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Inhibition of porcine reproductive and respiratory syndrome virus by interferon-gamma and recovery of virus replication with 2-aminopurine

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Summary. Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to a group of RNA viruses that establish persistent infections. A proposed strategy for evading immunity during persistent PRRSV infection is by preventing the induction of IFN activity in pigs and/or by blocking the activation of antiviral proteins in permissive cells. IFN-γ mRNA expression was observed in the lymph nodes and lungs of pigs infected with wild-type PRRSV strain SDSU-23983. Pretreatment of MARC-145 cells with IFN-γ inhibited wild-type (SDSU-23983 P6) and culture-adapted (SDSU-23983 P136) PRRS viruses in a dose-dependent manner and at relatively low concentrations. The effect of IFN-γ on virus replication included reductions in the number of infected cells, virus yield, and RNA content in single cells. Virus replication was partially restored by the addition of 2-aminopurine (2-AP), an inhibitor of dsRNA inducible protein kinase (PKR). The addition of 2-AP also restored the viral RNA content per cell to near normal levels, suggesting that inhibition of viral RNA synthesis was through PKR. The principal difference between P6 and P136 isolates was the recovery of P136 replication with lower concentrations of 2-AP. Immunostaining with anti-PKR antibody showed a redistribution of PKR from the cytoplasm into nucleoli of infected cells.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by an enveloped positive-stranded RNA virus placed in the family *Arteriviridae*, order *Nidovirales* [7, 13, 19, 53]. Other members in this family include lactate dehydrogenase-

elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). The arteriviruses structurally resemble togaviruses, but similar to coronaviruses, replicate via a nested 3′-coterminal set of subgenomic mRNAs possessing a common leader and a poly-A tail [37].

The outcome following PRRSV infection depends on the age and reproductive status of the pig [41]. Infection of adult pigs generally produces a nonfatal disease characterized by flu-like symptoms, including mild interstitial pneumonia, elevated temperature, and inappetance. In sharp contrast, the infection of pregnant gilts and sows results in abortion and the birth of dead and weak-born piglets. Surviving piglets exhibit the severest form of respiratory distress with mortality often approaching 100% within 3 weeks after farrowing. Surviving pigs continue to suffer the negative effect of PRRSV by exhibiting increased susceptibility to secondary infection.

During acute infection, PRRSV replication is found in all organs and tissues, which is consistent with the macrophage as the principal cell population supporting virus replication [42, 29]. Non-macrophage cells, including type II pneumocytes [49], bronchiolar epithelial cells and arteriolar endothelial cells [40] may provide additional sources of virus in infected pigs. In culture, PRRSV replicates in porcine alveolar macrophages, blood monocytes, monocyte-derived macrophages and monkey kidney lines derived from MA-104 cells [19, 22, 28, 50].

Pigs surviving acute PRRS support a long-term asymptomatic infection, which has contributed to the worldwide spread of the disease [1, 16, 17, 23, 54]. Even though PRRSV-specific humoral and cell-mediated immune responses appear early [4, 55], persistently infected pigs continue to shed virus for several months [16, 49]. The mechanistic basis for PRRSV persistence is not known. Similar to LDV, PRRSV may sustain replication in a subpopulation of renewable macrophages, while avoiding host-defenses [36] or restrict the localization of virus replication within "immunoprivileged" anatomical sites, such as testes [3, 16, 49]. The amino acid variability among PRRSV field isolates within the ectodomain of the ORF5 envelope protein suggests another strategy; antigenic drift [44].

Persistent viruses frequently incorporate evasion strategies that block the production or inhibit the activation of proteins up-regulated by interferon (IFN; [12, 24, 32, 34, 38]). Studies suggest that PRRSV may avoid IFN by preventing its induction in macrophages. Both type-I and type-II IFNs efficiently inhibit PRRSV replication in macrophages; however, small amounts of IFN are found in PRRSV-infected pigs and infected macrophages in culture [2, 5, 52]. Since PRRSV-specific cell-mediated immunity is present during infection, it is assumed that IFN-γ, a product of activated T cells and NK cells, is present in infected pigs [4, 10]. However, nothing has been reported regarding the induction of IFN-γ in PRRSV-infected pigs or the mechanism of IFN-γ-inhibition of virus replication. The purpose of this study was to evaluate the production of IFN-γ in pigs following infection with PRRSV and to characterize the mechanism of IFN-γ action following infection of MARC-145 cells with wild-type and culture

adapted PRRSV isolates. The results show that IFN-γ efficiently inhibits PRRSV replication in MARC-145 cells, and at least part of the antiviral activity may be through PKR.

Materials and methods

Viruses, cells and reagents

The North American macrophage-tropic strain, SDSU-23983-P6, is a low-passage isolate possessing attributes similar to other North American field strains. The MARC-145 cell-adapted strain, SDSU-23983-P136, was obtained by passaging P6 an additional 130 times on MARC-145 cells. Adaptation was performed by infecting MARC-145 cells on 24-well plates with serial 1:10 dilutions of virus. Medium from the last well showing CPE was retained, diluted 1:20, and 2 ml added to a new T-25 flask of MARC-145 cells. After incubation at 37 °C for 2 hr, the monolayer was washed with PBS, and fresh medium added. After 2 days the flask was freeze/thawed at −80 °C, and medium was again end-point diluted on a new 24 well plate. This process repeated until the total number of passages reached 136.

MARC-145 cells [28] were maintained in MEM supplemented with 8% heat-inactivated fetal bovine serum (FBS) and antibiotics at 37 °C in 5% CO_2. The Indiana strain of vesicular stomatitis virus (VSV) was obtained from Christopher Chase at South Dakota State University. Unless otherwise indicated, confluent MARC-145 cells were infected at an MOI of approximately 0.1 $TCID_{50}$/cell. Increasing the MOI of SDSU-23983 beyond 0.1 does not produce a further increase in the number of cells infected during the first round of infection or the virus yield (personal observation). Recombinant human IFN-γ (Boehringer Mannheim) and IFN-α (Prepo) were stored at −20 °C. A 200 mM solution of the nitrate salt of 2-aminopurine (2-AP; Sigma) was prepared in MEM and pH adjusted by adding 1 M Tris until the cell culture medium changed to pink. IFN-γ and 2-AP were added at approximately 15 h and 2 h, respectively, prior to infection with virus. After 2 h the medium was removed and cells washed twice with PBS. Incubation was continued in fresh medium, containing freshly prepared IFN-γ and 2-AP. Cytopathic effect (CPE) was observed by phase contrast microscopy of live cells and after staining acetone-fixed monolayers with 1% crystal violet.

RT-PCR of mRNA in pigs

Cytokine mRNA expression in lung and lymph nodes was evaluated in pigs that were exposed to PRRSV in utero. This model is based on observations that infected neonates exhibit the severest form of disease [41, 42]. Seronegative sows were infected at 90 days gestation with wild-type P6 or attenuated P136. Sows were allowed to farrow and live-born piglets sacrificed at 10 days after birth. Samples of lung and lymph node tissues were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from tissues according to Christopher-Hennings et al. [15] and stored at −80 °C. RT-PCR of porcine cytokine and β_2-microglobulin mRNAs was performed according to Reddy et al. [39]. cDNA was prepared from 1 ug of total RNA by reverse transcription using Molony murine leukemia virus reverse transcriptase and random hexamers as primers. PCR amplification of IFN-γ, IL-10 and β_2-microglobulin cDNAs was performed using the primers described in Table 1. PCR amplification consisted of 35 cycles (45 sec at 94 °C, 45 sec at 55 °C, and 45 sec at 72 °C) and DNA products electrophoresed on a 1.5% agarose gel and visualized using ethidium bromide. The identity of each amplified product was confirmed by cloning and sequencing the gel-purified fragment.

Table 1. PCR primers used for amplification of β_2 microglobulin, IFN-γ and IL-10

Primer name	Primer sequence (5′-3′)	Size (bp)	GenBank accession no.
β-2-m Forward	CTGCTCCACTGTCTGG	284	L13854
Reverse	ATCGAGAGTCACGTGCT		
IFN-γ Forward	CCAGAATGCAAGTACCTCAG	358	X53085
Reverse	TCTCTGGCCTTGGAACATAG		
IL-10 Forward	GCTCTATTGCCTGATCTTCC	404	L20001
Reverse	GCACTCTTCACCTCCTCCAC		

Measurement of virus replication in cells

Percent-infected cells were determined at 48 h after infection by spotting trypsinized cells, diluted in culture medium onto 10-well microscope slides (Cel-Line). After cells reattached (approximately 2 h), the slides were fixed in 100% acetone for 10 min then stained for 2 h at room temperature, or overnight at 4 °C, with a 1:100 dilution of FITC-labeled SDOW-17 anti-PRRSV nucleocapsid monoclonal antibody, diluted in PBS with 5% FBS [33]. Slides were viewed under a fluorescence microscope equipped with a 488 nm excitation filter. A minimum of 100 cells were counted in at least 5 randomly chosen microscope fields. The percentage of infected cells was calculated by dividing the number of SDOW-17-positive cells by total cells and multiplying by 100.

Virus yield was measured by end-point titration of cell culture medium on MARC-145 cells [19]. Serial 10-fold dilutions of virus were placed on 96-well tissue culture plates containing MARC-145 cells. After 3 days, plates were fixed in 80% acetone then stained with SDOW-17. Results were reported as $TCID_{50}$/ml.

PKR antigen localization in cells

The intracellular localization of PKR was determined by staining with anti-PKR MAb (anti-p68 from Transduction Laboratories). Acetone-fixed cells were incubated for 2 h at room temp with a 1:100 dilution of anti-PKR antibody, followed by a 1 h incubation with biotinylated anti-mouse IgG (Sigma), diluted 1:50. A final one hr incubation included Texas red-strepavidin (Molecular Probes), diluted 1:5000 and FITC-labeled SDOW-17, diluted 1:100. Antibodies and stepavidin were diluted in PBS-FBS and slides washed twice for 5 min in PBS between the addition of each reagent. FITC-labeled SDOW-17 and Texas Red-labeled anti-PKR antibodies were visualized under a fluorescence microscope using 488 nm and 540 nm (rhodamine) excitation filters, respectively.

Northern blot and in situ hybridization analysis of viral RNA

For Northern blots total RNA was extracted from cells at 12 h after infection using the acid phenol guanidinium technique of Chomcynski and Sachi [14]. RNA was electrophoresed on 1% agarose following denaturation with glyoxal and dimethyl sulfoxide [46], then transferred to a nylon membrane. The amount of RNA added to each well was adjusted to reflect the viral RNA from the same number of infected cells (determined by the percentage of SDOW-17-postive cells in a small sample of cells removed from culture prior to RNA extraction). The [^{32}P] dCTP-labeled PRRSV-specific probe, designed to detect genomic and subgenomic RNAs, was prepared by random-primed labeling a 484 bp cDNA template derived from RT-PCR amplification of the ORF7 region [15]. Nylon membranes were hybridized with

the probe overnight at 42 °C, using a hybridization buffer prepared according to Church and Gilbert [18]. Nylon membranes were washed in 1× SSC at 42 °C followed by a high stringency wash in 0.1× SSC at 56 °C for 15 min, then exposed to X-ray film.

The relative viral RNA content in single cells was determined using a modification of the in situ hybridization technique described by Peng et al. [35]. Approximately 104 cells in 50 ul of tissue culture medium were spotted onto Denhardt's-treated slides. Fixation, pre-treatment, hybridization, and post-hybridization procedures were performed according to Lawson et al., [29] using a modification of the original method described by Anderson et al. [3]. The [^{35}S] dCTP-labeled random-primed probe was prepared from the same 484 bp ORF7 template used in Northern blot hybridization. After the last post-hybridization wash, slides were dipped in NBT-2 autoradiography emulsion (Kodak), exposed for 2 h in the dark, developed, then H and E stained with Mayer's hematoxylin and 5% eosin Y in ethanol. PRRSV-infected cells were identified as having a silver grain content above that of mock-infected cells. Histogram distributions were constructed by counting the number of silver grains over the cytoplasmic and nuclear regions of 50 infected cells. Statistical analysis was performed using a two-tailed student's T test.

Results

Expression of IFN-γ mRNA in lung and lymph nodes

Previous studies [2, 52] identified the reduced ability of pigs and pig macrophages to produce IFN-α. The purpose of this experiment was to characterize IFN-γ mRNA expression in acutely infected pigs. Sows, at 90 days gestation, were infected with wild-type P6 or attenuated P136 isolates. All P6-infected piglets (n = 7) were positive for PRRSV at the time of necropsy (10 days after birth) and exhibited typical congenital PRRSV pathology, including microscopic lesions in lungs and lymph nodes. The piglets born to the P136-infected sow (n = 7) showed no signs of PRRS and were negative for virus at the time of necropsy, confirming that this virus was rapidly cleared from the mother and did not cross the placenta. Three pigs from each of the P6 and P136 groups were randomly chosen for analysis of cytokine mRNA expression in lungs and mandibular lymph nodes. IFN-γ mRNA expression was detected in the lymph nodes from two of the 3 pigs infected with the wild-type P6 virus (Fig. 1). A third P6 pig showed no IFN-γ mRNA in lymph node; however, IFN-γ mRNA was detected in the lungs. There was no evidence of IFN-γ expression in the lymph nodes or lungs of the P136

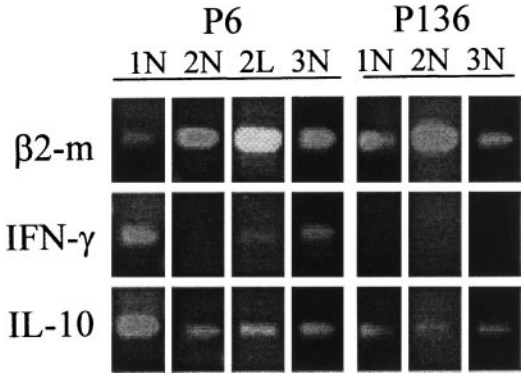

Fig. 1. Induction of IFN-γ mRNA during PRRSV infection of pigs. IFN-γ, IL-10 and β_2-microglobulin mRNA expression detected by RT-PCR amplification of total RNA from three infected (P6 virus) and three control pigs (P136 virus). Infection of pregant sows with SDSU-23983 wild-type P6 and attenuated P136 isolates is described in Materials and methods. *N* Lymph node; *L* lung

pigs. β_2 microgloblin mRNA was amplified to nearly equal levels in all tissues. IL-10 mRNA was also amplified from all tissues, a cytokine mRNA we frequently find it in lymphocytes and lymphoid tissues of un-infected pigs.

Inhibition of PRRSV replication by IFN

The effect of IFN-γ on the growth of PRRSV in MARC-145 was studied by incubating 24-well plates of confluent MARC-145 cells with human IFN-γ overnight followed by infection with PRRSV P6 or P136 isolates. The results of a representative experiment, presented in Fig. 2A, showed loss of crystal violet staining in P6 and P136 cultures not treated with IFN-γ. The effect of IFN-γ on CPE was apparent at concentrations as low as 10 U/ml IFN-γ. Intact cell monolayers were observed in wells incubated with 100 or 1,000 U/ml of IFN-γ. Phase-contrast microscopy, performed prior to crystal violet staining, confirmed the absence of CPE. For the purpose of comparison, IFN-γ-treated MARC-145 cells were also infected with VSV, a virus sensitive to interferon [21, 45]. The experimental

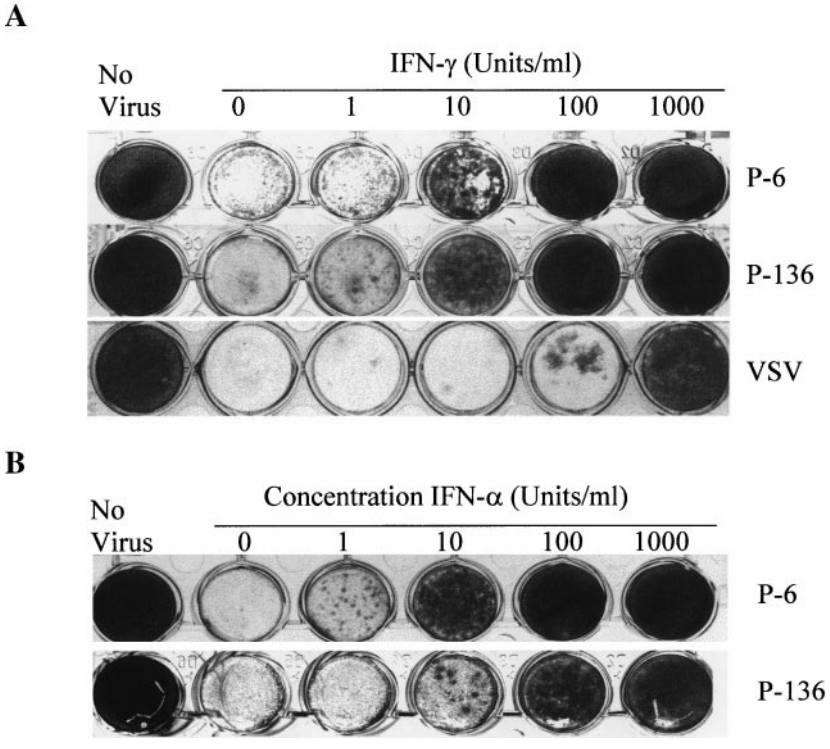

Fig. 2. Inhibition of PRRSV and VSV CPE by IFN. **A** MARC-145 cells on 24-well plates were infected with P6 (top row), P136 (middle row) or VSV (bottom row). Plates were incubated overnight with the indicated concentrations of human IFN-γ then infected with approximately 0.1 $TCID_{50}$/cell of each virus. After 3 days the cells were fixed and stained with crystal violet. **B** Inhibition of PRRSV infection by IFN-α. MARC-145 cells were treated overnight with human IFN-α prior to infection with P6 (top row) or P136 (bottom row). Infection of MARC-145 cells and crystal violet staining were performed in the same manner as described in **A**

conditions were the same as those used for PRRSV infection. Intact cell monolayers were only observed in VSV-infected cultures treated with 1,000 U/ml IFN-γ (Fig. 2A).

The effect of human IFN-α on PRRSV replication was also investigated. The experimental conditions were the same as described for the IFN-γ experiments. Representative results, presented in Fig. 2B, showed IFN-α inhibition of P6 and P136 isolates. Similar to IFN-γ, IFN-α activity was apparent at concentrations as low as 10 U/ml. Unlike IFN-γ, the P6 isolate was more sensitive to IFN-α than P136 (compare wells treated with 10 and 100 ug/ml IFN-α). This difference was confirmed in subsequent experiments.

Further assessments of IFN-γ inhibition of virus replication were made by counting the number of infected cells and by measuring virus yields in cultures incubated with IFN-γ overnight. IFN-γ treatment produced dramatic decreases in the percent-infected cells and virus yield for both P6 and P136 viruses (Fig. 3). Similar to the CPE data the effect was evident at concentrations as low 10 U/ml. IFN-γ, reduced virus yields at all time points and consistently reduced peak yields of P6 and P136 by greater than 3 to 4 log$TCID_{50}$/ml. IFN-γ also reduced the number of infected cells.

The growth curve experiments also revealed differences between P6 ad P136 isolates. Improved adaptation of P136 to MARC-145 cells was evident by increased virus yields and percent infected cells. Another difference was the timing of peak virus yield for P136, which was observed at 48 h after infection versus

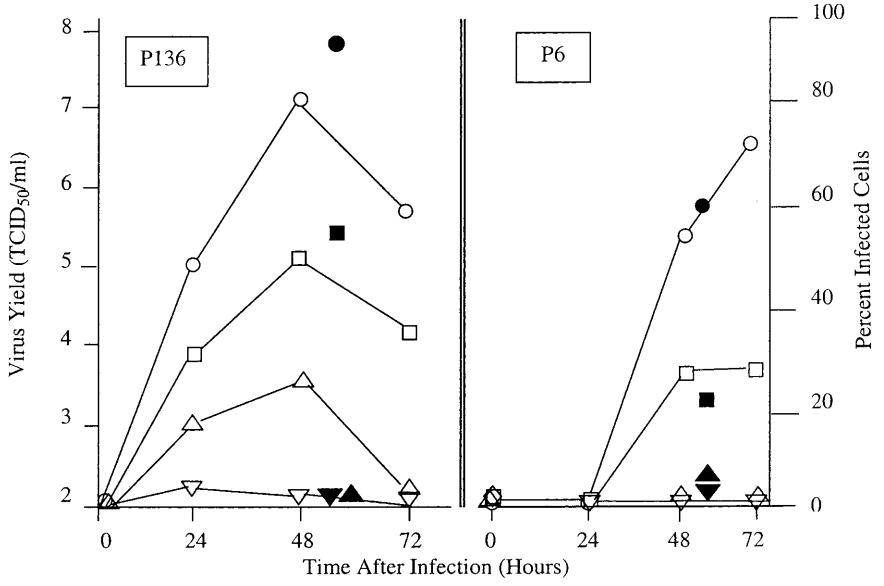

Fig. 3. Virus yield and percent infected cells in IFN-γ-treated cultures. MARC-145 cells were incubated overnight with 0 (circles), 10 (squares), 100 (triangles) or 1,000 (inverted triangles) U/ml IFN-γ. Virus yield was measured at 0, 24, 48 and 72 h after infection with P136 (left) and P6 (right) viruses. Percent-infected cells for each treatment (solid symbols) were determined at 48 h after infection

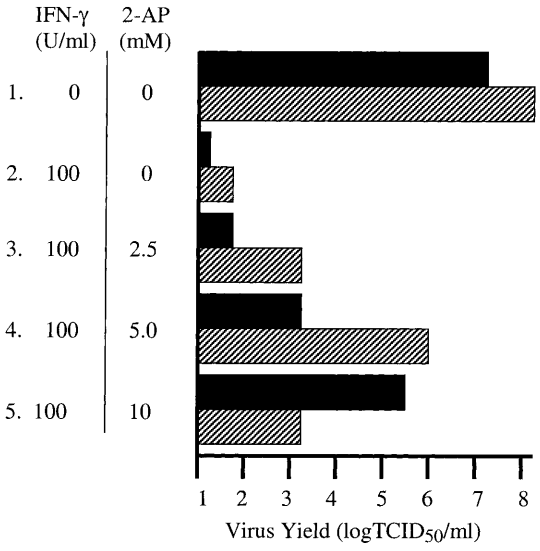

Fig. 4. Recovery of PRRSV replication with 2-AP. P6 (solid bars) and P136 (hatched bars) virus yields were measured following overnight incubation with 100 U/ml IFN-γ. 2 h after infection, incubation was continued in the presence of different concentrations of 2-AP. P6 and P136 yields were measured at three and two days after infection with P6 and P136 viruses, respectively. IFN-γ treatment, infection, and measurement of virus yields were performed in the same manner as described in Fig. 3

72 h for P6. The experiment presented in Fig. 3 also revealed what appeared to be an increased sensitivity of P6 to IFN-γ. However, due to the different growth characteristics of P6 and P136 isolates, a differential effect of IFN-γ was difficult to assess.

Recovery of virus replication with 2-AP

There are several approaches that can be used to study the activation of PKR. A role for activated PKR in the IFN-inhibition of PRRSV replication was studied by following the recovery of virus replication after the addition of 2-AP, an inhibitor of PKR phosphorylation [26]. Even though we did not evaluate the phosphorylation state of PKR, we assumed that reversal of IFN inhibition of virus replication following addition of 2-AP could only occur by preventing PKR phosphorylation. In these experiments 2-AP was added 2 h prior to infection and after overnight incubation with 100 U/ml IFN-γ. Inhibition of IFN-γ activity by 2-AP was dose-dependent and evident at concentrations as low as 1 mM for P136 (Fig. 4). The optimal concentration of 2-AP required for maximum virus recovery of P6 and P136 infection were 10 and 5 mM, respectively. Following several experiments, it was noted that 2-AP never recovered virus replication to the same levels obtained in infected cultures not treated with IFN-γ (Fig. 4; compare treatments 1 and 4 for P136 and treatments 1 and 5 for P6). And as shown in Fig. 4 further increasing 2-AP to 10 mM in P136-infected cultures actually inhibited virus yield. A similar effect was observed for the P6 virus incubated 20 mM 2-AP (data not shown).

Intracellular localization of PKR

PKR is typically found in both cytoplasmic and nuclear compartments [8]. In uninfected MARC-145 cells PKR was distributed throughout the cytoplasm with

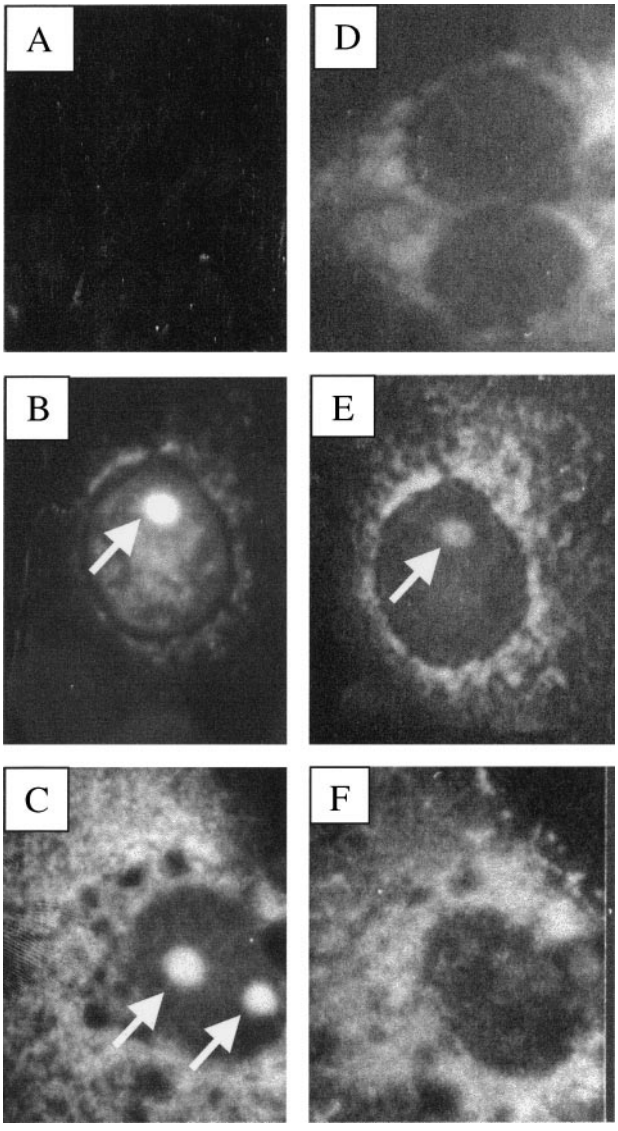

Fig. 5. Intracellular localization of PKR and PRRSV nucleocapsid protein in the same cells. N protein fluorescence (**A**, **B**, **C**) and PKR immunofluorescence (**D**, **E**, **F**) in MARC-145 cells mock-infected (**A** and **D**), infected with P6 (**B** and **E**), or transfected with pEGFP-123 (**C** and **F**), an EGFP fusion construct, which contained the full-length nucleocapsid protein. Two days after infection cells were fixed and stained with both FITC-labeled SDOW-17 and Texas red anti-P68 MAbs (**A**, **B**, **D** and **E**). Transfected cells (**C** and **F**) were stained at 24 h after transfection with only anti-P68-Texas red antibody. Green fluorescence for identification of the N protein localization was provided by EGFP. Photomicrographs of green (**A**, **C** and **E**) and red (**B**, **D** and **F**) fluorescence are shown for the same in individual cells. White arrows identify fluorescence within the nucleoli

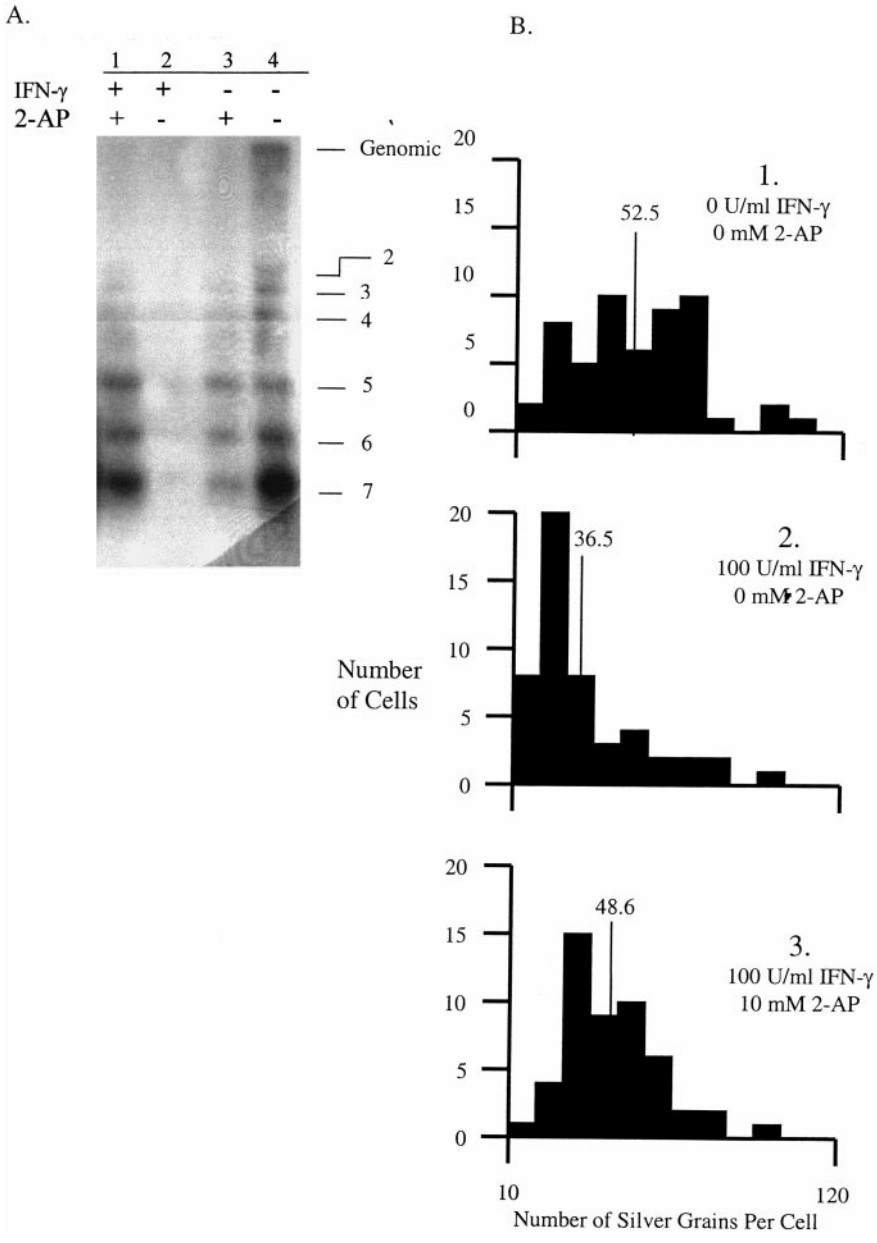

Fig. 6. PRRSV RNA content following treatment of MARC-145 cells with IFN-γ and 2-AP. MARC-145 cells were treated with IFN-γ, 2-AP and infected with P6 in the same manner as described in Fig. 4. **A** Northern blot of genomic and subgenomic mRNA expression at 12 after infection. **B** shows the distribution of silver grains following in situ hybridization of cells at 8 h after infection. Both **A** and **B** were hybridized with probes prepared from the same 484 bp ORF7 cDNA

increased anti-PKR fluorescence in the perinuclear region (Fig. 5D). We did not observe a significant increase or redistribution in PKR immunofluorescence in cells following overnight incubation with 100 U/ml IFN-γ. However, the intracellular distribution of PKR was dramatically different in PRRSV-infected cells. In a representative experiment (Fig. 5E) PKR immunofluorescence was still present in the cytoplasm, but with increased fluorescence around the perinuclear region and within the nucleoli of P6-infected cells (Fig. 5E). Similar results were observed in cells infected with P136. Incubation with 10 mM 2-AP prior to infection with P6 did not alter the pattern of PKR staining, suggesting that PKR activation was not required for nucleolar localization.

Interestingly, the intracellular distributions of PKR and PRRSV nucleocapsid (N) proteins were almost identical (compare Fig. 5B and E). To determine a possible association between PKR and N protein in the absence of infection, we performed a similar experiment that expressed the N protein fused to enhanced green fluorescent protein (EGFP). A representative result, presented in Fig. 4C and F, showed N-EGFP fluorescence in the nucleolus and cytoplasm; whereas, PKR remained in the cytoplasm. Since the N-EGFP fusion construct may not be representative the native N protein, we repeated the experiment using a construct that expressed only the N protein, which was detected using FITC-SDOW-17. Under these conditions the N and PKR proteins did not co-localize to the nucleolus (data not shown).

Inhibition of viral RNA synthesis by IFN-γ

The inability of 2-AP to completely recover virus replication following IFN-γ-treatment suggested that other anti-viral pathways, up-regulated by IFN-γ, were activated during PRRSV infection. For example, the 2′,5′ oligoadenylate synthetase (2–5 A synthetase) pathway, activated by dsRNA, inhibits translation by degrading mRNA [47]. Northern blots were used to evaluate the integrity of P6 subgenomic mRNAs. The results of a single experiment, presented in Fig. 6A, showed decreased expression of all PRRSV RNAs following treatment with 100 U/ml IFN-γ. We did not observe degraded RNA in the IFN-treated cultures. Viral RNA expression was also reduced in the presence of 10 mM 2-AP alone; however, the addition of 2-AP to IFN-γ treated cells increased the level of viral RNA expression above the IFN-γ-treated culture.

To further confirm that IFN-γ treatment decreased viral RNA expression, relative changes in viral RNA content in single cells were measured using a modified in situ hybridization technique [35]. Histograms showing the number of P6-infected cells versus number of silver grains/cell in infected cells at 8 hr r are presented in Fig. 6B. The mean silver grain content in infected cultures was 52.5 grains/cell (Fig. 6B, Panel 1). Pre-treatment with 100 U/ml of IFN-γ shifted the distribution towards cells containing significantly less viral RNA (mean = 36.5 silver grains/cell, $P = 0.0002$, Fig. 6B Panel 1 vs Panel 2). The addition of 2-AP restored the mean RNA content per cell to near normal levels (Fig. 6B, Panel 1 vs Panel 3).

Discussion

This study confirms previous reports describing IFN inhibition of PRRSV [2, 52, 56]. Furthermore, we extend these observations by demonstrating that IFN inhibits the replication of both wild-type and tissue culture-adapted isolates derived from a North American PRRSV isolate. Inhibition of PRRSV was observed following pre-incubation with either type-I or type-II IFNs. Furthermore, the sensitivity of PRRSV to IFN-γ was greater than VSV (Fig. 2A), a virus frequently used for the detection of IFN activity [45].

Consistent with other reports describing the presence of cell-mediated immunity during PRRSV infection [4, 31], we observed IFN-γ mRNA expression in lymph nodes and lungs of PRRSV-infected pigs (Fig. 1). Whether or not this amount of expression represents a biologically relevant quantity of IFN-γ remains to be determined. Even though we demonstrated the effectiveness of IFN in inhibiting PRRSV replication in MARC-145 cells, little is known regarding the properties of the macrophages supporting PRRSV replication in vivo. In infected pigs, PRRSV replication is initially found in all organs and tissues [29]. During the long asymptomatic stage of infection, virus replication is restricted to a subpopulation of cells located in tonsil and lymph nodes (personal observation), suggesting that these cells may be more resistant to IFN-γ, even when exposed to local elevations of cytokines from activated T cells. This observation is similar to studies of LDV infection of mice. LDV normally replicates in a subpopulation of macrophages, but in some strains of mice infects motor neurons causing paralysis. Injection of mice with IFN-γ prior to infection inhibits virus replication in motor neurons without significantly affecting the overall level of viremia [11]. We propose a model in which the induction of IFN-γ production during PRRSV infection is sufficient to suppress virus replication in permissive cells in non-lymphoid organs, but does not affect replication in a small subpopulation of permissive cells in lymph nodes and tonsil. The identity of this PRRSV-permissive population remains to be determined.

Anti-viral proteins, including PKR, 2-5A synthetase and Mx are up-regulated in response to IFN [9, 25, 27]. PKR, after binding viral RNA, is autophosphorylated on serine and threonine residues and inhibits translation by phosphorylating the alpha subunit of eIF2. Phosphorylation of PKR is inhibited by 2-AP, an ATP analog. The addition of 2-AP to cells pre-incubated with 100 U/ml IFN-γ partially recovered PRRSV replication in MARC-145 cells (Fig. 3). These results demonstrate that at least, in part, the interferon-induced anti-PRRSV activity may occur through the PKR pathway. The ability of 2-AP to partially restore viral RNA synthesis IN IFN-treated cells supports the work of deBoon et al. [20] who concluded that ongoing protein synthesis is required for the production of arterivirus RNAs. One explanation for the failure of 2-AP to completely restore virus replication is the activation of other antiviral pathways not regulated by PKR. For example the activation of the 2-5A synthetase pathway can be identified by the presence of degraded forms of both viral and ribosomal RNAs [48]. Degraded RNA was not detected in Northern blots (see Fig. 6A) in total

RNA from PRRSV infected cultures (personal observation). Recently, Zhang et al. [56] reported increased expression of Mx1 in cultured macrophages infected with PRRSV. A role for Mx in the inhibition of PRRSV replication has not been demonstrated. Another explanation for the failure of 2-AP to restore PRRSV replication is the inhibition of 2-AP sensitive phosphorylation event required for virus replication. For example, 25 mM 2-AP prevents the phosphorylation of the PE2/E2 envelope glycoprotein, which is required for the production of infectious progeny [30].

PKR is normally distributed between the cytoplasm and nucleus, which is consistent with its roles in regulation of translation and transcription. In MARC-145 cells, we found PKR primarily concentrated in the cytoplasm and perinuclear regions (Fig. 5D). An unexpected observation was the localization of PKR to the nucleoli of PRRSV-infected cells (Fig. 5E). Nucleolar localization was not affected by 2-AP, suggesting that PKR activation is not necessary for nucleolar localization. This observation confirms the result of Besse et al. [8] who identified a non-phosphorylated form of PKR in the nuclei of HeLa cells. Furthermore, PKR nucleolar localization during PRRSV replication is not the result of a physical association with the PRRSV nucleocapsid protein (Fig. 5C and F). From these results we can conclude that nucleolar localization of PKR in MARC-145 cells is dependent on PRRSV infection, but is independent of activation or an association with the nucleocapsid protein.

PRRSV infection of pigs continues to represent a worldwide disease problem. This study, combined with the results of others, clearly demonstrates an important role for IFN-γ in the control of PRRSV infection and suggests that the induction of IFN activity should be considered in the design of new vaccines. However, the mechanism for evasion of host defenses during persistent infection remains to be determined.

Acknowledgements

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