Beta interferon plus gamma interferon efficiently reduces acyclovir-resistant herpes simplex virus infection in mice in a T-cell-independent manner

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Acyclovir (ACV)-resistant herpes simplex virus type 1 (HSV-1) causes severe diseases in immunocompromised patients, so identification of new therapies is needed. Interferons (IFNs) are used to treat several other viral infections in the clinic, and IFN-β and IFN-γ are known to cooperatively reduce wild-type HSV-1 replication in the corneas of immunocompetent mice. Because IFN-γ has been shown to exert an antiviral effect mostly through T cells, whether combined IFN treatment can still inhibit ACV-resistant HSV-1 replication, especially in immunocompromised hosts, is unknown. The present study evaluated the efficacy of combined IFN treatment on ACV-resistant HSV-1 mutants. In vitro results showed that IFN-β acted synergistically with IFN-γ to inhibit HSV-1 replication in both human and mouse cell lines. Some ACV-resistant mutants were actually hypersensitive to combined IFN treatment. In vivo results showed that topical treatment with a low dose of IFN-β plus IFN-γ (200 U each) on mouse corneas efficiently reduced the viral loads by up to 4, 4 and 3 logs, respectively, in the eyes, trigeminal ganglia and brainstems of wild-type and also immunocompromised nude mice infected or co-infected with ACV-resistant HSV-1 in a manner independent of T cells. A highly efficient reduction in HSV acute replication by combined IFN treatment led to a dramatic decrease in subsequent virus reactivation from neural tissues, trigeminal ganglia, brainstems and spinal cords of latently infected mice. Thus, a combination of IFN-β and IFN-γ could be a potential treatment for ACV-resistant HSV-1 in immunocompromised patients.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) infects about 80% of young adults worldwide (Casrouge et al., 2006; Corey et al., 1988; Stanberry et al., 1997). After infection, the virus replicates in peripheral tissues, such as the eyes and skin, before spreading to replicate in peripheral sensory ganglia and the central nervous system. Subsequently, the virus establishes latency in both peripheral sensory ganglia and the central nervous system. The latent virus can reactivate periodically to cause recurrent infection. Both primary and recurrent infections can induce lesions in the peripheral and central nervous systems (Chen et al., 2006b; Roizman et al., 2007).

Immunodeficiency often promotes recurrent infection, which is a frequent cause of serious morbidity and even mortality in patients with AIDS or undergoing transplantation or cancer chemotherapy (Casrouge et al., 2006; Coen & Schaffer, 2003; Horsburgh et al., 1998; Roizman et al., 2007; Stranska et al., 2004; Whitley, 2002; Zhang et al., 2007). Acyclovir (ACV) and related nucleoside analogues are the predominantly prescribed drugs used prophylactically and therapeutically to treat these patients. However, ACV treatment is often hindered by the emergence of drug-resistant viruses, estimated to occur in ~6% of treated patients with immunodeficiency (Christophers et al., 1998; Coen & Schaffer, 2003). The majority of ACV-resistant clinical isolates contain mutations in the viral thymidine kinase (tk) gene, which encodes
the protein that activates ACV (Coen & Schaffer, 2003; Stranska et al., 2004). The remaining ACV-resistant clinical isolates contain mutations in the viral DNA polymerase (pol) gene, which encodes the protein that is inhibited by activated ACV (Coen & Schaffer, 2003; Stranska et al., 2004). ACV-resistant HSV is difficult to treat clinically, and thus identification of novel therapies that can be used for immunocompromised patients is important.

In addition to chemical compounds, interferons (IFNs) are natural cytokines with direct anti-HSV effects. Prophylactic treatment with leukocyte IFN has been shown to reduce HSV infection in patients who have undergone renal transplantation or a trigeminal root operation (Cheeseman et al., 1979; Pazin et al., 1979). Recent in vitro studies showed that IFN-β and IFN-γ act synergistically to reduce the replication of several viruses, including HSV-1 (Larkin et al., 2003; Peng et al., 2008; Sainz & Halford, 2002; Sainz et al., 2004, 2005). In the HSV-1 study, a wild-type virus was used for investigation (Sainz & Halford, 2002). In addition, the in vivo anti-HSV efficacy of combined IFN treatment was tested in immunocompetent, not immunocompromised, mice (Sainz & Halford, 2002). IFN-β and especially IFN-γ are known to activate immune cells, natural killer (NK) cells, dendritic cells, macrophages and particularly T cells (Biron et al., 1999; Le Bon & Tough, 2002; Sainz & Halford, 2002; Schoenborn & Wilson, 2007; Virelizier & Arenzana-Seisdedos, 1985), which in turn reduce HSV infection (Adler et al., 1999; Cheng et al., 2000; Kassim et al., 2006; Staats et al., 1991). Thus, whether combined IFN treatment would still inhibit ACV-resistant HSV-1 infection in immunocompromised hosts is unknown.

IFN-α is currently used to treat patients chronically infected with hepatitis B, C or D virus (Hooftnage & di Bisceglie, 1997). In contrast to chemical compounds, hepatitis B virus rarely develops resistance to IFN-α. Similarly, there are no reports showing that HSV develops resistance to IFNs. Some HSV-1 mutants are actually hypersensitive to IFN-α or IFN-β (Chou et al., 1995; Mossman et al., 2000). Therefore, in the present study, we evaluated the antiviral efficacy of combined IFN-β and IFN-γ treatment against ACV-resistant HSV-1 in vitro and in vivo, especially in immunocompromised nude mice deficient in T cells.

**RESULTS**

**IFN-β and IFN-γ act synergistically to reduce the replication of both wild-type and ACV-resistant HSV-1 in monkey, human and mouse cell lines**

We first examined how efficiently three types of IFN individually or in combination could suppress the replication of HSV-1, especially ACV-resistant HSV-1 in African green monkey kidney (Vero) cells. The challenge viruses were wild-type HSV-1 strain KOS; ACV-resistant, tk deletion (dlSptk), tk insertion (nLTRZ1) and pol (PAA5) mutants genetically engineered from KOS; and rescued virus (P5 AphbK2) of PAA5 (Coen et al., 1989; Davar et al., 1994; Fleming & Coen, 1984; Pelosi et al., 1998). Table 1 shows that the individual IFNs (100 U ml⁻¹) alone only modestly reduced the plaque formation of all viruses by <10-fold. In combination, IFN-α/β acted synergistically with IFN-γ to inhibit the replication of all viruses by ~20- to >250-fold, and IFN-β plus IFN-γ treatment was more potent than IFN-α plus IFN-γ treatment. Notably, all three ACV-resistant mutants, particularly PAA5, were hypersensitive to IFN-β plus IFN-γ treatment with reductions in plaque formation significantly higher than that of the wild-type virus, KOS (P<0.05, Mann–Whitney U test). We also tested combined IFN treatments on HSV-1 strain 294.1 and a tk deletion (615.9) isolated from the same patient, as well as a tk deletion mutant (294dlTKA) genetically engineered from 294.1 (Griffiths et al., 2003).

**Table 1. Reduction in HSV-1 replication as a result of treatments**

A plaque reduction assay was performed on wild-type (WT), thymidine kinase-negative (TK–), DNA polymerase-negative (Pol–) and rescued viruses in Vero cells as described in Methods. The data shown are fold reduction in p.f.u. calculated as number of p.f.u. in mock treatment/number of p.f.u. in treatment. The means ± SEM are from three independent experiments, each carried out in duplicate.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>IFN-α</th>
<th>IFN-β</th>
<th>IFN-γ</th>
<th>IFN-α + β</th>
<th>IFN-β + γ</th>
<th>IFN-α + γ</th>
<th>ACV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (KOS)</td>
<td></td>
<td>1.3±0.1</td>
<td>1.7±0.1</td>
<td>3.7±0.4</td>
<td>3.5±0.1</td>
<td>42.2±12.8*</td>
<td>30.6±10.0*</td>
<td>&gt;250*</td>
</tr>
<tr>
<td>TK– (dlSptk)</td>
<td></td>
<td>1.1±0.0</td>
<td>2.1±0.1</td>
<td>6.6±1.9</td>
<td>3.7±0.5</td>
<td>122.8±34.1*</td>
<td>19.7±5.4*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>TK– (nLTRZ1)</td>
<td></td>
<td>1.2±0.1</td>
<td>2.0±0.2</td>
<td>6.0±1.1</td>
<td>3.1±0.2</td>
<td>152.1±41.6*</td>
<td>25.0±2.9*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Pol– (PAA5)</td>
<td></td>
<td>1.5±0.1</td>
<td>2.5±0.3</td>
<td>9.6±1.7</td>
<td>10.1±0.1</td>
<td>&gt;250*†</td>
<td>&gt;250*†</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Rescued (P5 AphbK2)</td>
<td></td>
<td>1.4±1.0</td>
<td>2.0±1.1</td>
<td>4.2±0.2</td>
<td>6.1±0.3</td>
<td>54.4±19.0*</td>
<td>28.7±3.1</td>
<td>&gt;250*</td>
</tr>
</tbody>
</table>

*P<0.05, compared with mock treatment (Mann–Whitney U test).
†P<0.05, compared with KOS treated with IFN-β plus IFN-γ (Mann–Whitney U test).
IFN-β plus IFN-γ was still the most effective combination and significantly suppressed the replication of all viruses ($P<0.05$, Mann–Whitney U test) by $>51$-fold when compared with mock treatment (Table 2). IFN-β plus IFN-γ also significantly suppressed the replication of HSV-1 strain GG1 and a tk mutant (GG3) isolated from the same patient, as well as a tk deletion mutant (GGapk) genetically engineered from GG1 (Horsburgh et al., 1998), by $>138$-fold ($P<0.05$, Mann–Whitney U test) when compared with mock treatment. As expected, ACV treatment completely suppressed the replication of wild-type and rescued viruses, but barely affected the expected, ACV treatment completely suppressed the replication of wild-type (WT) and thymidine kinase mutant (TKA) viruses as described in Table 1. The means ± SEM are from three independent experiments, each carried out in duplicate. ND, Not done.

We next tested IFN-β plus IFN-γ treatment in human (A549) and mouse (L-929) cells. Fig. 1(a) shows that IFN treatment significantly reduced the plaque formation of KOS, tkLTRZ1 and 294dlTKA in A549 cells ($P<0.05$, Mann–Whitney U test) by $>15$-fold. Because HSV-1 failed to form plaques in L-929 cells, we harvested cultures at 24 h post-infection (p.i.) to determine viral titres. Fig. 1(b) shows that IFN treatment significantly reduced the yields of KOS, tkLTRZ1, 294.1 and 294dlTKA in L-929 cells ($P<0.05$, Mann–Whitney U test) by $~29$–$87$-fold.

**A low dose of IFN-β plus IFN-γ treatment efficiently reduces acute virus replication of ACV-resistant HSV-1 in mice**

We tested the *in vivo* antiviral efficacy of IFN-β plus IFN-γ against the ACV-resistant tk mutant 294dlTKA. This thymidine kinase deletion mutant was chosen because our previous study showed that it reactivated from latently infected mouse trigeminal ganglia (TG), although it failed to replicate in the TG during acute infection (Griffiths et al., 2003). ICR mice were treated with medium or IFN-β plus IFN-γ (200 U each) on scarified corneas at 12, 8, 4 and 0 h before infection with 294dlTKA. Viral growth in the mouse eyes, which peaked at day 1 p.i., was monitored (Table 3). IFN treatment significantly reduced the mean viral titre in mouse eyes ($P<0.01$, Mann–Whitney U test) by $>2000$-fold. At day 30 p.i., we found that IFN treatment given to mice during the acute phase significantly reduced the level of viral genomes in latently infected TG (see Supplementary Fig. S1, available in JGV Online), which was consistent with the results of a previous study (Sainz & Halford, 2002). There was no report on whether the reduction in HSV from establishing latency by IFN treatment would affect subsequent viral reactivation, so mouse TG were harvested and assayed for virus reactivation without adding IFNs during assay. Virus reactivation was observed in 67% of TG from mice treated with medium, but not in any of ten TG from mice treated with IFNs, during the acute phase.

Next, we tested the efficacy of administration of IFNs after infection. Administration of IFNs four times starting at the time of infection significantly reduced the mean viral titre in mouse eyes ($P<0.01$, Mann–Whitney U test) by 10-fold and also completely suppressed subsequent virus reactivation from mouse TG. Administration of IFNs four times starting at 4 h p.i. significantly reduced the mean viral titre in mouse eyes ($P<0.05$, Mann–Whitney U test) by $~4$t-fold and also reduced subsequent virus reactivation from mouse TG by 27%. No antiviral effect was observed when IFNs were given to mice at 8 h p.i.

In addition to 294dlTKA, a tk mutant genetically engineered from 294.1, we also tested a clinical tk mutant, 615.9, isolated from the same patient as 294.1 (Griffiths et al., 2003). IFN pre-treatment significantly reduced the mean viral titre in the eyes of C57BL/6 mice at day 1 p.i. ($P<0.01$, Mann–Whitney U test) by $>2500$-fold (Fig. 2). Thus, the highly effective anti-HSV-1 effect of IFN-β plus IFN-γ treatment observed *in vivo* was not a phenomenon specific to a particular tk mutant or mouse strain.

**Table 2. Reduction in HSV-1 replication as a result of treatments**

A plaque reduction assay was performed and the fold reduction in the number of viral plaques was calculated for wild-type (WT) and thymidine kinase mutant (TK−) viruses as described in Table 1. The means ± SEM are from three independent experiments, each carried out in duplicate. ND, Not done.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
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<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-β + IFN-γ</td>
<td>IFN-α + IFN-γ</td>
<td>IFN-α + IFN-β</td>
<td>ACV</td>
<td></td>
</tr>
<tr>
<td>WT (294.1)</td>
<td>54.6 ± 15.5*</td>
<td>14.6 ± 0.7</td>
<td>5.5 ± 0.1</td>
<td>$&gt;250^*$</td>
<td></td>
</tr>
<tr>
<td>TK− (615.9)</td>
<td>52.0 ± 12.5*</td>
<td>14.3 ± 1.8</td>
<td>4.8 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>TK− (294dlTKA)</td>
<td>51.2 ± 10.0*</td>
<td>14.2 ± 0.7</td>
<td>5.0 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>WT (GG1)</td>
<td>142.0 ± 36.0*</td>
<td>ND</td>
<td>ND</td>
<td>$&gt;250^*$</td>
<td></td>
</tr>
<tr>
<td>TK− (GG3)</td>
<td>142.4 ± 48.1*</td>
<td>ND</td>
<td>ND</td>
<td>2.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>TK− (GGapk)</td>
<td>138.3 ± 50.3*</td>
<td>ND</td>
<td>ND</td>
<td>4.3 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05, compared with mock treatment (Mann–Whitney U test).
IFN-β plus IFN-γ treatment significantly reduces ACV-resistant HSV-1 infection in T-cell-deficient mice

Immunocompromised patients display greater susceptibilities to HSV-1 infection and need ACV treatment, which often results in the emergence of ACV-resistant HSV-1 (Horsburgh et al., 1998; Stránská et al., 2004; Whitley, 2002). In addition, IFN-γ is known to inhibit virus replication mainly by activating T cells (Schoenborn & Wilson, 2007). Thus, we tested whether IFN-β plus IFN-γ treatment could still efficiently reduce 294dlTKA infection in immunocompromised nude mice deficient in functional T cells. ICR nude mice were pre-treated with medium or IFNs and then infected with 294dlTKA. Fig. 3(a) shows that IFN treatment significantly reduced the mean viral titres in mouse eyes from days 1 to 5 p.i. (P < 0.05, Mann–Whitney U test), especially at day 1 p.i. (by >2200-fold). At day 30 p.i., during latency, no infectious virus was detected in mouse TG and brainstems (data not shown), so these tissues were harvested and assayed for virus reactivation without adding IFNs during the assay. Fig. 3(b) shows that IFN treatment given to mice during the acute phase significantly reduced subsequent virus reactivation from TG by 49% and delayed the time to detection of infectious virus from explanted TG from a mean of 10.0 ± 3.3 to 13.6 ± 1.4 days (P <0.001, log-rank test). Fig. 3(c) shows that IFN treatment given to mice during the acute phase also significantly reduced subsequent virus reactivation from the brainstems by 48% and delayed the time to detection of infectious virus from explanted brainstems from a mean of 7.6 ± 2.6 to 11.3 ± 1.5 days (P <0.01, log-rank test).

**Table 3. Effects of IFN-β plus IFN-γ treatment on ACV-resistant HSV-1 infection in mice**

ICR mice infected with 294dlTKA were treated with medium or IFN-β plus IFN-γ (200 U each per mouse) four times at the times indicated. Mouse eyes were swabbed 1 day after infection to determine viral titres, and mouse TG were harvested 30 days after infection to assay for reactivation of latent virus.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viral titre in eye: log mean ± SEM (no. of mice assayed)</th>
<th>Ganglia reactivating/total ganglia (% reactivating)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>5.2 ± 0.1 (16)</td>
<td>20/30 (67)</td>
</tr>
<tr>
<td>IFN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12, 8, 4 and 0 h before infection</td>
<td>1.9 ± 0.3 (5)*</td>
<td>0/10 (0)*</td>
</tr>
<tr>
<td>0, 4, 8 and 12 h after infection</td>
<td>4.2 ± 0.2 (5)*</td>
<td>0/10 (0)*</td>
</tr>
<tr>
<td>4, 8, 12 and 16 h after infection</td>
<td>4.7 ± 0.1 (6)‡</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>8, 12, 16 and 20 h after infection</td>
<td>5.1 ± 0.1 (6)‡</td>
<td>6/10 (60)</td>
</tr>
</tbody>
</table>

*P < 0.01, compared with mock treatment (Mann–Whitney U test).
‡P < 0.05, compared with mock treatment (Fisher’s exact test).
§P < 0.05, compared with mock treatment (Mann–Whitney U test).
infection of ACV-resistant tk mutants and wild-type virus is also observed in humans (Horsburgh et al., 1998). Therefore, we tested IFN-β plus IFN-γ treatment on the co-infection of wild-type HSV-1 and tk mutant. ICR mice were pre-treated with medium or IFNs and then infected with $1 \times 10^8$ p.f.u. each of KOS and tkLTRZ1. The viral growth in mouse eyes and TG, which peaked at days 1 and 3 p.i., respectively, was monitored. In mice treated with medium, the mean viral titres in mouse eyes and TG were $10^{4.0}$ and $10^{1.7}$ p.f.u., respectively (Fig. 4a). IFN treatment completely suppressed viral growth in these two tissues. At day 30 p.i., mouse TG were harvested and assayed for virus reactivation without adding IFNs during the assay. Virus reactivated from 83% of the TG in mice treated with medium during acute infection. IFN treatment given to mice during the acute phase completely suppressed virus reactivation from TG. Using the challenge dose of $10^3$ p.f.u., virus failed to replicate in the mouse central nervous system. However, when mice were infected with a 10-fold higher inoculation dose, viruses replicated in mouse brainstems (Fig. 4b) and spinal cords at day 7 p.i. At this high inoculation dose, IFN treatment also significantly reduced the mean viral titres in mouse eyes and TG by $\sim 1500$- and 23 000-fold (4.3 logs), respectively ($P<0.05$, Mann–Whitney U test), and completely suppressed viral growth in mouse brainstems with a 635-fold (2.8 logs) reduction. At day 30 p.i., mouse brainstems and spinal cords were harvested and assayed for virus reactivation without adding IFNs during the assay. IFN treatment given to mice during the acute phase significantly reduced virus reactivation by 36% in both tissues ($P<0.05$, Fisher’s exact test). Collectively, IFN-β plus IFN-γ treatment could efficiently reduce viral loads up to 4, 4.3 and 2.8 logs in the eyes, TG and brainstems, respectively, of mice co-infected with wild-type and ACV-resistant HSV-1.

ACV-resistant mutants are known to be the predominant population in viruses recovered from patients treated with ACV (Horsburgh et al., 1998; Wang et al., 2007). To determine the ratios of tkLTRZ1 and KOS replicating in mouse eyes co-infected with $1 \times 10^7$ p.f.u. of each virus, we employed plaque autoradiography to distinguish plaques with or without TK activity as described previously (Horsburgh et al., 1998). The mean percentages of tkLTRZ1 and KOS replicating in mouse eyes treated with IFNs or medium (37.2 and 42.7, respectively) were not much different. This result suggests that IFN-β plus IFN-γ is a better treatment than ACV for the infection of ACV-resistant HSV because it will not enrich the population of ACV-resistant mutants.

**DISCUSSION**

In the clinic, ACV-resistant HSV-1 infection in immunocompromised patients is a serious problem and needs
alternative therapies. In the present study, we showed that IFN-β acts synergistically with IFN-γ to inhibit HSV-1 replication in vitro and that some wild-type and ACV-resistant HSV-1 are hypersensitive to combined IFN-β and IFN-γ treatment. In vivo analyses showed that a combination of a low dose of IFN-β and IFN-γ (200 U each) effectively reduced the acute replication of ACV-resistant tk mutants in multiple tissues of nude and wild-type mice. Thus, IFN-β plus IFN-γ could be a potential treatment used therapeutically and prophylactically to inhibit ACV-resistant HSV-1 in immunocompromised patients.

HSV-1 mutants deficient in the viral protein ICP 0 or 34.5, which counteract IFN antiviral pathways, have been shown to be hypersensitive to IFN-α or IFN-β (Chou et al., 1995; Mossman et al., 2000). Our results showed that HSV-1 strains vary in their susceptibility to combined IFN-β and IFN-γ treatment, with the hypersensitivity phenotype observed in strain GG1 and GG1-derived tk mutants when compared with strains KOS and 294.1. In addition, ACV-resistant tk mutants, especially the pol mutant (PAA’5) derived from KOS, were hypersensitive to the combination of IFN-β and IFN-γ, but not to individual IFNs. Moreover, PAA’5 was also hypersensitive to the combination of IFN-α and IFN-γ. These observations are novel as well as intriguing and have not been reported previously. Why strain GG1 and KOS-derived tk as well as pol mutants are hypersensitive to combined IFN therapies and whether HSV-1 thymidine kinase and DNA polymerase counteract IFN antiviral pathways are interesting issues that need further investigation.

Although IFN-β has been shown to act synergistically with IFN-γ to inhibit several viruses, there is little information concerning the induction of antiviral proteins after combined treatment. RNase L degrades viral RNAs and has been shown to mediate the anti-HSV-1 effect observed in mouse corneas treated with IFN-β (Al-Khatib et al., 2004). We employed Western blot analysis and found that IFN-β and IFN-γ cooperatively induced a slightly higher level of RNase L protein than either IFN alone (see Supplementary Fig. S2, available in JGV Online). In addition to its effect on RNase L, the combined IFN-β plus IFN-γ treatment has been shown to cooperatively induce several mRNAs encoding factors that regulate viral infections (Peng et al., 2008). It is likely that the synergistic effect of IFN-β plus IFN-γ is mediated by multiple antiviral effectors.

Our in vitro data showed that combined IFN-β and IFN-γ treatment reduced the yield of ACV-resistant HSV-1 by up to 87-fold. However, our in vivo data showed that combined treatment efficiently reduced viral titres in mouse tissues by up to 23000-fold. Evidently, combined IFN treatment exerts a greater anti-HSV-1 effect in vivo than in vitro. IFNs, particularly IFN-γ, have been shown to reduce viral infections by activating dendritic cells, NK cells, macrophages and T cells (Biron et al., 1999; Le Bon & Tough, 2002; Schoenborn & Wilson, 2007; Virelizier & Arenzana-Seisdedos, 1985). Thus, the greater anti-HSV-1 effect seen in vivo might be mediated by effector cells other than T cells, because the effect is not lost in T-cell-deficient nude mice.

In the clinic, HSV reactivation is frequently observed in immunocompromised patients. A short-term prophylaxis therapy using leukocyte IFN or ACV reduces infection in these patients (Cheeseman et al., 1979; Horsburgh et al., 1998). In the present study, topical treatment with a low dose of IFN-β plus IFN-γ efficiently reduced the replication of ACV-resistant HSV-1 in mouse tissues, particularly when the treatment was given before or early after infection. Therefore, combined IFN treatment could potentially be a prophylactic therapy for ACV-resistant HSV. For therapeutic usage, higher doses of IFNs or more
frequent IFN treatments may be needed to increase anti-HSV efficacy. Alternatively, IFN-β and IFN-γ could be combined with the second lines of anti-HSV drugs, such as foscarnet, which targets viral DNA polymerase (Whitley, 2002). The long-term use of a very high dose of IFN-α to treat chronic hepatitis B or C virus infection can cause side effects in some patients (Dienstag, 2008; Hoofnagle & Di Bisceglie, 1997). Further investigations are needed to determine whether the combination of low doses of IFN-β and IFN-γ could be a potential treatment to limit the replication and pathogenesis of ACV-resistant HSV-1 and whether it has any side effects in humans.

METHODS

Cells, viruses and IFNs. Vero, human A549 and mouse L-929 cells were maintained according to the instructions of ATCC. Viruses used in this study, HSV-1 strains KOS, dstpK, tkLTRZ1, PAA5, PSAPb, K2, 294, 615-9, 294dTKA, GG1, GG3 and GGdtk (Coen et al., 1989; Davar et al., 1994; Fleming & Coen, 1984; Griffiths et al., 2003; Horsburgh et al., 1998; Pelosi et al., 1998), were propagated and titrated in Vero cells. Human IFN-α, IFN-β and recombinant IFN-γ (provided by the National Institute of Allergy and Infectious Diseases, MD, USA) were used to treat Vero and A549 cells. Recombinant mouse IFN-β and IFN-γ (R&D Systems) were used to treat L-929 cells and mice.

Plaque reduction and virus replication assays. Vero, A549 and L-929 cells (4 × 10⁴ cells per well in six-well plates seeded the day before) were treated with or without individual IFNs (100 U ml⁻¹) or combinations of IFNs for 24 h before the cell monolayers were inoculated with ~250 p.f.u. HSV-1. Infected Vero and A549 cells were incubated in medium containing 0.8 % methylcellulose with or without IFNs or 20 μM ACV for 3 days before the plates were stained with crystal violet. Infected L-929 cells were incubated in medium with or without IFNs and harvested at 24 h p.i. to determine viral titres.

Infection of mice and tissue collection. All mouse experimental protocols were approved by the Laboratory Animal Committee at the National Cheng Kung University. Six- to 8-week-old male ICR and 6- to 8-week-old C57BL/6 mice purchased from the Laboratory Animal Center of our college were anaesthetized and treated with medium or IFN-β plus IFN-γ (200 U of each per mouse) before infection. In brief, both cornes of mice were scarified with a 26-gauge needle and given treatments three times by placing 4 μl medium with or without IFNs on the cornes with a 4 h interval between two treatments. Mouse cornes were scarified again 4 h after the last treatment, treated with medium or IFNs, and infected with 2 × 10⁶ p.f.u. 294dTKA or 1 × 10⁴ or 1 × 10³ p.f.u. each of KOS and tkLTRZ1 immediately in ICR mice or with 2 × 10⁶ p.f.u. of 615-9 in C57BL/6 mice. In some experiments, mouse cornes were scarified, infected with 294dTKA and then treated with medium or IFNs four times on mouse cornes after infection without scarification. Six-week-old male ICR nude mice purchased from the National Laboratory Animal Center in Taiwan were treated with medium or IFNs at 12, 8, 4 and 0 h on mouse cornes before infection with 294dTKA. In addition, infected mice were treated with medium or IFNs on cornes without scarification once daily for another 6 days. After infection, both eyes of the mice were swabbed to determine viral titres. The eyes of ICR and C57BL/6 mice were swabbed on day 1 p.i. and those of ICR nude mice were swabbed on days 1–7 p.i. Mouse TG and brainstems were harvested at days 3 and 7 p.i., respectively, to determine viral titres. At 30 days p.i., mice were sacrificed, and mouse TG, brainstems and spinal cords were harvested to assay for reactivation of latent virus using a dissociation method as described previously (Chen et al., 2006b).

Statistics. Data are presented as means ± SEM. All viral titres were transformed by adding a value of 1 so that all data could be analysed on a logarithmic scale. For statistical comparison, in vitro results and viral loads in mouse tissues were analysed by the Mann–Whitney U test. Virus reactivation percentages were analysed by Fisher’s exact test. Kaplan–Meier curves were analysed by the log-rank test. All P values are for two-tailed significance tests. A P value of < 0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

We thank Donald M. Coen for providing all the viruses used in the study and Robert N. Lausch and Robert Anderson for critical reading of the manuscript. This work was supported by a grant from the National Science Council in Taiwan (NSC-97-2320-B-006-010-MY3).

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