Synergistic inhibition of pseudorabies virus replication by porcine alpha/beta interferon and gamma interferon *in vitro*

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ABSTRACT. Interferon (IFN) is crucial for initiating the innate immune response and for the generation of the adaptive response. IFN, in most species, comprises IFN-alpha (IFN-α), IFN-beta (IFN-β) and IFN-gamma (IFN-γ). In this study, we compared the capacity of porcine IFN-α, -β and -γ, or a combination of them, to protect IBRS-2 cells (porcine kidney cells) from infection with pseudorabies virus (PRV). The results demonstrated that porcine IFN-β (PoIFN-β) was the most efficient of the three IFNs in conferring resistance PRV infection; 100 U/mL PoIFN-β inhibited PRV plaque formation 5.3-fold. Compared with PoIFN-β, porcine IFN-γ (PoIFN-γ) was less capable of inhibiting PRV plaque formation (3.3-fold inhibition). Porcine IFN-α (PoIFN-α) had the least capability of the three PoIFNs, and inhibited PRV plaque formation only 1.26-fold. The inhibitory capacity increased to only 2.3-fold with a treatment of 12,800 U/mL PoIFN-α. A combination of PoIFN-γ and PoIFN-α or PoIFN-β inhibited PRV plaque formation 12.8-fold or 100-fold, respectively. Treatment of IBRS-2 cells with PoIFN-α/β and PoIFN-γ inhibited PRV replication 29- or 146-fold. Additionally, real-time PCR analyses of the PRV immediate early (IE) gene revealed that IE mRNA expression was profoundly decreased in cells stimulated with PoIFN-α/β and PoIFN-γ (23.8–133.0-fold) compared with vehicle-treated cells. All the findings indicate that PoIFN-γ acts synergistically with other PoIFNs (PoIFN-α and -β) to potently inhibit PRV replication *in vitro*.

Keywords: pseudorabies virus, porcine interferon-alpha, porcine interferon-beta, porcine interferon-gamma, immediate-early gene

Pseudorabies virus (PRV) is a porcine alpha herpes virus related to the human pathogens *Herpes simplex* virus type 1 (HSV-1), HSV-2, and *Varicella zoster* virus [1]. It causes severe disease in piglets and leads to latent infection in all surviving pigs. PRV infection inflicts serious losses on the swine industry worldwide [2].

The PRV genome is a linear, duplex DNA molecule coding for three classes of genes: immediate-early (IE), early, and late genes [3]. The IE genes are transcribed immediately upon infection and do not require *de novo* protein synthesis. Transcription of early genes depends on IE protein expression and occurs before viral DNA replication. The late genes are transcribed after the onset of viral protein and DNA synthesis. Thus, the IE gene (IE180) of PRV functions to allow continuous transcription of late genes and shuts off the synthesis of its own RNA [4], which indicates that the IE gene is necessary for productive lytic infection.

The interferon (IFN) system has become recognized as a major natural defense mechanism against viral disease [5-8]. Most species have three IFNs: IFN-α and IFN-β belong to type I IFN, and IFN-γ belongs to type II IFN. The three IFNs are important components of the host immune response to viral infections. Recent reports have indicated that IFNs used in combination have synergistic antiviral activity against HSV-1 [9], HCV [10], severe acute respiratory syndrome-associated coronavirus (SARS-CoV) [11], Lassa virus [12] and HCMV [13]. Although it has been found that IFN-α/β/γ have anti-PRV activity [14-16], little is known about the anti-PRV activities of the different IFNs, or a combination of the specific porcine IFNs, on porcine cells. The objective of this study was to compare the sensitivity of PRV to the antiviral effects of porcine IFN-α (PoIFN-α), PoIFN-β, PoIFN-γ, and a combination of type I PoIFN and type II PoIFN *in vitro*.

METHODS

Cells, virus and interferons

IBRS-2 cells (porcine kidney cells) and MDBK cells (Madin-Darby bovine kidney cells) were provided by the China Institute of Veterinary Drug Control and grown in Dulbecco's Modified Eagle Media (DMEM, Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies Inc), 100 μg of streptomycin/mL, and 100 IU of penicillin/mL. All the cell lines were free of BVDV, which was monitored by the BVDV-specific primers.

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The pseudorabies virus (PRV) stock (strain Ea) contained 1.2×10^8/ml PFU as titrated in IBRS-2 cells. This virus was stored in our laboratory [17]. PoIFN-α, PoIFN-β and PoIFN-γ were prepared by our laboratory. The full-length genes, including the signal peptides of PoIFN-α, PoIFN-β and PoIFN-γ, were cloned from spleen cells or peripheral blood lymphocytes of the Chinese local breed of pig, Meishan porcine. A 3' nine-His tag was added by PCR, and the DNAs of PoIFN-α, PoIFN-β and PoIFN-γ were individually subcloned into the eukaryotic expression vector pcDNA3.1(+). Proteins of PoIFN-α, PoIFN-β and PoIFN-γ were produced by IBRS-2 cells transfected with pcD-PoIFNα, pcD-PoIFNβ, and pcD-PoIFNγ. The proteins were then purified on a Ni-nitrilotriacetic acid agarose column (Qiagen, Hilden, Germany). The purified PoIFN-α, PoIFN-β and PoIFN-γ were found to be 98%, 96% and 95% pure, respectively. The values in international units (IU), were determined by anti-vesicular stomatitis virus (VSV) titers in MDBK cells. The supernatant from IBRS-2 cells transfected with the pcDNA plasmid had no antiviral activity. A reference standard of PoIFN-α (PBL InterferonSource) was also 2-fold diluted, with an initial concentration of 160 U/mL. Concentrations of 100 IU/mL of each IFN were used in all experiments unless stated otherwise.

Plaque reduction and viral replication assays

For plaque reduction assays, IBRS-2 cells were seeded in 6-well plates at a density of 1.0×10^6 cells per well, and 12 h later different PoIFNs were added to the culture medium. IBRS-2 cells were inoculated with PRV 18 h later, and 1 h later the medium was replaced with complete DMEM containing 0.8% LWA (aMReSCO) and the respective PoIFN(s) was added to the cells. Plaques were counted two to three days later.

For viral replication assays, vehicle- and PoIFN-treated IBRS-2 cells were infected with PRV at a multiplicity of infection (MOI) of 0.1. After 1 h adsorption, the inoculum was removed, the monolayers were washed twice with 1×PBS, and fresh PoIFN-containing medium was returned to each well. The cultures were freeze–thawed 24 h after infection, and the viral titer was determined on IBRS-2 cells [9].

Viral entry assay

Vehicle- and PoIFN-treated IBRS-2 cells were inoculated with PRV at MOIs of 0.1, 0.2, 0.3, 0.6, 1.2, 2.5, 5, 10, and 20. After 1 h adsorption, the inoculi were removed. The cells were washed twice with 1×PBS and subsequently treated with 0.05% trypsin for five minutes to ensure elimination of virus particles that had adhered to but had not penetrated the cells [18-20]. Cells were pelleted and washed twice with 1x PBS to remove trypsin and non-adherent virus. DNA was isolated from each sample by a standard phenol:chloroform DNA extraction procedure [21], and PRV-specific oligonucleotide primers were used to amplify a 146 bp product corresponding to the partial PRV IE180 gene, as described previously [22]. PCR products were resolved in a 2% agarose gel and imaged using an Alpha Innotech gel documentation system (Alpha Innotech, Corp., San Leandro, CA, USA).

Real-time PCR

Vehicle- and PoIFN-treated IBRS-2 cells were infected with PRV at a MOI of 0.1. At 24 h p.i., total RNA was prepared using a RNeasy Mini Prep kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Samples were treated with DNase I (Promega GmbH, Mannheim, Germany), RNA concentration and purity were determined spectrophotometrically (A260/A280), and 250 ng were reverse transcribed in a total volume of 20 μL using the ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan), according to the manufacturer's instructions. For real-time PCR, 1 μL of cDNA was amplified in SYBR Green Realtion PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) containing specific primer pairs. The optimal primer concentrations and sequences were as follows: 200 nM IE180, sense 5′ AGACCGAGGGCAACTTC AGC 3′, antisense 5′ GGGGCCAAAGAGGAGATCC 3′; 200 nM GAPDH, sense 5′GTCAAGCTCATTTCCTG-GTA 3′, antisense 5′ AAACTGGAAGTCAGGAGATG 3′. All samples were run on the same plate; those for the reference gene (GAPDH) and those for the genes of interest were each run in triplicate in independent wells for each of three independent RNA preparations. PCR parameters were as follows: an initial step to denature at 95°C for 2 minutes followed by 40 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds. The threshold cycles (CT), at which an increase in reporter fluorescence above the baseline signal could first be detected, were determined. Relative quantification of the target genes in comparison to the GAPDH reference gene was determined by calculating the relative expression ratio (R) of each target gene as follows: $R = (Etarget)^{\Delta CT(vehicle\text{-}sample)}/(EGAPDH)^{\Delta CT(vehicle\text{-}sample)}$ [23]. Differences in gene expression between the PoIFN-treated cells and the vehicle-treated control cells were expressed as x-fold inhibition.

Statistical analysis

Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA) and Tukey's *post hoc* t-test using SPSS software.

RESULTS

PoIFN-α/β, PoIFN-γ and a combination inhibit PRV plaque formation

The abilities of PoIFN-α, PoIFN-β, and PoIFN-γ to inhibit the replication of PRV were initially compared in a plaque reduction assay on IBRS-2 cells. Viral plaque formation was reduced 1.26-, 5.3- or 3.3-fold in IBRS-2 cells treated with 100 IU/mL of PoIFN-α, PoIFN-β, or PoIFN-γ, respectively *(table 1)*. To test the effects of combined PoIFN-treatments on viral plaque formation, IBRS-2 cells were pre-treated with 100 IU/mL each of (1) PoIFN-α and PoIFN-β, (2) PoIFN-α and PoIFN-γ or (3) PoIFN-β and PoIFN-γ. As expected, the level of inhibition achieved with both PoIFN-α and PoIFN-β was not greater than the level of inhibition achieved by both PoIFNs separately. In contrast, pre-treatment with both type I (PoIFN-α or PoIFN-β) and type II IFN (PoIFN-γ) reduced PRV plaque

Table 1
PoIFN-α, PoIFN-β and PoIFN-γ inhibit PRV plaque formation in IBRS-2 cells

Treatment (IU/mL)[a]	Mean no. of plaques[b] ± SEM	*x*-fold reduction[c]
Vehicle	200 ± 0.9	
PoIFN-α (100)	167 ± 2.5*	1.26
PoIFN-β (100)	38 ± 1.1*	5.3
PoIFN-γ (100)	61 ± 0.9*	3.3
PoIFN-α (100) + PoIFN-β (100)	40 ± 1.3*	5.0
PoIFN-α (100) + PoIFN-γ (100)	**17 ± 0.6***[d]	**12.8**
PoIFN-β (100) + PoIFN-γ (100)	**2 ± 0.4***	**100**
PoIFN-α (200)	154 ± 3.0*	1.34
PoIFN-β (200)	31 ± 0.6*	6.5
PoIFN-γ (200)	34 ± 0.8*	5.8

[a] IBRS-2 cells were treated continuously with PoIFN-α, PoIFN-β, PoIFN-γ, or combinations of these cytokines, from 18 h before infection until the end of the experiment.
[b] The number of plaques formed in IBRS-2 cells inoculated with 200 PFU of PRV strain Ea (n = 4 per group). * $p<0.05$, as determined by one-way ANOVA and Tukey's *post hoc* t-test comparison of this treatment to vehicle.
[c] The *x*-fold reduction in each group was calculated as follows: number of plaques in vehicle/number of plaques in treatment.
[d] Boldface type indicates a >10-fold reduction in PRV plaque formation.

formation efficiency 12.8- and 100-fold, respectively *(table 1)*. To eliminate the possibility that this effect was merely a result of doubling the total amount of PoIFNs per culture, we tested the inhibitory effects of 200 IU/mL of each PoIFN separately. Two hundred IU/mL of PoIFN-α, PoIFN-β or PoIFN-γ reduced PRV plaque formation 1.34-, 6.5- or 5.8-fold, respectively *(table 1)*. Of the three PoIFNs, PoIFN-α gave the least inhibition of the PRV plaque formation. To further evaluate the effects of dose, variable concentrations of PoIFN-α were used. With a pretreatment of 12800 IU/mL of PoIFN-α, the reduction in plaque formation was 2.3-fold *(figure 1)*.

PoIFN-α/β and PoIFN-γ synergize to inhibit PRV replication in IBRS-2 cells

PRV replication was compared in IBRS-2 cells treated with vehicle, PoIFN-α, PoIFN-β, PoIFN-γ or both PoIFN-α and PoIFN-γ or both PoIFN-β and PoIFN-γ.

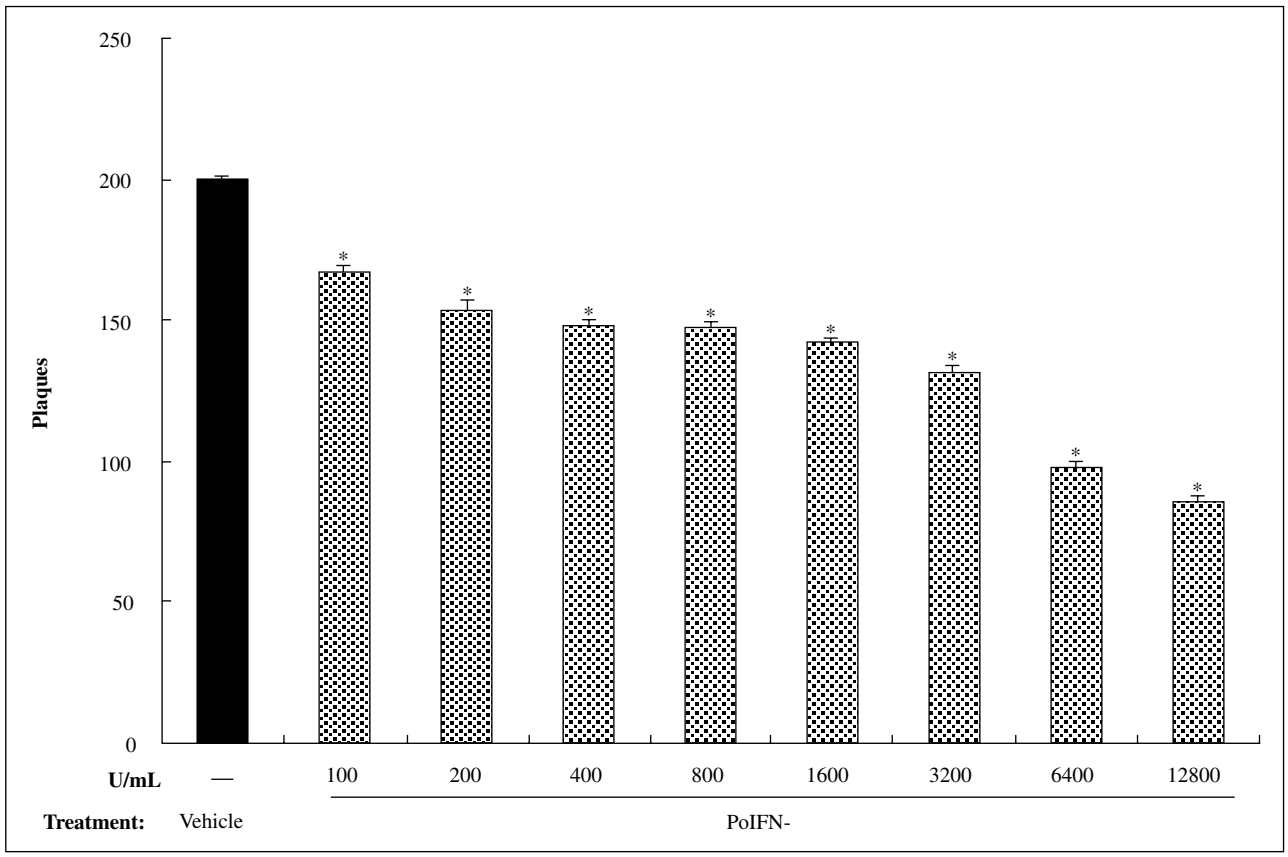

Figure 1
PoIFN-α inhibits PRV plaque formation on IBRS-2 cells at variable concentrations (n = 4 per group). Significant reductions in plaque counts relative to vehicle-treated cells are denoted by a single asterisk ($p < 0.05$).

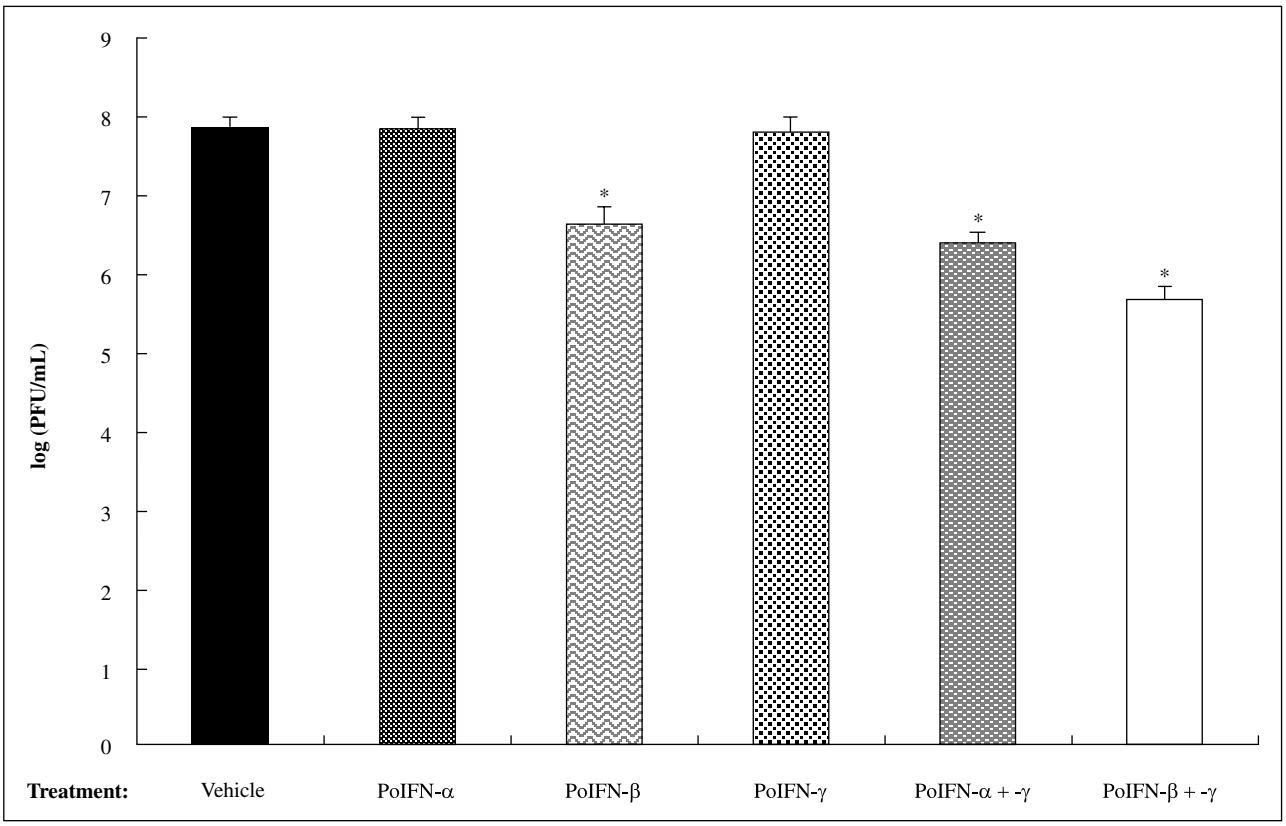

Figure 2

PoIFN-α, PoIFN-β and/or PoIFN-γ inhibit PRV replication in IBRS-2 cells. IBRS-2 cells were treated with vehicle or 100 IU/mL of PoIFN 18 h prior to infection with PRV at a MOI of 0.1. Virus titers were determined 24 h after infection (n = 4 per group). Treatments that significantly reduced virus titer relative to vehicle-treated cultures are indicated with an asterisk ($p < 0.05$, one-way ANOVA and Tukey's *post hoc t*-test).

In vehicle-treated cultures, PRV strain Ea replicated to a titer of 7×10^7 PFU/mL over a 24-h period of incubation *(figure 2)*. PRV replicated to titers of 6.8×10^7, 4.4×10^6, and 6.4×10^7 PFU/mL in cultures treated with 100 IU of PoIFN-α, PoIFN-β, or PoIFN-γ/mL, respectively *(figure 2)*. Treatment with PoIFN-β alone significantly reduced PRV replication by 16-fold ($p < 0.05$, *figure 2*). In cultures treated with both PoIFN-α/β and PoIFN-γ, PRV replicated to titers of $2.4 \times 10^6 – 4.8 \times 10^5$ PFU/mL and was significantly inhibited, by 29- or 146-fold, relative to vehicle-treated cultures. This effect was far greater than a possible additive effect, and indicated synergistic inhibition ($p < 0.05$, *figure 2*).

Treatment with PoIFN-α/β and PoIFN-γ does not inhibit PRV adsorption to IBRS-2 cells

The PRV replication cycle is a multi-step process, beginning with viral attachment and entry into the host target cell. To investigate the mechanisms by which PoIFN-α/β and PoIFN-γ inhibit PRV replication, we first examined the effect of PoIFNs on PRV entry into IBRS-2 cells. Cells were treated with vehicle or PoIFNs for 18 hours (h) prior to infection with PRV. One h after viral adsorption, DNA was isolated from the PRV-infected cells and PCR was used to amplify a 146 bp fragment of the partial PRV IE180 gene *(figure 3)*. For each treatment group, the PCR product yield increased as a function of viral multiplicity of infection (MOI). At all MOIs tested, the amount of PCR product amplified from IBRS-2 cells treated with PoIFNs was comparable to that of vehicle treated IBRS-2 *(figure 3)*. Co-amplification of a GAPDH 207 bp PCR product served as an internal loading control for normalization of PCR product between treatment groups (data not shown). The amplification of similar levels of PCR products from IBRS-2 cells suggests that the inhibitory effect of PoIFN-α/β and PoIFN-γ does not occur at the level of viral adsorption.

PoIFN-α/β and PoIFN-γ inhibit PRV IE mRNA expression

IE protein expression plays a pivotal role in controlling subsequent viral and cellular gene expression during productive PRV infection [24], such that an inhibitory effect at this level significantly impairs viral replication. It has been demonstrated that PRV replication in Vero cells is suppressed by treatment with human, natural IFN-α. In addition, messenger RNA transcribed from the PRV IE gene is reduced in IFN-alpha-treated cells [15]. However, the mechanisms of the antiviral activities of PoIFNs against PRV are still unclear. To assess the effect of PoIFN treatment on IE180 gene expression, real-time PCR analyses of IE180 mRNA levels in PoIFN-treated cells were performed. *Figure 4* summarizes the repression in IE180 mRNA levels in PoIFN-treated cultures compared with vehicle-treated controls. At 24 h p.i., statistically significant differences were found in IE180 mRNA levels between treated and non-treated cells (data not shown). In addition, mRNA levels in IBRS-2 cells pretreated individually with either PoIFN-α, PoIFN-β, or PoIFN-γ were inhibited < 20-fold, whereas in cells pretreated with both

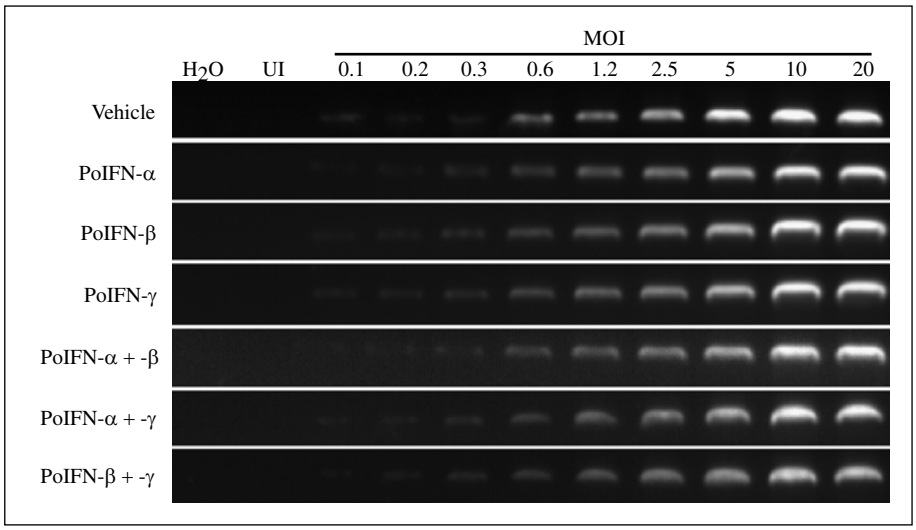

Figure 3
PoIFN-α, PoIFN-β and/or PoIFN-γ do not inhibit PRV adsorption to IBRS-2 cells. Ethidium bromide stained IE180 products amplified from PRV-infected IBRS-2 pre-treated with either vehicle or 100 IU/mL of PoIFN-α, PoIFN-β, PoIFN-γ, PoIFN-α and PoIFN-β, PoIFN-α and PoIFN-γ, or PoIFN-β and PoIFN-γ. From left to right, PCR products were amplified form H_2O control, 100 ng of uninfected (UI) IBRS-2 DNA or 100 ng of PRV-infected IBRS-2 DNA harvested from cells inoculated for 1 h at MOIs of 0.1 to 20. GAPDH PCR products were run alongside IE180 products and served as internal loading controls (data not shown).

PoIFN-α and PoIFN-γ, IE180 mRNA expression was inhibited 23.8-fold. A more enhanced inhibitory effect was observed in IBRS-2 cells treated with both PoIFN-β and PoIFN-γ. In these cultures, IE180 mRNA expression was repressed 133-fold *(figure 4)*.

Interestingly, the degree of IE mRNA inhibition observed in IBRS-2 cells treated with PoIFN-γ plus PoIFN-β was greater than that observed in cultures treated with PoIFN-γ plus PoIFN-α, suggesting that type II IFN-mediated inhibition of IE mRNA expression is better facilitated by treatment with PoIFN-β than PoIFN-α.

DISCUSSION

The immune response of the host is responsible for preventing viral dissemination and replication following viral infection. As part of the non-specific immune response, type I IFNs are secreted by infected cells and function to induce an antiviral state in neighboring, uninfected cells. Type I IFNs also contribute to the overall antiviral response by stimulation of infiltrating immune cells, such as natural killer (NK) cells and macrophages, to secrete numerous chemokines and cytokines. With the activation of the spe-

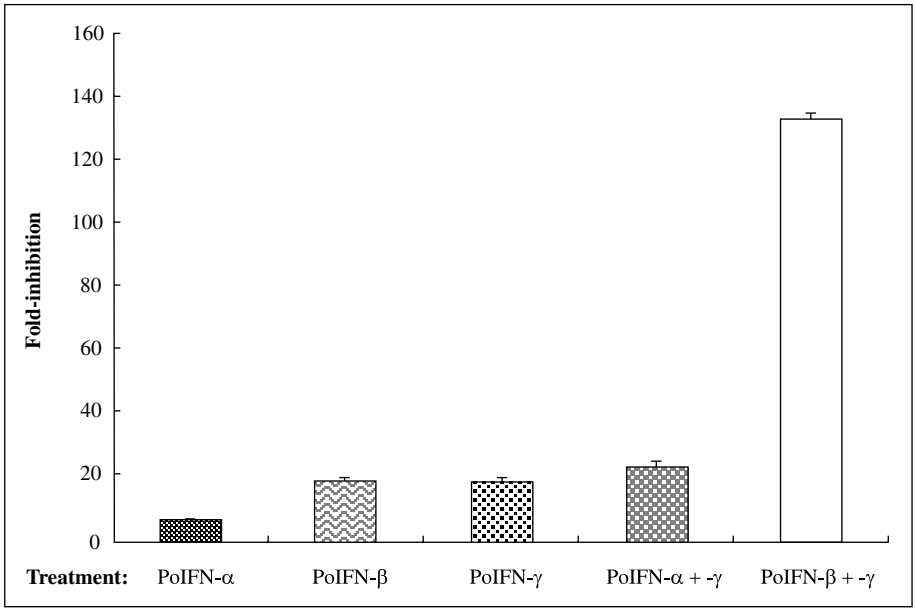

Figure 4
PoIFN-α, PoIFN-β and/or PoIFN-γ inhibit PRV IE mRNA expression. SYBR green real-time PCR analyses of IE mRNA expression in vehicle- or PoIFN-treated IBRS-2 cells 24 h p.i. (n = 4 per group). Differences in IE180 mRNA levels between treated and non-treated cells were statistically significant (data not shown). Presented are *x*-fold inhibition ± S.E. of IE mRNA expression in each treatment group.

cific immune response, T-cells can further add to the milieu of immune cytokines present at the site of viral infection by secreting additional cytokines, including IFN-γ [13]. Thus, both type I and type II IFNs are important for the antiviral defenses of the host. Because the proteins used in this study were purified and the supernatant of IBRS-2 transfected with the pcDNA3.1(+) plasmid had no anti-VSV activity, the antiviral activities of the proteins were induced by PoIFNs and not by other biologically active molecules.

Because pigs are the natural host of PRV, it is necessary to investigate the antiviral effects of porcine IFNs in porcine cell lines. The results of the plaque formation and viral replication assays established that PoIFN-β had the highest potency of the three PoIFNs to inhibit PRV *in vitro*, which is consistent with the results of inhibition of IE180 mRNA. Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) activating distinct but related Jak/STAT signal cascades resulting in the transcription of several hundred IFN-stimulated genes [25]. Although similar genes are activated by all three IFNs, Der *et al.* [26] have identified numerous genes differentially regulated by IFN-α, IFN-β or IFN-γ. In particular, IFN-β stimulation induces twice as many genes as IFN-α. This differential regulation of IFN-induced genes may partially explain the fact that the level of inhibition observed in IBRS-2 cells treated with PoIFN-β was consistently greater than that observed in cells treated with PoIFN-α, although both PoIFN-α and PoIFN-β bind to the same receptor.

It has previously been demonstrated that treatment of cells with both IFN-α and IFN-β potently inhibits PRV replication [15, 16]; however, these studies did not determine whether the effect was synergistic or identify the mechanism of inhibition. Recent studies have shown that type I and type II IFNs function, in synergy, to inhibit both RNA and DNA viruses, including HCV [10], HSV-1 [9], SARS-CoV [11], Lassa virus [12] and HCMV [13]. The results presented here are consistent with this hypothesis.

The inhibitory effect of PoIFN-α/β and PoIFN-γ was synergistic, and the degree of inhibition was not matched by increasing the concentrations of each individual PoIFN. These results indicate that the observed PoIFN-induced antiviral effects were a direct result of the presence of two distinct types of PoIFN. The mechanism(s) by which PRV replication is inhibited remain unclear. Type I and type II IFNs may act synergistically by acting on one or more different stages of the PRV lytic cycle, such as (1) viral attachment, (2) viral entry, (3) IE gene expression, (4) early gene expression, (5) DNA replication, (6) late gene expression, (7) virus assembly, or (8) viral egress and maturation. To address the question of attachment and entry, PCR was used to amplify viral DNA from IFN-treated and vehicle-treated cultures shortly after infection. Consistent with earlier observations [9, 13], PoIFN treatment did not prevent viral entry into cells, as indicated by equal PCR product yields from all treatment groups. These data indicate that PoIFNs exert their inhibitory effects at a step after viral attachment and entry.

The PRV genome expresses a single, immediate-early (IE) protein species from two copies of the IE gene that are present in each inverted repeat region of the viral genome [15]. The product of the PRV IE gene, IE180, functions to allow continuous transcription of late genes and shuts off the synthesis of its own RNA [27]. Using real-time PCR, we showed that while PoIFN-α, PoIFN-β, or PoIFN-γ treatment inhibited IE mRNA expression 7.3- to 19.3-fold at 24 h p.i., a combination of PoIFN-α and PoIFN-γ, or PoIFN-β and PoIFN-γ, inhibited IE mRNA expression 23.8–133-fold. These data suggest that PoIFN-α/β and PoIFN-γ have a synergistic inhibitory action on the regulation of IE gene expression.

Here, we have demonstrated that PoIFN-β was the most efficient of the three PoIFNs in promoting resistance to PRV, and PoIFN-γ together with the PoIFN-α/β act synergistically to inhibit the replication of PRV *in vitro*.

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