–2), 53–100.

Massa, P.T., Whitney, L.W., Wu, C., Ropka, S.L., Jarosinski, K.W., 1999. A mechanism for selective induction of 2′-5′oligoadenylate synthetase, anti-viral state, but not MHC class I genes by interferon-beta in neurons. J. Neurovirol. 5 (2), 161–171.

Meurs, E., Chong, K., Galabru, J., Thomas, N.S., Kerr, I.M., Williams, B.R., Hovanessian, A.G., 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell 62 (2), 379–390.

Meurs, E.F., Watanabe, Y., Kadereit, S., Barber, G.N., Katze, M.G., Chong, K., Williams, B.R., Hovanessian, A.G., 1992. Constitutive expression of human double-stranded RNA-activated p68 kinase in murine cells mediates phosphorylation of eukaryotic initiation factor 2 and partial resistance to encephalomyocarditis virus growth. J. Virol. 66 (10), 5804–5814.

Moriya, A., Yoshiki, A., Kita, M., Fushiki, S., Imanishi, J., 1994. Heat shock-induced reactivation of herpes simplex virus type 1 in latently infected mouse trigeminal ganglion cells in dissociated culture. Arch. Virol. 135 (3–4), 419–425.

Nemerow, G.R., Stewart, P.L., 1999. Role of alpha(v) integrins in adenovirus cell entry and gene delivery. Microbiol. Mol. Biol. Rev. 63 (3), 725–734.

Pestka, S., Langer, J.A., Zoon, K.C., Samuel, C.E., 1987. Interferons and their actions. Annu. Rev. Biochem. 56, 727–777.

Peters, G.A., Khoo, D., Mohr, I., Sen, G.C., 2002. Inhibition of PACT-mediated activation of PKR by the herpes simplex virus type 1 Us11 protein. J. Virol. 76 (21), 11054–11064.

Poppers, J., Mulvey, M., Khoo, D., Mohr, I., 2000. Inhibition of PKR activation by the proline-rich RNA binding domain of the herpes simplex virus type 1 Us11 protein. J. Virol. 74 (23), 11215–11221.

Roninson, I.B., 2002. Oncogenic functions of tumour suppressor p21 (Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. Cancer Lett. 179 (1), 1–14.

Silverman, R.H., 1997. 2–5 A-dependent RNase L: a regulated endoribonuclease in the interferon system, in: Riordan, G.D.A.J.F. (Ed.), Ribonucleases: Structure and Function, Academic Press, New York, pp. 515–551.

Soudais, C., Laplace-Builhe, C., Kissa, K., Kremer, E.J., 2001. Preferential transduction of neurons by canine adenovirus vectors and their efficient retrograde transport in vivo. FASEB J. 15 (12), 2283–2285.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Stojdl, D.F., Abraham, N., Knowles, S., Marius, R., Brasey, A., Lichty, B.D., Brown, E.G., Sonenberg, N., Bell, J.C., 2000. The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. J. Virol. 74 (20), 9580–9585.

Taylor, J.L., Witt, P.L., Irizarry, A., Tom, P., O'Brien, W.J., 1995. 2′,5′-oligoadenylate synthetase in interferon-alpha- and acyclovir-treated herpes simplex virus-infected cells. J. Interferon Cytokine Res. 15 (1), 27–30.

Williams, B.R., 2001. Signal integration via PKR. Sci STKE 2001 (89), RE2.

Yang, Y.L., Reis, L.F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B.R., Aguet, M., Weissmann, C., 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J. 14 (24), 6095–6106.

Yokota, S., Yokosawa, N., Kubota, T., Suzutani, T., Yoshida, I., Miura, S., Jimbow, K., Fujii, N., 2001. Herpes simplex virus type 1 suppresses the interferon signaling pathway by inhibiting phosphorylation of STATs and janus kinases during an early infection stage. Virology 286 (1), 119–124.

Zheng, X., Silverman, R.H., Zhou, A., Goto, T., Kwon, B.S., Kaufman, H.E., Hill, J.M., 2001. Increased severity of HSV-1 keratitis and mortality in mice lacking the 2-5A-dependent RNase L gene. Invest. Ophthalmol. Vis. Sci. 42 (1), 120–126.

Zhou, A., Paranjape, J., Brown, T.L., Nie, H., Naik, S., Dong, B., Chang, A., Trapp, B., Fairchild, R., Colmenares, C., Silverman, R.H., 1997. Interferon action and apoptosis are defective in mice devoid of 2′,5′-oligoadenylate-dependent RNase L. EMBO J. 16 (21), 6355–6363.

Zhou, A., Paranjape, J.M., Der, S.D., Williams, B.R., Silverman, R.H., 1999. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. Virology 258 (2), 435–440.