Background: Microbe-induced over-activation of cytokines, especially tumor necrosis factor (TNF)-α, is key to the pathogenesis of hantavirus infection leading to severe inflammation with high mortality rate. Although ribavirin showed promise in inhibiting viral replication in vitro, its clinical efficacy remains controversial.

Methods: Various concentrations of ribavirin were used to determine its effect on cytokine activation in our infectious model system.

Results: Ribavirin decreased the virus load and dose-dependently inhibited the accumulation of RANTES messenger RNA in Andes-virus (ANDV)-infected human endothelial cells, but failed to suppress TNF-α-induced activation of RANTES and interleukin-6 in ANDV-inoculated cultures. This report also shows, for the first time, that the deleterious over-stimulation by TNF-α is mediated by nuclear factor-κB, and describes the effect of ribavirin on cytokine production following ANDV infection.

Conclusions: Although highly effective in preventing ANDV replication and suppressing activation of select inflammatory mediators, the therapeutic efficacy of ribavirin is limited due to its inability to fully inhibit cytokine outburst triggered by hantavirus infection.

Ribavirin (1β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic nucleotide analogue [1] that has a broad spectrum of antiviral activity [2–10]. Various mechanisms of action were suggested to explain antiviral activity, including enzyme suppression [10–15] and the ‘error catastrophe’ effect in which a lethal accumulation of minor mutations leads to a stop in viral replication [16,17]. Although highly effective in vitro, ribavirin’s therapeutic efficacy remains controversial and even conflicting clinical results are often seen with the same viral infection, such as with hantavirus [18,19]. For example, ribavirin has been shown to be effective for treatment of patients with hantavirus haemorrhagic fever with renal syndrome (HFRS), whereas no efficacy was documented in patients with hantavirus pulmonary syndrome (HPS) [20].

Although highly effective against many RNA viruses in vitro, clinically ribavirin’s efficacy has been proven only in treatment of chronic HCV and respiratory syncytial virus [21]. It appears that direct antiviral activity of ribavirin has little effect on its overall clinical efficacy in many virus infections. Therefore, it has been suggested that mechanisms of therapeutic efficacy of ribavirin may involve its immunomodulatory properties [22]. A number of studies have reported cytokine activation in peripheral blood mononuclear cells, human pulmonary epithelial cells and human endothelial cells treated with ribavirin in vitro [18,19,23]. Current data suggests that ribavirin may facilitate Th1 type immune response activation promoting clearance of virus-infected cells [18,19,23].

Clinical studies have shown early cytokine activation in HFRS and HPS patients. High serum levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1 were found during the acute phase of HFRS [24].
hantaviruses are not cytopathic, it has been suggested that pathogenesis of HFRS is mainly attributed to the action of virus-activated cytokines [24–27]. Therefore, inhibition of hantavirus-induced cytokines may benefit patients by preventing excessive inflammation, which is believed to be the main cause for cardio-pulmonary shock and kidney failure in HPS patients. Although ribavirin has been shown to be effective for treatment of the subset of HFRS patients, its effect on cytokine activation during hantavirus infection remains largely unknown. We hypothesized that low efficacy of ribavirin therapy of HPS may be caused by its failure to inhibit cytokine activation caused by hantavirus infection.

Endothelial cells are the prime target for hantavirus infection in vivo [28,29]. Therefore, we applied an in vitro model using human endothelial cells to determine the effects of ribavirin on cytokine activation caused by hantavirus infection. Andes virus (ANDV) was utilized in this study. ANDV has been shown to be a causative agent in several HPS outbreaks in South America [24,25]. We have found that ribavirin suppresses ANDV replication in human umbilical cord vein endothelial cells (HUVEC) in vitro. Antiviral effects of ribavirin correlated with inhibition of RANTES (chemokine ligand 5 [CCL5]) secretion caused by ANDV replication in HUVECs. TNF-α activates IL-6 and augmented RANTES activation in ANDV-infected HUVECs. Ribavirin failed to inhibit TNF-α-caused RANTES and IL-6 activation in ANDV-infected HUVECs. We believe that activation of nuclear factor (NF)-κB by TNF-α led to upregulation of IL-6 and RANTES in ANDV-infected HUVECs. Although ribavirin inhibited ANDV replication, it did not affect TNF-α-triggered NF-κB activation, which sustained transcriptional activation of IL-6 and RANTES.

Methods

Cell lines and reagents
HUVEC and Vero clone E6 (Vero E6) cells were obtained from ATCC (Manassas, VA, USA). HUVECs were grown in MCDB 131 medium supplemented with human vascular endothelial cell growth factor, hydrocortisone, 2% fetal bovine serum (FBS), human fibroblast growth factor (0.5 ml, 1 μg/ml), ascorbic acid, heparin (0.5 ml, 1 μg/ml) and gentamicin (Clonetics Corporation, Walkersville, VA, USA). Cells were used between passages 2 and 4. Vero E6 cells, isolated from monkey kidney epithelial cells and generally used as host cells to grow viruses, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) medium containing 20% FBS and 50 μg/ml gentamicin. Ribavirin was obtained from Sigma-Aldrich (St Louis, MO, USA). TNF-α was purchased from R&D Systems (Minneapolis, MN, USA).

Virus
ANDV strain 23 used in this study was kindly provided by T Ksiazek (CDC, Atlanta, GA, USA). Viral stock (3–6×10⁶ plaque-forming units [PFU]) was prepared using Vero E6 cells and stored at -80°C. In experimental studies, cells were infected at a multiplicity of infection (MOI) of 1. Virus was concentrated by ultra-centrifugation (1 h, 22,000×g, 4°C) and re-suspended in DMEM (10% FBS, 50 μg/ml gentamicin). Virus was allowed to attach to cells for 1 h at 37°C in a 5% CO₂ atmosphere. Non-attached virus was removed, cells were washed with Hank’s balanced salt solution, and new medium was added. In some experiments new medium was supplemented with ribavirin and TNF-α.

To determine ANDV titre, serial 10-fold dilutions of ANDV (1 ml) were added onto 3–7-day old Vero E6 cell monolayers and incubated for 1 h at 37°C in a 5% CO₂ atmosphere. Medium was removed and 3 ml of overlay DMEM (10% FBS, 50 μg/ml gentamicin) supplemented with 0.6% agarose (Invitrogen, Carlsbad, CA, USA) was added. Monolayers were incubated at 37°C for 7 days and then stained by adding 2 ml/well of overlay medium containing 5% FBS and 5% neutral red solution (Gibco, Rockville, MD, USA). Plaques were counted after 2 days at 37°C.

Real-time PCR
Total RNA was extracted from hantavirus-infected cells at selected time points (1, 3, 24 and 72 h post-infection [PI]) using Trizol reagent (Gibco) according manufacturer’s recommendations and stored at -80°C.

TaqMan Minor Groove Binder (MGB) fluorogenic probes and primers were designed using Primer Express software (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer’s recommendations. The sequences of primers and probes used are summarized in Table 1. Primers were obtained from Invitrogen and probes were synthesized by ABI (Applied Biosystems). Complementary DNA (cDNA) synthesis was performed using random primers (Invitrogen) and stored at -20°C before use. A total of 1 μg RNA was used for cDNA synthesis. RNA was first incubated with random primers at 70°C for 15 min and then quickly chilled at 4°C for 15 min. Mixture of 1× reverse transcriptase buffer, 20 mM nucleoside triphosphates, RNAsin, and reverse transcriptase was added and reaction was extended at 42°C for 1 h. At the end of the reaction, enzymes were inactivated at 70°C for 15 min.

TaqMan was performed using TaqMan MGB probes on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each PCR reaction (25 μl) consisted of 1 μl of cDNA, 1× Platinum qPCR Supermix-UDG (Invitrogen), 200 nM of each primer and 100 nM of probe. 18S ribosomal RNA was used as an internal control according to the manufacturer’s
instructions. cDNA samples were diluted 1:1,000 with nuclease-free water before use in the 18S ribosomal RNA-specific TaqMan reactions. Standard curves for relative quantitation of the ANDV S segment RNA, RANTES mRNA, IL-6 mRNA and 18S RNA were created using serial dilutions of cDNA purified from infected and uninfected control samples depending on specifics of experimental design. In each experiment, the TaqMan values obtained for the ANDV S segment RNA, as well as the values obtained for the RANTES and IL-6 mRNAs, were normalized to the TaqMan values of the 18S RNA of the corresponding sample and presented as relative units. In addition, for some experiments these normalized values were calculated as the relative values to the same mRNAs produced in the corresponding control group. All TaqMan reactions were performed in duplicate.

Electrophoretic mobility shift assays

Gel shift assays were performed according to the manufacturer’s instructions for the Gel Shift Assay Core System (Promega, Madison, WI, USA) using double-stranded NF-kB consensus oligonucleotides. Briefly, 3.5 pmol of consensus oligonucleotides were end-labelled with 1 μCi [γ-32P]ATP using 5 units of T4 polynucleotide kinase in 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, and 5 mM DTT at 37°C for 10 min. The reaction was stopped by the addition of 10 mM EDTA and diluted with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Unincorporated label was removed by size exclusion chromatography with Sephadex G-50 spin columns. DNA binding reactions (15 μl) were performed using 1 μl of labelled consensus oligonucleotides and 4 μg of nuclear extract in binding buffer containing 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5) and 0.05 mg/ml poly(dI-dC). The reactions were incubated at room temperature for 20 min and stopped with an appropriate volume of 10× gel loading buffer containing 250 mM Tris-HCl (pH 7.5), 0.2% bromphenol blue and 40% glycerol. Samples were loaded onto 1.0 mm, 10×12 cm nondenaturing 4% acrylamide gels and electrophoresed at 100 V. Gels were dried overnight at room temperature between cellophane sheets and exposed to a phosphorimager screen.

Nuclear protein extraction

Nuclear protein extractions were performed according to standard protocols [30]. Briefly, treated cells were rinsed twice and then scraped in 100 μl of ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) containing 1 μg/ml aprotonin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 1 μg/ml pepstatin A. The cells were pelleted by centrifugation at 2,000×g for 5 min at 4°C. The pellet was washed twice with 1 ml of buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF) and centrifuged as just described. The supernatant was discarded, and the cellular proteins remaining in the pellet were released by hypotonic lysis in 100 μl of buffer A containing 0.1% Nonidet P-40 and centrifuging at 10,000×g for 10 min at 4°C. The supernatant containing the cytosolic fraction was transferred to a new tube, while the nuclear proteins remaining in the pellet were obtained by extraction in a high-salt buffer B (20 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM Na₂EDTA, pH 8.0, 25% glycerol, 0.5 mM DTT and 0.5 mM PMSF) and centrifugation at 10,000×g for 10 min at 4°C. The supernatant containing nuclear proteins was then diluted with an equal volume of buffer C (20 mM HEPES [pH 7.9], 50 mM KCl, 25% glycerol, 0.5 mM DTT and 0.5 mM PMSF). Protein concentrations for all extracts were determined by the bicinchoninic acid method (Sigma-Aldrich) according to the manufacturer’s recommendations. Bovine serum albumin was used as the standard.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed using an in situ cell death detection kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s recommendations.

Statistical analyses

Statistical analyses were performed using Student’s t-test for comparisons between individual experimental groups (infected and non-infected). Significance was established at a value of P<0.05. Data are presented as mean ±S.E.
Results

Effect of ribavirin on Andes-virus replication in HUVECs

To determine the in vitro effect of ribavirin on ANDV replication, HUVECs were infected with ANDV at an MOI of 1. Mock-infected HUVECs were used as control. At 1 h PI, ribavirin (50 μg/ml) was added to the culture medium. Accumulation of the ANDV S segment RNA was measured using real-time PCR. Additionally, the titre of ANDV in cell culture supernatants was determined by plaque assay at 72 h PI.

Results indicated that in infected cells without ribavirin, ANDV S segment RNA levels were significantly increased at 12 h PI, reaching a maximum at 24 h PI (Figure 1). Ribavirin treatment (50 μg/ml) significantly reduced ANDV S segment RNA levels at 12, 24 and 72 h PI (Figure 1). TUNEL assay was performed to demonstrate that ribavirin (50 μg/ml) treatment did not induce apoptosis in HUVECs (data not shown). Likewise, apoptosis was not detected in ANDV-infected HUVECs treated with ribavirin (50 μg/ml; data not shown). Therefore, we concluded that ribavirin inhibition of ANDV replication in HUVECs was not due to its cytotoxic activity.

Ribavirin inhibition of ANDV replication was also demonstrated in plaque assay at 72 h PI. ANDV titre in HUVEC culture supernatants was 10^2 ± 20 PFU/ml. ANDV titre in HUVECs treated with ribavirin was 2.75 ± 1.9 PFU/ml, which is significantly (P < 0.001) lower compared to that in supernatants of HUVECs without ribavirin treatment.

Ribavirin inhibits RANTES expression in Andes-virus-infected HUVECs

Previously, we have shown that hantavirus infection activates RANTES in HUVECs [31, 32]. Therefore, ribavirin’s effect on cytokine activation was evaluated using TaqMan by assessing RANTES mRNA levels in ANDV-infected HUVECs.

RANTES mRNA levels were significantly increased in the ANDV-infected cells at 12, 24, and 72 h PI when compared to mock-infected cells (Figure 2). Ribavirin treatment did not affect RANTES mRNA levels in the mock-infected cells at each selected time point (3, 12, 24 and 72 h PI; Figure 2). However, ribavirin significantly reduced levels of the RANTES mRNA in ANDV-infected cells at 12, 24 and 72 h PI when compared with infected cells without ribavirin treatment (Figure 2).

These data demonstrate that ribavirin inhibits RANTES activation in ANDV-infected cells. It appears that by inhibiting ANDV replication, ribavirin prevents ANDV-caused activation of cellular RANTES.

Dose-dependent effect of ribavirin on Andes-virus S segment RNA and RANTES messenger RNA accumulation in HUVECs

HUVECs were infected with ANDV at an MOI of 1 or mock-infected. Ribavirin (1, 10 and 50 μg/ml) was added to the culture medium of infected and mock-infected cells 1 h after infection. Ribavirin demonstrated a dose-dependent effect on accumulation of the ANDV S segment RNA (Figure 3A). At concentrations of 10 and 50 μg/ml, ribavirin significantly inhibited ANDV S segment RNA levels at 12, 24 and 72 h PI (Figure 3A). By contrast, ribavirin at a concentration of 1 μg/ml affected virus RNA level only at 48 h PI (Figure 3A).

Similarly, ribavirin had a dose-dependent effect on RANTES gene activation in the infected cells. For instance, ribavirin treatment (50 and 10 μg/ml) down-regulated RANTES mRNA level at 24 and 72 h PI compared to untreated infected cells (Figure 3B). However, ribavirin applied at 1 μg/ml had no effect on RANTES mRNA level (Figure 3B).

This data demonstrates that inhibitory effects of ribavirin on RANTES activation correlate with its antiviral activity.

Ribavirin modulates cellular responses activated by tumour necrosis factor-α

TNF-α is an important mediator of innate inflammation and up-regulates expression of adhesion molecules in vivo and in vitro [33, 34]. Increased levels of
TNF-α have been demonstrated in sera of hantavirus-infected patients [24,35]. Our data and others have shown that hantavirus infection does not activate TNF-α in HUVECs. However, we have demonstrated activation of TNF-α in hantavirus-infected pulmonary macrophages in vivo [36]. Therefore, we used TNF-α supplemented medium to determine effects of ribavirin on cytokine activation in ANDV-infected HUVECs. At 1 h PI with ANDV of HUVECs, TNF-α (10 ng/ml) and ribavirin (50 µg/ml) were added to the culture medium. Total RNA was collected at selected time points, 24 and 72 h PI. Real-time PCR was employed to estimate accumulation of the ANDV S segment RNA.

ANDV S segment RNA accumulation pattern in HUVECs was similar to that observed previously when levels of viral RNA reached its maximum at 24 h PI (Figure 4A). Ribavirin decreased ANDV S segment RNA accumulation at 24 and 72 h PI compared to ANDV-infected control cells (Figure 4A). Similarly, ANDV S segment RNA accumulation in HUVECs was decreased by TNF-α alone and in combination with ribavirin at 24 h PI (Figure 4A). Since, levels of ANDV S segment RNA were decreased in ANDV-infected control cells to the same level as in cells treated with ribavirin and its combination with TNF-α at 72 h PI (Figure 4A), effects of ribavirin, TNF-α, and their combination on ANDV replication were further determined by the virus titre in cell culture supernatants. In a plaque assay, ribavirin (50 µg/ml) inhibited ANDV replication in HUVECs at 72 h PI (Table 2). TNF-α treatment alone did not affect ANDV replication. However, combination of TNF-α and ribavirin suppressed ANDV replication (Table 2).

Ribavirin (50 µg/ml) did not affect levels of RANTES mRNA in mock-infected HUVECs at 24 h, whereas RANTES mRNA levels were significantly lower in cells treated with ribavirin at 72 h (Figure 4B). TNF-α (10 ng/ml) significantly increased RANTES mRNA levels in mock-infected HUVECs at 24 and 72 h compared to that in control cells (Figure 4B). Similarly, TNF-α combined with ribavirin significantly increased RANTES mRNA levels in mock-infected HUVECs at 24 and 72 h compared to control cells (Figure 4B). Thus ribavirin does not alter TNF-α induced RANTES mRNA accumulation in HUVECs.

ANDV infection significantly increased RANTES mRNA levels in HUVECs compared to control cells at 24 and 72 h PI (Figure 4B). As it had been expected, ribavirin significantly decreased RANTES mRNA levels in ANDV-infected cells at 24 and 72 h PI compared to that in infected cells without ribavirin (Figure 4B). TNF-α significantly up-regulated levels of RANTES mRNA in ANDV-infected HUVECs at 24 and 72 h PI compared to mock-infected cells (Figure 4B). Interestingly, TNF-α did not alter ANDV-induced RANTES mRNA accumulation in HUVECs.
at 24 and 72 h PI. Ribavirin combined with TNF-α significantly decreased RANTES mRNA levels only at 72 h PI compared to that in ANDV-infected cells treated with TNF-α alone (Figure 4B), although average values remained significantly higher compared to that in infected HUVECs treated with ribavirin alone (Figure 4B).

TNF-α (10 ng/ml) increased IL-6 mRNA levels in mock-infected HUVECs at 24 and 72 h (Figure 4C). Ribavirin decreased IL-6 mRNA levels only at 72 h PI in mock-infected HUVECs. Ribavirin did not alter IL-6 mRNA levels in cells treated with TNF-α at 24 and 72 h (Figure 4C). ANDV infection increased IL-6 mRNA levels in HUVECs at 24 h PI (Figure 4C). However, IL-6 mRNA levels were increased in HUVECs treated with TNF-α (10 ng/ml; Figure 4C). Ribavirin did not affect IL-6 mRNA levels in ANDV-infected HUVEC (Figure 4C). Similarly, there was no effect of ribavirin on IL-6 mRNA level in ANDV-infected HUVECs treated with TNF-α (Figure 4C).

Figure 3. Ribavirin dose-dependent effect on Andes-virus S segment RNA and RANTES messenger RNA accumulation in HUVECs

Error bars represent standard error. Human umbilical cord vein endothelial cells (HUVECs) were infected with Andes-virus (ANDV) at a multiplicity of infection of 1. Ribavirin (1, 10 and 50 µg/ml) was added into the culture medium 1 h after infection. TaqMan was performed using TaqMan Minor Groove Binder probes on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Standard curves for relative quantitation of ANDV S segment, RANTES and 18S RNA were created using serial dilutions of complementary DNA from infected and uninfected control samples, respectively. All TaqMan reactions were performed in duplicate. *P-value < 0.05 between control and ribavirin-treated groups. (A) Dose-dependent effect of ribavirin treatment on ANDV S segment RNA accumulation. (B) Dose-dependent effect of ribavirin treatment on RANTES mRNA accumulation. A, ANDV-infected cells; M, mock-infected cells.
These data demonstrate that ribavirin fails to inhibit TNF-α-stimulated RANTES and IL-6 activation in ANDV-infected HUVECs.

Effect of TNF-α and ribavirin on NF-κB activation

Our data revealed that TNF-α increases intracellular levels for RANTES and IL-6 mRNA in ANDV-infected HUVECs. NF-κB regulates RANTES and IL-6 gene activation [37,38]. Therefore, we sought to determine whether NF-κB is activated in response to TNF-α and/or ANDV infection. HUVECs were infected with ANDV at MOI of 1 for 72 h and used for collection of the nuclear fractions, which were used in an electrophoretic mobility shift assay.

In mock-infected HUVECs, little binding of the nuclear proteins was seen in the presence of an oligonucleotide encoding the consensus DNA sequence for NF-κB (Figure 5; lane 1). This binding is ablated in the

Figure 4. Ribavirin effect on RANTES and interleukin-6 activation by tumor necrosis factor-α in Andes-virus-infected HUVECs

Table 2. Andes-virus titre in supernatant of HUVECs treated with tumor necrosis factor-α, ribavirin and their combination

<table>
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<tr>
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<th>ANDV</th>
<th>ANDV plus ribavirin</th>
<th>ANDV plus TNF-α</th>
<th>ANDV plus ribavirin plus TNF-α</th>
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<tr>
<td>Titre 72 h PI, PFU</td>
<td>3.0 × 10^3 ± 2</td>
<td>2.5 ± 1.2</td>
<td>2.7 × 10^3 ± 3</td>
<td>1.5 ± 0.5</td>
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Data are mean ± se. ANDV, Andes-virus; HUVECs, human umbilical cord vein endothelial cells; PFU, plaque-forming units; PI, post-infection; TNF, tumor necrosis factor.
presence of excess unlabelled NF-κB oligonucleotide, indicating that the increase in binding seen is specific for NF-κB (Figure 5; lane 2). Ribavirin did not affect nuclear protein binding to the NF-κB oligonucleotide (Figure 5; lane 3) while TNF-α significantly increased nuclear protein binding to the NF-κB oligonucleotide (Figure 5; lane 4). Nuclear protein binding was slightly decreased in mock-infected HUVECs treated with combination of TNF-α and ribavirin (Figure 5; lane 5).

ANDV infection did not affect nuclear protein binding to the NF-κB oligonucleotide compared to mock-infected cells (Figure 5; lane 6). Similarly, nuclear protein binding to the NF-κB oligonucleotide was not altered in ANDV-infected HUVECs treated with ribavirin (50 μg/ml; Figure 5; lane 7). TNF-α (10 ng/ml) increased nuclear protein binding to the NF-κB oligonucleotide in ANDV-infected HUVECs (Figure 5; lane 8). Interestingly, it appears that TNF-α induced more nuclear protein binding to the NF-κB oligonucleotide in ANDV-infected HUVECs compared to that of mock-infected HUVECs (Figure 5; lane 4 versus 8). Nuclear proteins binding to the NF-κB oligonucleotide remained increased in ANDV-infected HUVECs treated with TNF-α and ribavirin (Figure 5; lane 9).

Discussion

A number of studies have reported that ribavirin exhibits diverse effects on cytokine production by various cell types [18,19,23]. Therefore, we sought to determine whether ribavirin treatment affects RANTES activation in hantavirus-infected cells. Our data demonstrate that its dose-dependent inhibition of RANTES in ANDV-infected cells correlates with its inhibition of ANDV replication.

RANTES is known to attract mononuclear immune effector cells to the site of infection [39–41]. Therefore, ANDV-caused RANTES activation may explain pathogenesis of the interstitial mononuclear pneumonia commonly diagnosed in HPS patients [29]. Our data on ribavirin inhibition of RANTES activation in hantavirus-infected cells suggest potential therapeutic efficacy of ribavirin in treatment of HPS patients. However, clinical studies have demonstrated that ribavirin shows no significant improvement in the clinical manifestation or mortality rate of HPS patients [20,42]. It has been suggested that virus infection and cytokine activation play equal roles in hantavirus pathogenesis. Therefore, we hypothesize that ribavirin, although a strong antiviral agent, has less effect on virus-caused cytokine activation.

Increased serum concentration of TNF-α and IL-6 in hantavirus patients’ serum has been reported [24,35]. We have shown activation of TNF-α in human alveolar macrophages infected with Sin Nombre virus [36]. Additionally, high numbers of TNF-α-positive cells were described in lung biopsies of HPS patients [25,26]. These data suggest that TNF-α may play a significant role in hantavirus pathogenesis. TNF-α is a potent activator of an array of cytokines in endothelial cells including IL-6 [43]. Therefore, we sought to further investigate effects of TNF-α on cytokine activation in ANDV-infected HUVECs. Our data revealed that TNF-α up-regulates IL-6 in ANDV-infected HUVECs. TNF-α and IL-6 share several physiological effects that are often synergistic. Both cytokines up-regulate expression of adhesion molecules, promote leukocyte migration, and activate acute phase proteins in the liver [44,45]. Thus, it appears that TNF-α may promote inflammatory responses in ANDV-infected cells by up-regulating IL-6 and augmenting RANTES activation. Overall, we have shown here that ribavirin failed to avert RANTES and IL-6 activation in TNF-α-treated ANDV-infected cells. Thus these data suggest that ribavirin has a limited effect on TNF-α-caused cytokine activation in ANDV-infected cells.

RANTES and IL-6 gene expression is regulated by activation of the transcription factor NF-κB [37]. It is also known that TNF-α stimulates NF-κB signalling [46]. However, there is no data on NF-κB activation in hantavirus-infected cells. Our data demonstrates that ANDV infection does not activate nuclear protein binding to NF-κB at 72 h PI. However, TNF-α increased NF-κB activation in hantavirus-infected cells. NF-κB complex is formed inactive monomer (NF-κB p65 and p50) in the cytoplasm. In resting cells, the NF-κB complex is bound by the inhibitor of NF-κB (IκB) protein, which keeps NF-κB in the inactive state. When certain stimuli activate the NF-κB pathway, the IκB protein is phosphorylated, thereby releasing NF-κB from its inhibited state. The activated NF-κB complex then translocates to the nucleus, where it binds to specific DNA sequences and regulates transcription of target genes.

TNF-α has been shown to activate NF-κB in various cell types, including endothelial cells [47]. The activation of NF-κB by TNF-α is mediated through the IKK complex, which phosphorylates IκB, leading to its degradation and subsequent release of NF-κB into the nucleus. Once in the nucleus, NF-κB binds to the DNA and activates the transcription of target genes, including cytokines such as TNF-α itself, IL-6, and IL-1β.

Figure 5. Effects of ribavirin on tumour necrosis factor-α activation of nuclear factor-κB in HUVECs

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<thead>
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<th>NF-κB</th>
<th>Non-specific</th>
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<tbody>
<tr>
<td>Ribavirin</td>
<td>+</td>
</tr>
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<td>Mock</td>
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TNF-α has been shown to activate NF-κB in various cell types, including endothelial cells [47]. The activation of NF-κB by TNF-α is mediated through the IKK complex, which phosphorylates IκB, leading to its degradation and subsequent release of NF-κB into the nucleus. Once in the nucleus, NF-κB binds to the DNA and activates the transcription of target genes, including cytokines such as TNF-α itself, IL-6, and IL-1β.
binding in mock-infected and ANDV-infected cells at 72 h. Therefore, it could be suggested that TNF-α caused activation of RANTES and IL-6 in ANDV-infected cells is regulated by NF-kB.

Our data has shown that ribavirin significantly reduces ANDV replication in HUVECs. Antiviral effect of ribavirin correlates with inhibition of RANTES activation in HUVECs. Although highly effective in inhibiting ANDV replication when combined with TNF-α, ribavirin failed to suppress TNF-α induced IL-6 and RANTES activation in ANDV-infected cells. It is generally accepted that proinflammatory cytokines such as TNF-α and IL-6 play a significant role in hantavirus pathogenesis [26,28,29,47]. Therefore, we believe that, although, ribavirin decreases virus load and suppresses virus replication in HPS patients, it fails to inhibit cytokine activation causing lung inflammation. As a result, ribavirin therapeutic efficacy could be limited in HPS patients, as observed during ribavirin clinical trials [42]. However, early initiation of ribavirin treatment could be therapeutically beneficial due to early inhibition of hantavirus replication, which will prevent activation of proinflammatory cytokines such as TNF-α. Recently, combination of ribavirin with antibodies against proinflammatory cytokines was shown to significantly reduce mortality and morbidity in virus-infected animals [48,49]. This approach combines inhibition of virus replication and ongoing inflammatory responses, as both are involved in development of the morbidity and mortality characteristic of the HPS.

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Disclosure statement

The authors declare no competing interests.

References


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