Interferons Regulate the Phenotype of Wild-type and Mutant Herpes Simplex Viruses In Vivo

By David A. Leib,*‡ Travis E. Harrison,* Kathleen M. Laslo,*
Michael A. Machalek,* Nathaniel J. Moorman,* and Herbert W. Virgin‡§‖

From the *Departments of Ophthalmology and Visual Sciences and the ‡Department of Molecular Microbiology, the §Center for Immunology, and the ‖Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Summary

Mechanisms responsible for neuroattenuation of herpes simplex virus (HSV) have been defined previously by studies of mutant viruses in cultured cells. The hypothesis that null mutations in host genes can override the attenuated phenotype of null mutations in certain viral genes was tested. Mutants such as those in infected cell protein (ICP) 0, thymidine kinase, ribonucleotide reductase, virion host shutoff, and ICP34.5 are reduced in their capacity to replicate in nondividing cells in culture and in vivo. The replication of these viruses was examined in eyes and trigeminal ganglia for 1–7 d after corneal inoculation in mice with null mutations $(-/-)$ in interferon receptors (IFNR) for type I IFNs (IFN-α/βR), type II IFN (IFN-γR), and both type I and type II IFNs (IFN-α/β/γR). Viral titers in eyes and ganglia of IFN-γR$^{-/-}$ mice were not significantly different from congenic controls. However, in IFN-α/βR$^{-/-}$ or IFN-α/β/γR$^{-/-}$ mice, growth of all mutants, including those with significantly impaired growth in cell culture, was enhanced by up to 1,000-fold in eyes and trigeminal ganglia. Blepharitis and clinical signs of infection were evident in IFN-α/βR$^{-/-}$ and IFN-α/β/γR$^{-/-}$ but not control mice for all viruses. Also, IFNs were shown to significantly reduce productive infection of, and spread from intact, but not scarified, corneas. Particularly striking was restoration of near-normal trigeminal ganglion replication and neurovirulence of an ICP34.5 mutant in IFN-α/βR$^{-/-}$ mice. These data show that IFNs play a major role in limiting mutant and wild-type HSV replication in the cornea and in the nervous system. In addition, the in vivo target of ICP34.5 may be host IFN responses. These experiments demonstrate an unsuspected role for host factors in defining the phenotypes of some HSV mutants in vivo. The phenotypes of mutant viruses therefore cannot be interpreted based solely upon studies in cell culture but must be considered carefully in the context of host factors that may define the in vivo phenotype.

Key words: herpes simplex virus mutants • interferons • mice • pathogenesis

In humans and experimental animals the pathogenesis of herpes simplex virus (HSV)-1[1] occurs in a series of discrete stages (1). Acute viral replication at a peripheral site such as the cornea is followed by viral entry into neuronal termini. Corneal infection is followed by intra-axonal transport, which moves the virus to the trigeminal ganglia, where further replication may occur before clearance of infectious virus and the establishment of latency. Failure to clear the virus may result in central nervous system infection, encephalitis, and death. Latency may periodically break down in response to certain stimuli, leading to viral reactivation and shedding. The host factors that regulate the discreet stages of pathogenesis are incompletely defined.

In this paper, the role of host IFNs in determining the phenotypes of herpesvirus mutants in vivo is examined. The ability to create recombinant viruses coupled with the availability of a variety of laboratory animal models of HSV infection have elucidated the roles of viral genes in specific stages in pathogenesis (2–6). Studies with mutant viruses have shown that many HSV genes are dispensable for viral replication in dividing cells (7). However, such mutants often fail to replicate efficiently in nondividing cultured cells. In animal models, such genes often play key roles, particularly in promoting acute replication either at the primary site of infection or in the nervous system (2, 6, 8–10). To date, studies of HSV mutants have generally assumed, first, that in vivo phenotypes observed are determined solely by

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[1]*Abbreviations used in this paper:* ICP, infected cell protein; HSV, herpes simplex virus; PKR, double-stranded RNA-dependent protein kinase R; *rr*, ribonucleotide reductase; *tk*, thymidine kinase; *vhs*, virion host shutoff.

specific virus-cell interactions, and second, that the function of the gene (for example, promotion of efficient replication in nondividing cells) demonstrated in cell culture must correlate with and account for phenotypic differences observed in vivo. Only a few studies have examined a role for the host immune system in influencing the in vivo phenotypes of HSV strains and mutants. For example, infected cell protein (ICP)47 has been shown to influence neurovirulence by blocking CD8$^+$ T cell responses, and certain large genomic fragments of HSV-1 and HSV-2 strains have been shown to confer resistance to IFNs, although specific genes have not been identified (5, 11, 12). This line of investigation is especially relevant to those viruses whose growth in culture is significantly reduced due to mutation of a transactivation function such as ICP0 (13, 14), or a DNA replication function such as ribonucleotide reductase (*rr*) or thymidine kinase (*tk*), without which the virus cannot thrive in nondividing cells (15, 16). Restoration of normal virulence to attenuated viral mutants by deletion of specific host factors defines these factors as likely targets of the viral gene in question. With these considerations in mind, the hypothesis was tested that the host IFN response may be an additional, or even strongly determining, influence on the phenotype of herpesvirus mutants.

For these studies, HSV mutants were selected to represent genes from each kinetic class and a variety of functions (Table I). All mutants examined have been intensively characterized in vitro and in vivo and their phenotypes proven to be due to mutation of the targeted gene either through marker-rescue or examination of independently isolated mutants. These mutants are capable of replication in cell under some conditions. However, in nondividing cells some of these mutants are significantly impaired for replication (Table I). All of them have been shown to play important roles in pathogenesis and replication in vivo.

Infected cell protein (ICP) 0, an immediate-early gene, nonspecifically stimulates the expression of all classes of HSV and cellular genes at the transcriptional level (17–21). ICP0 is packaged in the virion and after infection colocalizes with and redistributes nuclear domain 10 antigens, and

also attenuates DNA-dependent protein kinase activity (22, 23). ICP0 null mutant viruses exhibit significantly reduced replication in culture, especially at low multiplicities of infection and at certain points in the cell cycle (24). The critical role of ICP0 in regulating viral and host gene expression is thought to be sufficient to explain the fact that ICP0 mutant viruses are severely compromised in animal models, exhibiting impaired replication, establishment of latency, and reactivation (2, 25). A role for host factors in determining the in vivo phenotype of ICP0 mutants has not been demonstrated.

The early genes *tk* and *rr* are two of several nucleotide metabolism enzymes that allow HSV to replicate autonomously of the cell-cycle status of the host cell. Viral *tk* phosphorylates deoxypyrimidine nucleosides and *rr* catalyzes the reduction of ribonucleoside triphosphates (26, 27). Both genes are dispensable in dividing cells, but are essential for viral replication in nonmitotic cells, where it is thought that cellular homologues are not present in sufficient quantity to compensate for *rr* or *tk* null mutations (10, 15). In mice, mutants in *rr* or *tk* are significantly impaired for replication in the cornea, although the extent of impairment for *rr* mutants depends on the nature of the mutation (4, 6, 28). However, in all cases mutant viruses are cleared more rapidly than are wild-type virus. Reduced replication in eyes has been proposed to be due to an inability of *rr* mutants to replicate efficiently at 38°C in mouse cells, coupled with the nonmitotic status of much of the corneal epithelium (28). In the adult nervous system, replication of *rr* and *tk* mutants is undetectable and these mutants can establish latency but are unable to reactivate. The lack of replication in the nervous system is clearly consistent with the limited nucleotide metabolism in neurons, and a role for host factors in determining the in vivo phenotypes of *rr* or *tk* mutants has not been demonstrated.

Mutants in virion host shutoff (*vhs*) and ICP34.5 were of particular interest in these studies, primarily because the highly significant degree of attenuation of such mutants in vivo suggests a strong role for host factors in determining the mutant phenotype given their near wild-type replica-

Table I. *Summary of HSV-1 Mutants Used in this Study*

Virus (parent strain)	Mutated gene	Kinetic class	Abbreviation in this study	Growth defect in culture	Growth defect in vivo	Reference
KOS	Wild-type	N/A	KOS	None	None	64
*dlx*3.1 (KOS)	ICP0	Immediate-early	Δ0	All cells	Cornea/neurons	13
*hr*R3 (KOS)	*rr*	Early	ΔRR	Nondividing cells	Cornea/neurons	15, 65
*dl*8.36tk (KOS)	*tk*	Early	ΔTK	Nondividing cells	Neurons	4, 47
UL41NHB (KOS)	*vhs*	Late	Δvhs	None	All cells	29
17TermA (17)	ICP34.5	Late	Δ34.5	Nondividing cells	Neurons	9
17TermA^R (17)	Marker rescue of 17TermA	N/A	Δ34.5^R	None	None	9

N/A, not applicable.

tion in culture (9, 29). *vhs* is a tegument protein that induces the destabilization of host mRNA, leading to rapid cessation of host protein synthesis after infection (30). *vhs* also degrades viral mRNAs and serves to facilitate the transition of viral gene expression from one kinetic class to the next. The effect of *vhs* deletion on viral replication in cell culture is modest, but is very significant in mice, in the cornea, trigeminal ganglion, and the brain (29). The poor replication at the periphery probably explains the requirement for *vhs* for the efficient establishment of latency (31). ICP34.5 shows homology to the DNA damage protein GADD34 and acts by precluding the shutoff of protein synthesis associated with apoptosis by regulation of the IFN-inducible double-stranded RNA-dependent protein kinase R (PKR) pathway (32, 33). The effect of deletion of ICP34.5 upon replication in permissive cells is minimal, but ICP34.5 mutants are impaired for replication in the cornea (9, 34, 35). More importantly, these mutants are profoundly neuroattenuated in trigeminal ganglia after corneal infection, and in the brain after intracranial injection (9, 35, 36). It is possible that the replication of ICP34.5 mutants may be cell cycle dependent, explaining why such mutants fail to replicate efficiently in postmitotic or quiescent cells. Specific host factors that influence the phenotypes of *vhs* or ICP34.5 mutants in vivo have not been identified fully.

The immune response to acute HSV infection involves both innate and acquired immunity. The fact that all mutants examined in this study show striking attenuation within 1 to 2 d after infection suggested a pivotal role for mediators of innate immunity. Key mediators of innate resistance to viral infection include the IFNs, although the precise mechanisms by which they exert their effect upon HSV infection is not fully understood. IFN-α has been shown to inhibit the onset of immediate-early HSV gene expression, and in mice IFN-α is a potent inhibitor of replication in the cornea (37–40). In addition, IFN-α/β serves to activate host defenses such as NK cells, which have themselves been shown to be important in controlling HSV infection and pathology (41). IFN-α/β has also been suggested to be important for limiting progress of infection from peripheral tissues to the nervous system (42). IFN-γ has been perhaps the most extensively studied of the IFNs in HSV infection. IFN-γ has been shown to vary widely in its efficacy in reducing HSV growth depending on its target cell, and in culture, HSV replication appears less sensitive to inhibition by IFN-γ than to IFN-α/β (43). However, it appears that IFN-γ may play an important role in the clearance of HSV from the cornea and in resistance to encephalitis, possibly by inhibiting apoptosis of neurons (44, 45).

In this study, mice that are genetically altered in their abilities to respond to IFNs by virtue of null mutations in IFNRs were corneally infected with mutant viruses whose ability to replicate in normal mice has been shown to be significantly reduced either in corneas or trigeminal ganglia, or both. The data indicate that the phenotypes of a variety of viruses is significantly impacted by the ability of the infected mice to respond to IFNs. The phenotype of HSV

mutants therefore must always be interpreted with careful regard to the host's immune response.

Materials and Methods

Viruses and Cells. African green monkey kidney (Vero) cells were propagated and growth and assay of all virus strains was done as previously described (46). The KOS strain of HSV-1 was the background for mutant viruses *dlx*3.1 (ICP0 deletion termed here Δ0, provided by Dr. Priscilla Schaffer, University of Pennsylvania, Philadelphia, PA [13]), *hr*R3 (ribonucleotide reductase deletion termed here ΔRR, provided by Dr. Sandra Weller, University of Connecticut, Farmington, CT [15]), UL41NHB (*vhs* nonsense mutation termed here Δvhs [29]), and *dl*8.36*tk* (*tk* deletion, termed here Δtk [47]). The ICP34.5 mutant, 17TermA (termed here Δ34.5) and its marker-rescued virus, 17TermA^R (termed here Δ34.5^R), were made in the background of strain 17 and were provided by Dr. Richard Thompson, University of Cincinnati, Cincinnati, OH (9). A summary of information and abbreviations on all seven viruses used in this study is provided in Table I.

Mouse Strains. All animal handling and procedures were in compliance with Federal and University policies. Mice were housed and bred in the Washington University School of Medicine biosafety level 2 animal facility, where sentinel mice were screened every 3–6 mo for mouse pathogens and determined to be negative. All mice used in this study were in a pure 129 Ev/Sv (referred to as 129) background and 129 mice were used as immunocompetent controls. Mice deleted for IFNRs used were IFN-α/βR^−/−, IFN-γR^−/−, and IFN-α/β/γR^−/−, and were all originally obtained from Michel Aguet (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland; 48).

Animal Procedures. 6–8-wk-old mice were anesthetized with xylazine and ketamine, corneas were scarified with 10 interlocking strokes with a 27-gauge needle, and 5 μl of medium containing 2×10^4 or 2×10^6 PFU of virus was added per eye. Lids were massaged together briefly to promote adsorption of virus. Eye swab and trigeminal ganglion assays of acute infection were performed as previously described (29). In brief, for eye swabs, eyes were proptosed and moistened cotton swabs used to sample the surface of the eye, going three times in a circular fashion around the eye and twice across the eye making an "x", before placing the swab in 1 ml of tissue culture medium. Sampled trigeminal ganglia were homogenized in 1 ml of tissue culture medium using 1-mm beads in a Mini-Beadbeater-8 (Biospec Products). All samples were titered using standard plaque assay on Vero cells. All data presented are logarithmic means ± SEM and the limit of detection was 10 PFU. Clinical signs of infection (eyelid swelling and loss of facial hair) were measured on a semiquantitative scale of 0–4 as previously described (49). All mouse procedures conformed to protocols approved by the Washington University Animal Studies Committee.

Results

Infection of Immunocompetent 129 Mice. The replication of KOS and other mutants used in this study has not been studied previously in strain 129 mice. Therefore, it was important to determine that the patterns of growth seen in other wild-type mouse strains were reproduced in strain 129. The replication in eyes of Δvhs, ΔRR, and Δ0 mutant viruses was reduced relative to KOS in 129 wild-type ani-
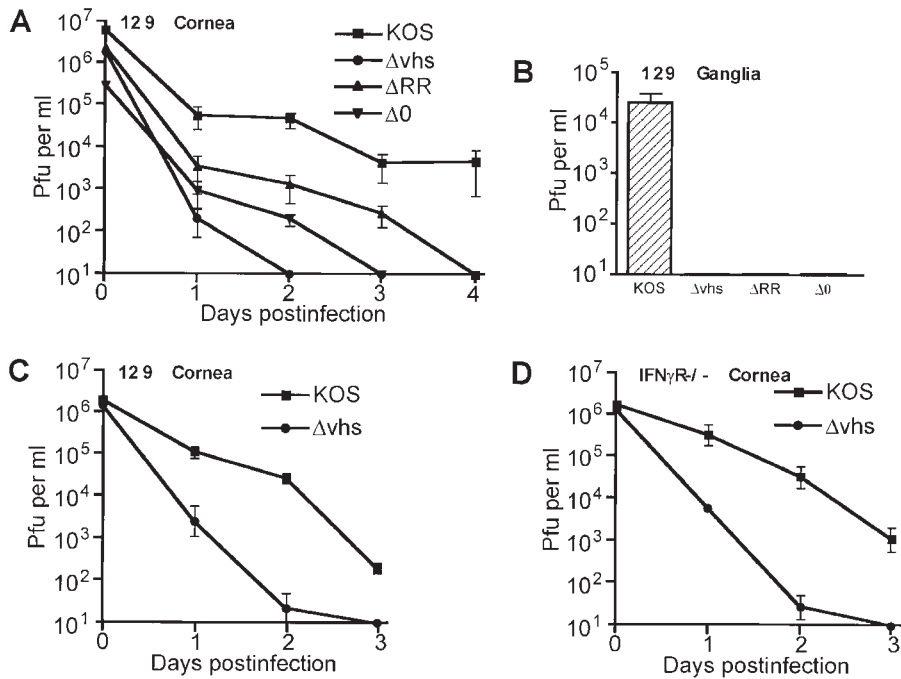
Figure 1. (A) Replication of wild-type KOS and mutants in *vhs* (Δvhs), *rr* (ΔRR), and ICP0 (Δ0) in eyes of control 129 mice from day 1 to day 4 after infection. Values are logarithmic means for at least eight samples for each virus. (B) Replication of KOS, and Δ0, Δ34.5vhs, and ΔRR viruses in trigeminal ganglia of control 129 strain mice 3 d after infection. Values are logarithmic means for at least eight samples for each virus. (C) Replication of wild-type KOS and Δvhs mutant viruses in eyes of 129 mice from days 1 to 3 after infection. Values are logarithmic means for four samples for each virus. (D) Replication of KOS and Δvhs viruses in eyes of IFN-γR$^{-/-}$ mice from days 1 to 3 after infection performed in parallel with the experiment shown in C. Values are logarithmic means of four samples for each virus.

mals (Fig. 1 A). The replication of the ΔRR mutant was reduced by ∼10–1,000-fold and was cleared more rapidly than KOS. The growth of the Δ0 and Δvhs mutants was more significantly impaired, up to 10,000-fold relative to KOS. This is especially notable for the Δvhs mutant, whose replication in tissue culture is essentially identical to that of KOS. In trigeminal ganglia of 129 mice, the replication of all viral mutants was undetectable on day 3 after infection, at which time the titer of KOS was >10^4 PFU per ml (Fig. 1 B), day 3 being the peak of viral replication in the trigeminal ganglion. Taken together, these data show that these mutants exhibit impaired replication in 129 mice rel-

ative to wild-type virus in both corneas and trigeminal ganglia, consistent with previously published data (Table I and references 2, 6, 29).

Infection of IFN-γR$^{-/-}$ Mice. Previous work from other laboratories showed that the deletion of IFN-γR had little impact upon early acute wild-type HSV infection in mice (50), while others have argued for an important role (44, 45). To examine this further, IFN-γR$^{-/-}$ mice were infected with 2 × 10^6 PFU with either KOS or Δvhs. Consistent with these previous studies, the early acute replication patterns of KOS and Δvhs were unaltered whether examined in 129 or IFN-γR$^{-/-}$ mice (Fig. 1, C and D).
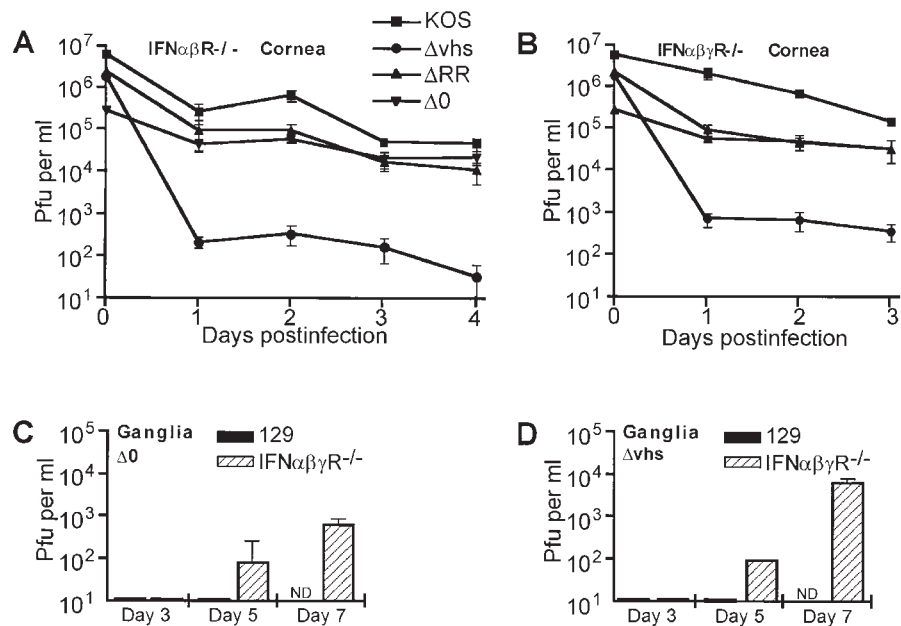

Figure 2. (A) Replication of KOS and mutants in ICP0 (Δ0), *rr* (ΔRR), and *vhs* (Δvhs), and in eyes of IFN-α/βR$^{-/-}$ mice from day 1 to day 4 after infection. Values are logarithmic means for at least eight samples for each virus. (B) Replication of KOS, Δ0, ΔRR, and Δvhs in the eyes of IFN-α/β/γR$^{-/-}$ mice from days 1 to 3 after infection. Values are logarithmic means for at least four samples for each virus. (C) Replication of ICP0 mutant virus Δ0 in trigeminal ganglia of control 129 strain and IFN-α/β/γR$^{-/-}$ mice from days 3, 5, and 7 after infection. Values are logarithmic means for eight samples for each virus. (D) Replication of *vhs* mutant virus Δvhs in trigeminal ganglia of control 129 strain and IFN-α/β/γR$^{-/-}$ mice from days 3, 5, and 7 after infection. Values are logarithmic means for eight samples.

Infection of IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} Mice. To determine the role of IFN-α/β in the reduced growth phenotypes of the mutant viruses, IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} mice were infected in parallel with 129 mice, with wild-type or mutant viruses (Fig. 2, A and B). In the eyes of the IFNR^{−/−} mice there was an increase in replication of ∼10-fold on days 1–4 after infection for KOS, relative to growth in 129 mice. For Δ0 and ΔRR in IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} mice, there was a very significant enhancement of viral replication, with 10^4–10^5 PFU per ml in eye swab samples. At comparable times in 129 mice, titers for these mutants were 10^0 PFU per ml or undetectable. These enhanced titers were such that they were within 10-fold of the titers seen for KOS. Titers for Δvhs were enhanced by 10–100-fold in IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} mice. Although this enhancement was significant ($P < 0.001$ by Student's *t* test), it was reproducibly less than was seen for Δ0 and ΔRR. Overall, the patterns of viral replication were very similar in IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} mice. Given that the IFN-α/β/γR^{−/−} mice are derived from crossing IFN-α/βR^{−/−} with IFN-γR^{−/−} mice, these data are consistent with observations of previous work (50) as well as this study, indicating that IFN-γ plays only a minor additional role to IFN-α/β in controlling early acute infection.

Initial experiments in IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} mice showed that the replication of Δ0 and Δvhs was undetectable in trigeminal ganglia on day 3 after infection, normally the peak of viral replication in the ganglion (2). However, longer time course experiments in IFN-α/βR^{−/−} (data not shown) and IFN-α/β/γR^{−/−} (Fig. 2, C and D) mice revealed that Δ0 and Δvhs were capable of replication to readily detectable levels in trigeminal ganglia of IFN-α/β/γR^{−/−} mice when sampled from day 3 onwards, ranging from 10^1 to 10^4 PFU per ml of ganglion homogenate. At these times, both mutant viruses were undetectable in the trigeminal ganglia of 129 mice.

Taken together, these data show that all viruses tested, even those with severe growth restrictions in vivo due to disparate mutations, can replicate with significantly increased efficiency in IFN-α/βR^{−/−} and IFN-α/β/γR^{−/−} mice in both eyes and trigeminal ganglia. Furthermore, patterns of replication seen in IFN-α/βR^{−/−} mice were comparable to those seen in IFN-α/β/γR^{−/−} mice.

Characterization of tk and ICP34.5 Mutants in IFNR^{−/−} Mice. Viruses containing mutations in *tk* and ICP34.5 have been extensively characterized in vivo in terms of replication, neurovirulence, and establishment and reactivation of latency. As described in the introduction, explanations for their neuroattenuation have been formulated from a large body of data. Thus, these mutants were examined more closely in this study to assess the impact of IFNs on their phenotypes.

The replication of Δtk was significantly enhanced (10–100-fold) in the corneas of IFN-α/β/γR^{−/−} compared with 129 mice (Fig. 3 A). In 129 mice, Δtk was cleared by day 5, at which time there was >10^3 PFU per ml persisting in the IFN-α/β/γR^{−/−} mice. In addition, blepharitis and periocular lesions became evident in the IFN-α/β/γR^{−/−} mice by day 7, whereas the 129 mice looked essentially uninfected. Moreover, Δtk replication was readily detected in trigeminal ganglia of IFN-α/β/γR^{−/−} mice on days 3, 5, and 9, whereas, consistent with previous observations in wild-type mice, no virus was detectable in the 129 mice (Fig. 3 B).

The ICP34.5 mutant, Δ34.5, was the only mutant tested in this study whose corneal replication was only modestly higher in IFN-α/βR^{−/−} compared with 129 mice, with a <10-fold increase over the first 3 d of infection (Fig. 4, A and B). However, the mutant was cleared more rapidly from 129 than IFN-α/βR^{−/−} mice. Similar to wild-type KOS, the marker-rescued virus Δ34.5[R] showed >10-fold increased replication in IFN-α/βR^{−/−} eyes compared with 129 mice. Replication of Δ34.5 was not detectable in ganglia of 129 mice at any time (Fig. 4 C). In contrast, replication of Δ34.5 in the trigeminal ganglia of IFN-α/βR^{−/−} mice was robust with a titer of 10^4 PFU per ml on day 3 and 3×10^4 on day 5, these titers being at most 10-fold less than its marker-rescued virus Δ34.5[R] (Fig. 4 D). Moreover, all IFN-α/βR^{−/−} mice infected with Δ34.5 that were not killed for trigeminal ganglion data died on or before day 9 after infection with symptoms consistent with viral encephalitis. Data comparable to those seen for Δ34.5 and Δ34.5R replication in IFN-α/βR^{−/−} mice were also observed in corneas and ganglia of IFN-α/β/γR^{−/−} mice (data not shown).

Infection of Scarified and Nonscarified IFN-α/β/γR^{−/−} Mice. Corneal scarification is a prerequisite for efficient uptake of HSV and other materials in normal mice such as horseradish peroxidase from the cornea into the trigeminal ganglion
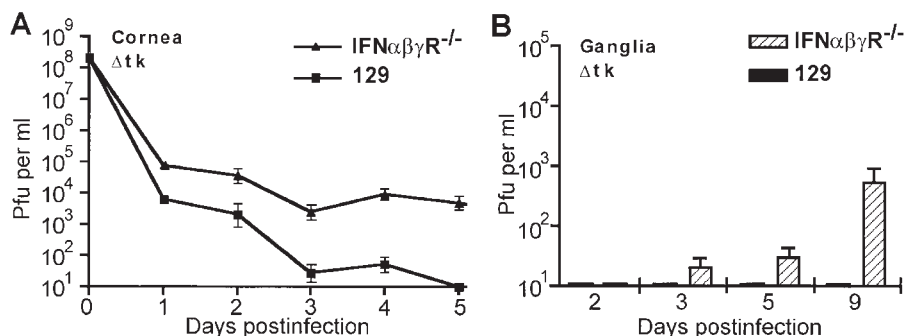

Figure 3. (A) Replication of a *tk* mutant virus, Δtk in corneas of control 129 and IFN-α/β/γR^{−/−} mice from days 1–5 after infection. Values are logarithmic means for eight samples. (B) Replication of Δtk in trigeminal ganglia of control 129 and IFN-α/β/γR^{−/−} mice from days 2, 3, 5, and 9 after infection. Values are logarithmic means for eight samples.
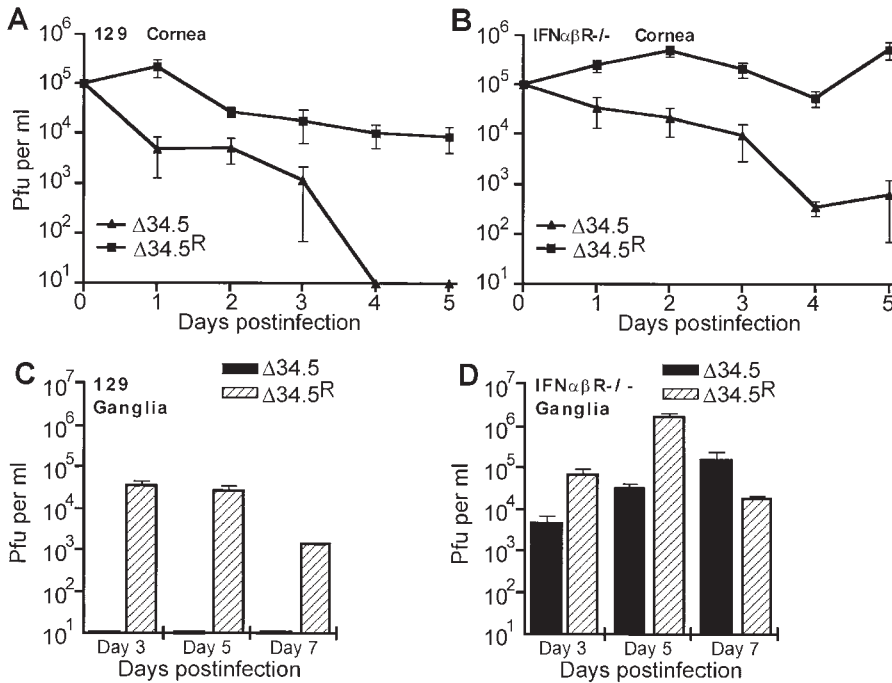
Figure 4. Replication of an ICP34.5 mutant virus, Δ34.5, and marker-rescued virus Δ34.5^R, in corneas of (A) control 129 strain and (B) IFN-α/βR^{−/−} mice from days 1 to 5 after infection. Replication of Δ34.5 and Δ34.5^R in trigeminal ganglia of (C) control 129 and (D) IFN-α/βR^{−/−} mice on days 3, 5, and 7 after infection are also shown. Values represent logarithmic means for eight samples.

(51). In the absence of scarification, replication of KOS both at the cornea and in ganglia is extremely limited (Strelow, L.I., and D.A. Leib, unpublished data, and Fig. 5). Therefore, infection of neurons is thought to be the result of direct uptake into axonal termini coupled with local amplification and subsequent retrograde axonal transport of virus. Since IFNs played a key role in determining the corneal phenotype of even very attenuated viruses, it was of interest to assess whether corneal scarification is actually needed for establishment of HSV corneal infection in the absence of IFN responsiveness. Control 129 and IFN-α/β/γR^{−/−} mice were either scarified or not and infected with 2×10^6 PFU of KOS. In 129 mice, there was robust replication of KOS in scarified corneas, with titers $>2 \times 10^4$ PFU per ml 4 d after infection (Fig. 5 A). In contrast, replication in the nonscarified group was undetectable with one virus-positive sample (out of eight) on day 2 after infection. However, in IFN-α/β/γR^{−/−} mice, replication was readily detected in both scarified and nonscarified mice, although there was a delay in onset of replication in the nonscarified group. The level of replication in the scarified IFN-α/β/

γR^{−/−} mice was significantly higher than in the nonscarified mice at all time points tested. A similar pattern of replication emerged in the trigeminal ganglia (Fig. 5 B). In 129 mice, replication was virtually undetectable in nonscarified mice, but robust in scarified mice. In IFN-α/β/γR^{−/−} mice, replication was readily detectable for both scarified and nonscarified groups, but there was a delay in onset of replication in the nonscarified group. By day 4, titers of virus in the nonscarified IFN-α/β/γR^{−/−} mice were comparable to those of scarified 129 mice. These data show that the IFNs play a key role in determining the outcome of viral infection of the intact cornea.

Discussion

The results presented in this study indicate that the acute replication patterns in eyes and trigeminal ganglia of both mutant and wild-type viruses tested are significantly impacted by IFNs. In particular, this study underscores the critical role played by IFN-α/β in the control of early acute replication of HSV, and the relatively minor role of IFN-γ.
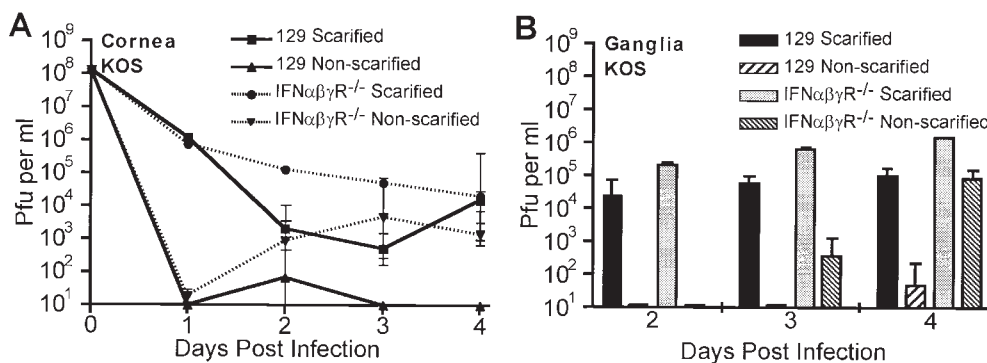

Figure 5. Replication of KOS in (A) corneas and (B) trigeminal ganglia of control 129 and IFN-α/β/γR^{−/−} mice from days 1 to 4 after infection. Corneas were either scarified or left undamaged before infection with 2×10^6 PFU of KOS. Values represent logarithmic means for four samples in a representative experiment.

These data are consistent with those of Su et al. (52), who showed that neutralizing antibody-mediated depletion of IFN-α/β led to a >1,000-fold increase in ocular viral titers and a corresponding increase in corneal opacity after infection with wild-type HSV-1. Correspondingly, the data of Cantin et al. (50) showed a less than twofold increase in viral titers in trigeminal ganglia of IFN-γR$^{-/-}$ mice compared with control isogenic mice. These protective effects of IFN-α/β in mice are likely to be due to induction of an antiviral state in target cells coupled with activation of other nonspecific host defenses such as NK cells and probably represents the first line of defense against HSV infection. With respect to NK cells, one study showed that resistance to acute wild-type HSV-1 infection correlated to early IFN production but not NK cell activity, suggesting that NK cell activation is not the primary mechanism of IFN-mediated resistance (53). A specific role for IFNs is further supported by the observation that, in contrast to data presented here for IFN-α/βR$^{-/-}$ mice, *tk* and ICP34.5 mutants are incapable of significant replication in the nervous system of SCID mice, which lack adaptive immunity but express and respond to IFNs (54–56).

The behavior of the *tk* and ICP34.5 mutants in IFN-α/βR$^{-/-}$ and IFN-α/β/γR$^{-/-}$ mice is of particular interest and worthy of further discussion. Both of these genes have been extensively studied and shown by several independent laboratories to be critical determinants of viral replication in the nervous system and to be essential for efficient peripheral neuroinvasion. *tk* mutants in the mouse eye model typically show robust (albeit reduced relative to wild-type virus) replication in the cornea, with little or no replication observed in the trigeminal ganglion (4). However, *tk* mutants do establish latency, as judged by the presence of viral DNA in the ganglion, although with 10-fold reduced efficiency (57). Reactivation, however, is minimal or absent. The lack of *tk* mutant virus replication and reactivation in neurons is consistent with the absence of complementary host tk activity in nondividing cells. How, then, is a tk mutant able to replicate in the nervous system of IFNR$^{-/-}$ mice as shown in this study? One clue comes from the observations that *tk* mutants can be made to reactivate by addition of thymidine to the culture medium of latently infected ganglia and that IFN-α can significantly reduce steady state levels of thymidine and thymidine metabolites in HSV-infected cells (58, 59). Therefore, it is possible that the lack of IFN-α/β receptors in the knockout mice leads to higher intracellular levels of thymidine in the nervous system, thereby allowing a *tk*-deleted virus to replicate to detectable and significant levels. An alternate hypothesis is that the increased viral replication and persistence in the cornea leads to a higher viral load in the ganglion with a concomitant rise in viral trans-acting factors such as VP16 and ICP0, thereby promoting a lytic infection. This hypothesis seems unlikely since increasing the infectious dose of mutant virus does not promote replication in the ganglion if the virus is inherently crippled for replication at that site (Strelow, L.I., and D.A. Leib, unpublished data). In addition, such increased levels of trans-

acting factors would probably not be able to overcome a block in thymidine metabolism and DNA replication.

ICP34.5 mutants exhibit significantly reduced replication in eyes of wild-type mice and minimal replication in the nervous system after peripheral infection. This lack of replication and neurovirulence is believed to be due to the ability of ICP34.5 to preclude the shutoff of protein synthesis by IFN-inducible PKR (32, 33). IFNs induce the synthesis of PKR in an inactive form, which subsequently becomes activated by binding of viral doubled-stranded RNA and homodimerization. Once activated, PKR causes the phosphorylation of the basal translation initiation factor eIF2α such that translation and protein synthesis are arrested. ICP34.5 interferes with this host protective pathway by binding to and redirecting the activity of protein phosphatase 1 to dephosphorylate the basal translation factor eIF-2α, thereby prolonging protein synthesis and viral replication. Cells infected with ICP34.5$^-$ viruses therefore exhibit premature shutoff of protein synthesis. Thus, one hypothesis to explain the data in this study is that the significantly increased neurovirulence of the ICP34.5 mutant in IFN-α/βR$^{-/-}$ and IFN-α/β/γR$^{-/-}$ mice is due to reduced intracellular levels of PKR that result from an inability to respond to IFNs. A lack of PKR would result in low efficiency phosphorylation of eIF-2α and an inability of the host to shut off protein synthesis, obviating the need for ICP34.5. This could explain why ICP34.5 mutants exhibit almost wild-type virulence and replication in IFNR-α/β$^{-/-}$ mice. This near wild-type replication and lethality of this severely neuroattenuated virus should be additionally considered in the light of ongoing phase 1 clinical trials using intracranial injection of ICP34.5 mutants for treatment of human glioma (60). The use of viruses singly deleted in the ICP34.5 locus may present a potentially lethal challenge to those individuals with any deficiencies in IFN responses. The use of viruses with double deletions, such as ICP34.5, in addition to ribonucleotide reductase may therefore be preferable (61).

The phenotypes of the ICP0 and *vhs* mutants in IFNR$^{-/-}$ mice are also worthy of discussion. With respect to ICP0, it has been shown that the growth of ICP0 mutants is compensated by a host function which is expressed 8 h after release from G_0/G_1 cell-cycle arrest (24). In wild-type mice, the expression and response to IFNs after virus infection may lead to a nonproliferative state in infected cells. In IFNR$^{-/-}$ mice, IFNs cannot induce such a nonproliferative state, allowing more cells to remain freely cycling. Therefore, it is possible that greater replication of ICP0 mutants observed in IFNR$^{-/-}$ mice is due, at least in part, to an enhanced level of expression of the complementary host factor due to a higher level of proliferation in infected cells.

The phenotype of the *vhs* mutant is enigmatic in the sense that it is the least compromised in terms of its replication in cell culture and yet the most compromised in mice (29). Moreover, it is the virus whose replication in the cornea is least enhanced by lack of IFN responsiveness. However, the observation of significant replication of Δvhs in

the trigeminal ganglion is consistent with the idea that the replication of this mutant is not compromised in the nervous system to the same extent that it is in the cornea. When Δvhs can gain access to the nervous system either by corneal infection of IFN-α/β/γR$^{-/-}$ mice or intracerebral infection of wild-type mice (29), the virus can replicate to significant levels. The fact that the mutants tested in IFNR$^{-/-}$ mice in this study do not behave in the same way strongly suggests that there is some degree of specificity to the phenotypes observed and not just a global enhancement of all viral replication to the same level. There is, therefore, an IFN-independent impairment of *vhs* mutant virus replication in vivo. Whether this is related to the observed alterations in MHC class I expression previously reported is an important issue for further experimentation (62).

The intact cornea is an effective barrier to HSV infection, and thus many experimental eye models employ corneal scarification as a means to promote peripheral infection and nervous system invasion (34, 46, 63). However, the extent to which virus can penetrate the cornea without scarification is virus strain dependent. Strain KOS, used in this study, has been shown in mice and rabbits to be virtually incapable of corneal and nervous system invasion without prior scarification, whereas HSV-1 strain McKrae can penetrate without scarification (34). The mechanism by which scarification promotes viral invasion is not known, but a common assumption is that the mechanical damage to the corneal epithelium permits direct access to the nerve termini, allowing a conduit into the peripheral nervous system. The data in this study suggest that major determinants of corneal resistance to viral invasion are the IFNs, since KOS was capable of robust replication in corneas and ganglia of nonscarified IFNR$^{-/-}$ mice. This is consistent with the idea that different HSV strains may be differentially susceptible to the effects of IFNs (11, 12).

In conclusion, this study has shown the critical role played by IFN, especially IFN-α/β, in controlling early acute HSV infection, and has also shown that in the absence of IFN responsiveness mice are highly susceptible to infection even by very attenuated viruses. These studies show that the attenuation of viral mutants must be carefully considered within the context of the immune response, especially early-onset innate immunity. Furthermore, any attenuation of growth of HSV mutants in culture should be examined with respect to IFNs. The use of these and other genetically altered mice will now allow study of the interactions of viral genes and cytokines during the establishment, maintenance, and reactivation of viral latency.

Address correspondence to David A. Leib, Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, Box 8096, 660 South Euclid Ave., St. Louis, MO 63110. Phone: 314-362-2689; Fax: 314-362-3638; E-mail: leib@am.seer.wustl.edu

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